In vitro activity of antifungal agents on yeasts isolated from vaginal secretion

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ABSTRACT. Vulvovaginal candidiasis (VVC) is the second most frequent cause of vaginal infection, having high incidence and recurrence, which requires longer treatments. The genus *Candida,* specially the species *C. albicans,* is the most common agent. However, other species, more difficult to eradicate, may also be involved. The aim of this study was to determine the frequency of vaginal colonization by yeasts and to evaluate susceptibility to three drugs, which are indicated for the VVC treatment. Vaginal secretion was collected from four hundred and twelve unselected women. The overall rate of yeast colonization was 20.15%. *Candida albicans* was the most frequent (84%), followed by *C. glabrata* (7%), *C. tropicalis* (4%), *Torulopsis* sp (2%), *C. parapsilosis* (2%) and *S. cerevisiae* (1%). *In vitro* susceptibility tests revealed that 96,4% of the isolated yeasts were susceptible to fluconazole, 71% to nistatin and 98,8% to amphotericin B.

Key words: *Candida* sp., vulvovaginal colonization, antifungal drugs, susceptibility test.

RESUMO. Atividade *in vitro* de agentes antifúngicos sobre leveduras isoladas de secreção vaginal. Candidíase vulvovaginal (CVV) é a segunda causa de infecção vaginal e apresenta alta incidência da forma recorrente, o que resulta em tratamentos prolongados. O gênero *Candida*, principalmente a espécie *C. albicans*, é o agente mais comum. Entretanto, outras espécies mais difíceis de serem erradicadas podem também estar envolvidas. A proposta deste estudo foi determinar a freqüência de colonização vaginal por leveduras e avaliar a susceptibilidade a três drogas indicadas para tratamento de CVV. Foram coletadas amostras de secreção vaginal de quatrocentas e doze mulheres, não triadas. A taxa total de colonização por leveduras foi de 20,15%. *Candida albicans* foi a mais freqüente (84%), seguida por *C. glabrata* (7%), *C. tropicalis* (4%), *Torulopsis* sp (2%), *C. parapsilosis* (2%) e *S. cerevisiae* (1%). Testes de susceptibilidade *in vitro* revelaram que 96,4% dos isolados foram considerados susceptíveis ao fluconazol, 71% à nistatina e 98,8% à anfotericina B.

Palavras-chave: *Candida* sp., colonização vulvovaginal, drogas antifúngicas, teste de susceptibilidade.

Introduction

Vulvovaginal candidiasis (VVC) is the second most frequent cause of vaginal infection (Osset *et al*., 2001). It is estimated that 90% of vulvovaginal candidiasis cases are attributed to *Candida albicans.* The remaining cases have the non-*albicans* species as causal agents, being *Candida glabrata* and *Candida tropicalis* the most common ones (Sobel, 1996). It is estimated that at least 75% of all women develop one episode of VVC at some time in their lives (Saporiti *et al*., 2001) and the recurrence rate is high (Sobel, 1996). On the other hand, 20 to 25% of the women in child-bearing age have vaginal colonization by yeast (Redondo-Lopez *et al*., 1990). The presence of yeast as colonization is the basis for infection development.

The other species called non-*albicans* are more difficult to eradicate (Gupta and Bluhm, 2002). It has been evident that there are differences in the susceptibility pattern to the antifungal drugs among different species, what is relevant to the clinical and epidemiological point of view (Odds, 1996). *In vitro* studies have demonstrated that most of non-*albicans* species from vaginal secretion have sensibility reduced in relation to *Candida albicans*, for both topical and systemic antifungal drugs (Lynch and Sobel, 1994).

The emergence of a large diversity of fungus considered pathogenic up to this moment can be a consequence of the increasing use of the azoles antifungicals, specially fluconazole, which may incite the more resistant yeasts, *Candida* non-*albicans,* to emerge by suppression of the normal endogenous

microorganisms. These agents can also change the resistance pattern of the isolated *C. albicans* (Milan *et al*., 1998).

The aim of this study was to determine the species distribution and susceptibility pattern of yeasts isolated from the vulvovaginal specimens to fluconazole, nistatin and amphotericin B.

Material and methods

Sample

The yeasts included in this study were obtained from patients from the Teaching and Research Clinical Analyses Laboratory, performing routine exams of the vulvovaginal secretion. Samples of vulvovaginal materials, obtained by swabs, were used for "fresh" bacteriological exam (in 0.85% sterile saline), Gram staining and plated on Sabouraud Dextrose Agar (Difco, Detroit, USA) with 50μg.mL^{-1} of chloranphenicol. All cultures were incubated at 30ºC for seven days.

Mycological investigation

Yeasts colonies were identified according to the method recommended by Kurtzman and Fell (1998). Negative germ tube test isolated were tested for pigment production on niger seed agar, capsule observation, microscopic morphology on cornmeal-Tween 80 agar, and carbohydrate fermentation and assimilation assays comprising 7 and 15 sugars, respectively. Organisms were also checked for urease production, nitrate assimilation and ascospore formation when necessary. *C. albicans* identification by the germ tube test was confirmed by the presence of chlamydoconidia on cornmeal-Tween 80 agar.

Antifungal susceptibility testing

The isolated yeasts were tested by broth microdilution method, performed according to the proposed NCCLS, M27-A standard guidelines (1997). Fluconazole powder (Galena Chemistry and Pharmaceutical), nistatin (Sigma) and amphotericin B (Bristol-Myers Squibb) were used to obtain final drug dilutions ranging from 1.25 to 640μg.mL^{-1} for fluconazole and nistatin and 0.3 to 160μg.mL^{-1} for amphotericin B. In brief, broth microdilution testing was performed in sterile, flat-bottomed 96-well microplates (Nunclon, Delta, Nunc., InterMed, Denmark), with RPMI-1640 (Sigma) with L-glutamine, without bicarbonate, and buffered with MOPS at pH 7.0. The microplates containing double the final concentration of drugs were prepared in advance and stored at –20ºC for no more than 3 weeks. On the testing day, the inoculum suspension had its turbidity adjusted by the spectrophotometer according to the 0.5 McFarland standard at 530nm wavelength. A volume of 100μL of the adjusted inoculum suspension was dispensed in each well, resulting in the desired final drug concentration and inoculum size between 0.5 and 2.5 x 10^3 cells.mL^{-1}. The plates were incubated at 35ºC for 48 h. A quality control organism (*C. parapsilosis* ATCC 22019) was included in each test, in order to check the accuracy of the drug dilutions and the reproducibility of the results.

The minimum inhibitory concentration (MIC) for fluconazole was defined as the first well with a significant reduction (approximately 80%) in growth, compared to the one of the positive control, while for amphotericin B and nistatin, the MIC was defined as the lowest concentration able to inhibit any visual growth. MIC_{50} and MIC_{90} were defined as the MIC for 50% and 90% of isolated yeasts, respectively. The yeasts with MICs between 16 and 32μg.mL^{-1} for fluconazole and 8 to 32μg.mL^{-1} for nistatin have reduced dose-dependent susceptibility (DDS). The ones with MICs ≤ 8μg.mL^{-1} for fluconazole, ≤ 4μg.mL^{-1} for nistatin and ≤ 1μg.mL^{-1} for amphotericin B were considered susceptible. MICs ≥ 64μg.mL^{-1} for fluconazole and nistatin and ≥ 2μg.mL^{-1} for amphotericin B were considered endpoints for resistance to these antifungal agents (Mcginni and Rinaldi, 1991; NCCLS, 1997).

Results

Four hundred and twelve unselected women, from whom 83 yeasts (20,15%) were extracted, were evaluated. All patients were attended at the Teaching and Research Clinical Analyses Laboratory, State University of Maringá, in Maringá, Paraná, Brazil. The following species were identified: *C. albicans* (84%), *C. glabrata* (7%), *C. tropicalis* (4%), *Torulopsis* sp. (2%), *C. parapsilosis* (2%) and *S. cerevisiae* (1%).

The variation of the MICs for 3 drugs, tested against the 83 yeasts, showed MICs among 0.125 to 64μg.mL^{-1} for fluconazole; 0.5 to 8μg.mL^{-1} for nistatin and 0.125 to 8μg.mL^{-1} for amphotericin B. There was high MICs variation for all antifungals used, but it was possible to observe that the MIC variation for fluconazole was higher when compared to the other two antifungals (Table 1).

Table 2 shows the MIC_{50} and MIC_{90} values of the three antifungal drugs tested against 83 yeasts isolated according gender and species.

Table 3 shows the interpretation of MICs results obtained in assays with 83 yeasts against those three drugs.

Resistance to the tested antifungals was observed in some cases: 2,4% of the yeasts showed *in vitro* resistance to fluconazol and 1,2% to amphotericin B.

Table 1. The minimum inhibitory concentration (MIC) range of the three antifungal drugs against 83 yeasts strains.

Organisms	N	MICs range (µg mL-1)		
		Fluconazole	Nistatin	Amphotericin B
C. albicans	69	≤0.125 – ≥64	0.5 – 8	0.125 – 8
C. glabrata	6	0.25 – 4	2 – 8	0.5 – 1
Torulopsis sp	2	8 –32	4 – 8	0.5 – 1
C. tropicalis	3	0.25 – 0.5	4 – 8	0.5
C. parapsilosis	2	0.5 – 64	8	1
S. cerevisiae	1	4	2	0.5

N = Number of isolated yeasts.

Table 2. In vitro susceptibility profile of 83 yeasts isolated against three antifungal drugs.

Organisms	N	MIC (µg mL-1)					
		Fluconazole		Nistatin		Amphotericin B	
		MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
C. albicans	69	0.125	0.25	4	8	0.5	0.5
C. glabrata	6	2	4	4	2	0.5	0.5
Torulopsis sp	2	-	-	-	-	-	-
C. tropicalis	3	0.25	0.25	4	4	0.5	0.5
C. parapsilosis	2	-	-	-	-	-	-
S. cervisiae	1	-	-	-	-	-	-
Total	83	0.25	2	4	8	0.5	0.5

N = Number of isolated yeasts; MIC_{50} = MIC for 50% of isolated yeasts; MIC_{90} = MIC for 90% of isolated yeasts.

Discussion

It was possible to determine, through the methodology proposed, that the frequency of the vaginal mucosa colonization by yeasts among 412 women was 20,15%. It was also possible to investigate the *in vitro* susceptibility of 83 yeasts isolated to fluconazole, nistatin and amphotericin B.

Among 83 patients carrying yeasts in the vaginal mucosa, 84% of them showed positive cultures for *C. albicans*. This result is superior to the ones registered by Tietz *et al*. (1995) in Madagascar (62,4%) and Angola (64,8%) and by Buitron *et al*. (2002) in Mexico, but is equivalent to the first study performed in Brazil by Ribeiro *et al*. (2000), which found *C. albicans* in 88% of the symptomatic patients and in 40% of the asymptomatic women, in the State of Espírito Santo. In the present study, the second most frequent yeast species was *C. glabrata* (7%), confirming the results found by the authors above.

All non-*albicans* species previously mentioned have been reported in other studies about VVC, including the species *Sacharomyces cerevisiae*, which not only shows industrial interest but also could be a human pathogenic agent (Lynch and Sobel, 1994; Nyirjesy *et al*., 1995; Hoog, 1996).

Vulvovaginal candidiasis may be treated with either topical or systemic antifungal agents. Fluconazole has been considered the choice drug, because it is well tolerated, has a good oral bioavailability and is efficacious against most of *Candida* spp yeasts (Odds, 1996). However, it is more commonly used for systemic candidiasis, due to its high price.

Nistatin has been widely used in Brazil as the first alternative to treat VVC in public health care, because of the high cost of fluconazole, but there aren't any publications about nistatin *in vitro* results.

Despite the fact that amphotericin B is the most efficacious fungicide, it shows some difficulties, such as: difficult intravenous via administration, association with a variety of adverse effects that include serious drug reactions as to nephrotoxicity and others. The use of the amphotericin B was limited on VVC only in some topical formulations.

In the present study, the microdilution broth method for antifungal susceptibility testing was used due to its standardization by the NCCLS (1997), its easiness of performance, generation of reproducible results and good correlation with clinical data (Rex et al., 1993; NCCLS, 1997). The analysis of results showed, through samples of yeasts from VVC, that the susceptibility rank is in accordance with data obtained from other authors (Kwok *et al*., 1998; Ribeiro *et al*., 2000). Taking into account all yeasts isolated from vaginal colonization, the incidence rate of resistance to fluconazol was 2,4%, 1,2% to amphotericin and there was no resistance to nistatin, agreeing with the literature. Microbiological studies have revealed azole resistance to *C. albicans* and infection by less sensitive non-albicans *Candida* species (Ribeiro *et al*., 2000; Sobel, 2001).

Table 3. Interpretation of MIC values of fluconazole, nistatin and amphotericin B for 83 yeasts strains.

Organisms	N	Fluconazole[a]			Nistatin[b]			Amphotericin B[c]	
		S❶	S-DD❷	R❸	S	S-DD	R	S	R
C. albicans	69	68 (98,5)	-	1 (1,5)	51 (73,9)	18 (26,1)	-	68 (98,5)	1 (1,5)
Torulopsis glabrata	6	6 (100)	-	-	4 (66,7)	2 (33,3)	-	6 (100)	-
Torulopsis sp	2	1 (50)	1 (50)	-	1 (50)	1 (50)	-	2 (100)	-
C. tropicalis	3	3 (100)	-	-	2 (66,7)	1 (33,3)	-	3 (100)	-
C. parapsilosis	2	1 (50)	-	1 (50)	-	2 (100)	-	2 (100)	-
S. cerevisiae	1	1 (100)	-	-	1 (100)	-	-	1 (100)	-
Total	83	80 (96,4)	1 (1,2)	2 (2,4)	59 (71)	24 (29)	-	82 (98,8)	1 (1,2)

N = Number of isolated yeasts; ❶ S = susceptibility - number (%); ❷ S-DD = susceptibility dose-dependent - number (%); ❸ R = resistance - number (%); [a]Fluconazole: **S** ≤ 8.0µg mL^{-1}; **S-DD =** 16-32µg mL^{-1}; **R** ≥ 64µg mL^{-1}; [b]Nistatin: **S** ≤ 4.0µg mL^{-1}; **S-DD =** 8-32µg mL^{-1}; **R** ≥ 64µg mL^{-1}; [c] Amphotericin B: **S** ≤ 1.0µg mL^{-1}; **R** ≥ 2.0µg mL^{-1}.

C. albicans and *C. parapsilosis* were the strains resistant to fluconazol and, surprisingly, one *C. albicans* sample was resistant to amphotericin in repeated assays. Although no case of *in vitro* resistance to nistatin was observed, 29% of the tested samples showed dependent susceptibility dose, suggesting limitations to the treatment with this drug in VVC.

These results showed that the yeasts isolated from vaginal colonization have high sensibility to *in vitro* assays, while the patients that developed VVC show not the same, what makes the treatment with antifungals difficult.

Nevertheless, while considerable progress in standardizing antifungal susceptibility testing has been made, additional efforts are necessary to develop simpler and more economical methods that could be routinely performed in clinical laboratories and could contribute to VVC diagnosis and therapeutic monitoring.

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