doi: 10.5216/rpt.v49i3.64233

ORIGINAL ARTICLE

ACCURACY IN CLINICAL EXAMINATIONS FOR THE DIAGNOSIS OF VULVOVAGINITIS BY *Candida* SPP. AND *in vitro* SUSCEPTIBILITY TO THE MAIN ANTIFUNGALS

Andressa Santana Santos[1], Ana Laura Sene Amâncio Zara[1], Fábio Silvestre Ataídes[1], Elisangela Gomes da Silva[2], Vivianny Aparecida Queiroz Freitas[1], Carolina Rodrigues Costa[1], Thaísa Cristina Silva[1] and Maria do Rosário Rodrigues Silva[1]

ABSTRACT

Vulvovaginal candidiasis (VVC) is a common infection. This work aims to determine the positive predictive value (PPV) of the clinical diagnosis of VVC and to characterize *Candida* species isolated from the vaginal mucosa. This cross-sectional study was conducted from February 2016 to February 2017 at the Gynecology and Obstetrics Outpatient Clinic of the Hospital das Clínicas, in Goiânia, Goiás State, Brazil. The study included samples of vaginal secretion from 55 women who complained of vaginal discharge and itching as their main symptoms. The PPV of the clinical diagnosis of VVC was estimated in comparison to the laboratory culture method. The phenotypic methods and molecular tests were performed to identify *Candida* spp. *In vitro* susceptibility of *Candida* spp. isolates to fluconazole, itraconazole, clotrimazole, nystatin, and amphotericin B was determined using the broth microdilution assay. Yeast growth using the enzymes protease, phospholipase, and hemolysin was carried out in media containing respectively bovine albumin, egg yolk, and sheep erythrocytes. A PPV of 61.8% (34/55) was determined. Among the 55 vulvovaginal samples collected, we identified 36 isolates in which *C. albicans* was the most common species. High resistance to fluconazole and low minimal inhibitory concentration (MIC) values for clotrimazole, nystatin and amphotericin B were observed. All isolates were proteinase and hemolysin producers, while seven strains were phospholipase negative. The clinical diagnosis of VVC presented a moderate PPV, which meant that cultures had to be conducted in the laboratory to confirm infection. The high resistance to fluconazole and itraconazole indicated the importance of the *in vitro* susceptibility test.

KEY WORDS: Vulvovaginal candidiasis; antifungal susceptibility; enzymatic activity

1. Instituto de Patologia Tropical e Saúde Pública, Universidade Federal de Goiás (UFG), Goiânia, Goiás, Brazil.
2. Instituto de Ciências Biológicas, UFG, Goiânia, Goiás, Brazil

Corresponding author: Maria do Rosário Rodrigues Silva, Sala 426, Rua 235, esquina c/ 1ª Avenida, s/n,, Setor Universitário, CEP 74605-050 Goiânia, Goiás, Brazil. E-mail: rosario@ufg.br

Received for publication: 6/7/2020. Reviewed: 27/7/2020. Accepted: 21/8/2020.

INTRODUCTION

Vulvovaginal candidiasis (VVC) is a common fungal infection among women of childbearing age. VVC is a global public health issue since an estimated two-thirds of adult women experience at least one episode of VVC during their lifetime, and 50% of them experience VVC more than once (Ghaddar et al., 2019). Recurrent episodes have been observed in approximately 9% of women (Foxman et al., 2013). The disease occurs because of predisposing factors such as uncontrolled diabetes, use of oral contraceptives, sexual activity, genetic predisposition, deficiencies in the immune system, hormone replacement therapy, and microbiota alterations that favor yeast multiplication (Sobel, 2013).

Although symptoms such as itching, burning, dyspareunia, dysuria, and the presence of whitish plaques are associated with candidiasis (Wölber et al., 2020), these are insufficient for the clinician to reliably determine the cause of infection by *Candida* sp. Itching is present in approximately 90% of patients, but only 35% to 40% of women with this symptom have VVC (Mendling, 2015). According to a Ministry of Health guideline the differentiation of bacterial vaginosis and vulvovaginitis by *Candida* should be determined. Fresh examination and Gram staining of vaginal secretion allow this distinction. However, examination of vaginal secretion by adding 10% KOH or Gram-stained smears or Giemsa smears shows that 50% of women with *Candida* spp vulvovaginitis exhibit false-negative results. Unfortunately, due to the lack of a fast, reliable, and inexpensive test to confirm the diagnosis of VVC clinicians adopt an empiric approach in which antimycotic therapy is prescribed (Ministério da Sáude, 2015).

Laboratory culture is considered the gold standard method for diagnosis, yet it is not routinely performed as it is time consuming and more expensive. According to Aniebue et al. (2018), clinical evaluation and laboratory culture of vulvovaginal specimens should be the standard diagnostic method to eliminate the risk of misdiagnosis and eliminate potential long-term consequences of incomplete treatment, balanitis in the males, and vulvodynia in the affected females (Buyukbayrak et al., 2010; Sobel, 2013; Mendling, 2015; Aniebue et al., 2018).

Although antifungal agents such as nystatin and azole derivatives are available to treat VVC, a reduction in the susceptibility of *Candida* species to these antifungal drugs has been observed, such as *Candida* spp resistance to azoles (Sobel et al., 2013; Sobel & Sobel, 2018; Bitew & Abebaw, 2018). This characteristic highlights the importance of *in vitro* susceptibility testing for this yeast. In this study, the objectives were to determine the positive predictive value (PPV) of the clinical diagnosis of VVC, characterize *Candida* species isolated from the vaginal mucosa, verify the *in vitro* susceptibility profile to antifungal agents, and determine the virulence factors considering the production of hydrolytic enzymes by *Candida* species.

MATERIALS AND METHODS

Study design

This cross-sectional study was conducted from February 2016 to February 2017 at the Gynecology and Obstetrics Outpatient Clinic of the Hospital das Clínicas, in Goiânia, Goiás State, Brazil. The study included samples of vaginal secretion from 55 women who complained of vaginal discharge and itching as their main symptoms. Previous use of antifungals was considered an exclusion criterion, except in cases of recurrent vulvovaginal candidiasis, when the patient's clinical history presented more than three episodes during the previous twelve months.

Questionnaires were used to obtain data on age, marital status, and personal history regarding use of contraception, recent use of antibiotics, pregnancy, and diabetes. The study was reviewed and approved by the Ethics Committee of Hospital das Clínicas da Universidade Federal de Goiás (HC/UFG) under the number 1.374.728 on December 17, 2015.

Collection, culture, and identification

For each patient with clinical diagnosis of vulvovaginal candidiasis (VVC), vulvovaginal secretion was collected with sterile cotton swabs. The samples were stored in sterilized tubes containing 0.85% saline solution, at 8˚C, until processing.

Vaginal swabs were streaked onto Sabouraud dextrose agar with chloramphenicol (ASD, Difco® Laboratories, USA) and incubated at 37˚C for seven days.

Phenotypic identification of Candida species

Phenotypic identification of the recovered isolates was performed by germ tube test, corn meal tween-80 agar growth, subculture in CHROMagar *Candida* spp. medium (Difco® Laboratories, USA), auxanogram and zymogram assays (Kurtzman et al., 2011).

Genotypic identification

DNA from *Candida* species was extracted as described by Casali et al. (2003). Species identification were performed by amplifying an RPS0 gene fragment according to Martinez et al. (2010). The primers used in the study were described as follows: INT1(5′-AAGTATTT-GGGAGAAGGGAAAGGG-3′) and INT2 (5′-AAAATGGGCATTAAGGAAAAGAGC-3′) for *C.albicans*, CG1(5′-ACATATGTTTGCTGAAAAGGC-3′) and

CG2 (5′-ACTTTTTCTTAGTGTTCAGGACTTC-3′) for *C. glabrata*, CPf (5´- AGGGATTGCCAATATGCCCA-3´) and CPr (5´-GTGACATTGTGTAGATCCTTGG-3´) for *C.parapsilosis*, CTf (5´-TGATAGTTAGGAAAGATCAGGTG-3´) and CTr (5´-AACATATCCCATGTGTGTGT-3´) for *C.tropicalis*, and CDf (5´-AGTATTGGGAGAGGGAAAGACC-3´) and CDr (5´-ACAGGGAAGTCGATTCTTGC-3´) for *C. dubliniensis*.

Amplification was performed in an automated thermocycler with a final volume of 25 µL containing 2.5 µL 10× buffer, 1 µl 50 mM of M_gCl_2, 2.5 U of Eco Taq polymerase (Ecogen®), 2.5 µl dNTPs (2.5 mM each; Sigma®, St. Louis, MO, USA), with each primer in 4-µM concentration. One microliter of DNA suspension (30-50 ng) was amplified in a PCR thermal cycler (Bio-Rad PTC-100®) with one cycle at 95°C for 5 min and then 35 cycles as follows: 30 s of denaturation at 95°C, 30 s of annealing at 56°C, and 90 s of primer extension at 72°C. PCR was completed by a final extension at 72°C for 10 min. The resulting fragments of amplified DNA were analyzed by electrophoresis through 1% agarose gel, stained with ethidium bromide, and visualized with UV light. A 100-bp ladder (Invitrogen® Life Technologies, USA) was used as a DNA Ladders.

Antifungal susceptibility testing

Antifungal susceptibility tests of the recovered isolates were performed according to Clinical and Laboratory Standards Institute (CLSI) documents M27-A3(2008) and M-59(2016). The drugs fluconazole (Pfizer®, United States), itraconazole (Janssen Pharmaceuticals®, Beerse, Belgium), clotrimazole (Medley®, Campinas, São Paulo), nystatin (Medley®, Campinas, São Paulo), and amphotericin B (National Chemical Pharmaceutical Union®, India) were evaluated. Each isolate was tested in duplicate and the visual readings were obtained after 48 h of incubation and compared with the growth control. The minimal inhibitory concentration (MIC) of azole derivatives was defined as the lowest concentration that inhibited 50% of the yeast growth and for the polyene derivatives as the lowest concentration that inhibited growth of the microorganisms. Based on the MIC results for fluconazole, itraconazole, and amphotericin B, the isolates were classified as susceptible or resistant according to the CLSI (2008, 2016). For clotrimazole, there are no established breakpoints; therefore, we considered MIC value thresholds, using a value 0.5 µg/mL for resistance (Pelletier et al., 2000). The MIC_{90}, which inhibited 90% of the isolates, was determined for nystatin. Quality control was performed using standard strain *C. parapsilosis* ATCC 22019.

Enzymatic activity

The evaluation of proteinase activity was performed according to Ruchel et al. (1982), using yeast carbon base (YCB) medium (1.7%) with 0.2% bovine serum albumin (BSA). The YCB-BSA was adjusted to pH 5.0, sterilized by filtration using Millipore membrane 0.22 μm, and added to previously autoclaved agar (1.8%). The medium was distributed in Petri dishes with surface inoculation of 10 μL yeast suspension containing 10^7 cells/mL, and incubated at 37°C for 7 days. The proteolytic activity results in a zone around the colony, which corresponds to the hydrolysis of the BSA present in the medium.

The Price et al. (1982) method was used to verify phospholipase activity. The Sabouraud dextrose agar medium (1.2%; SDA, Difco Laboratories®, USA) containing 5 mM $CaCl_2$, 1 M NaCl was autoclaved and added with 8% egg yolk emulsion. The medium was distributed in Petri dishes, with surface inoculation of 10 μL yeast suspension containing 10^7 cells/mL and incubated at 37°C for 7 days. The precipitation zone around the colony results by formation of a calcium complex with fatty acids. The activity of hemolysin was determined according to Manns et al. (1994). The medium containing Sabouraud dextrose agar supplemented with 3% glucose and 7% defibrillated sheep's erythrocytes (Newprov®, Brazil) was distributed in Petri dishes. Subsequently, 10 μL yeast suspension containing 10^8 cells/mL was inoculated in this medium. The plates were incubated for 24 h in 5% CO_2 atmosphere. Hemolytic activity was defined as the formation of a translucent halo around the colonies.

The enzyme activity tests were performed in triplicates and measured in terms of the ratio between the diameter of the colony and the total diameter of the colony, plus a precipitation zone around the colony, according to Price et al. (1982), and classified into four categories, according to Ramos et al. (2015). Pz value of 1 meant no enzymatic activity; between 0.99 and 0.70 low enzymatic activity; between 0.69 and 0.40 moderate activity and between 0.39 and 0.10 high activity.

Data analysis

The PPV was calculated by dividing the number of positive cases diagnosed clinically by the number of positive cases by laboratory culture, multiplied by 100.

The socio demographic and clinical information of the participants were inserted into a database and analyzed using SPSS 22.0 Statistical software (SPSS Inc.®, Chicago, IL, USA). The categorical variables were compared using the chi-square test or Fisher's exact test. Statistical differences were considered significant when $p < 0.05$.

RESULTS

Vulvovaginal secretions totaled 55 samples collected from women with clinical suspicion of VVC, aged 14 to 64 years, with a mean age of 32.0 years (standard deviation ± 12.0 years). Among the 55 women, 34 had diagnostic confirmation by laboratory culture, presenting a positive predictive value (PPV) of 61.8% (Table 1). Of the women with positive cultures, 16.4% had had more than four episodes of the disease, characterized as recurrent. Among women with VVC, 94.4% were infected with one species of *Candida*, and 5.6% were infected with two species (*C. albicans* and *C. glabrata* in one case and *C. albicans* and *C. tropicalis* in the other cases). *Candida albicans* was the predominant species (75%) followed by *C. glabrata* (14%) and *C. tropicalis* (11%) (Figure).

Table 1. Distribution of age group, marital status and specialty of care for women with clinical suspicion of vulvovaginal candidiasis coming from Hospital das Clínicas, in Goiânia-GO, 2016-2017.

Characteristics	Total (n=55)	Culture		p-value
		positive (n=34)	negative (n=21)	
Age (in years)				
Mean ± standard deviation	32.0 ±12.0	29.7 ±9,7	35.6 ±14.5	0.074*
Median (IIQ 25-75)	32 (21-38)	29 (21-35)	36 (22-43)	
Minimum/maximum	14/64	14/60	15/64	
Marital status	n (%)	n (%)	n (%)	
Married	36 (65.5)	23 (67.3)	13 (61.9)	0.430**
Single	18 (32.7)	11 (32.4)	7 (33.3)	
Divorced	1 (1.8)		1 (4.8)	
Clinical specialty	n (%)	n (%)	n (%)	
General gynecology	22 (40.0)	11 (32.4)	11 (52.4)	0.514**
Family planning	18 (32.7)	13 (38.2)	5 (23.8)	
Pregnant	10 (18.2)	7 (20.6)	3 (14.3)	
Adolescent	4 (7.3)	2 (5.9)	2 (9.5)	
Pelvic pain	1 (1.8)	1 (2.9)	-	
Positive Predictive Value	34/55 (61.8)			

* T test (p<0,05). ** Chi-Square Test (p<0,05).

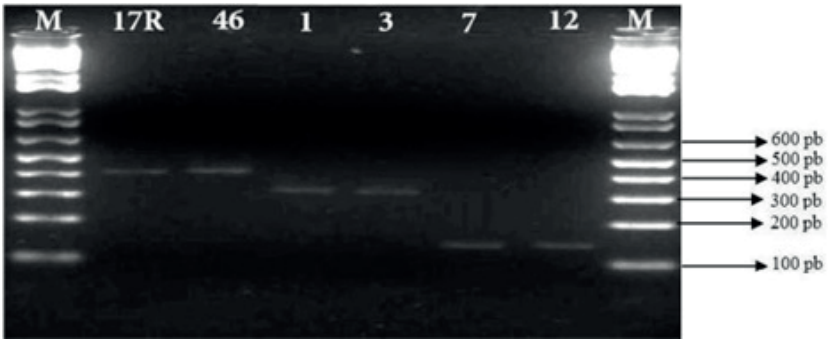

Figure. Agarose gel electrophoresis (1.2%), showing the amplification of the DNA of the species of *C. albicans* (1 and 3), *C. glabrata* (17R and 46) and *C. tropicalis* (7 and 12). M: 100 bp molecular marker (Invitrogen).

The main signs and symptoms observed were itching, burning, leukorrhea, dysuria, and dyspareunia. A higher proportion of women with pruritus was found among those diagnosed with candidiasis than with other diagnoses (p=0.020) (Table 2).

Table 2. Signals and symptoms associated to vulvovaginal candidiasis in Women from Hospital das Clínicas, in Goiânia-GO, 2016-2017.

Signals and symptoms	Total n (%)	Culture		
		Positive (34) n (%)	Negative (21) n (%)	p-value
Itching	41 (74.5)	29 (70.7)	12 (29.3)	**0.020***
Burning	29 (52.7)	20 (69.0)	9 (31.0)	0.249*
Like-curd discharge	54 (98.2)	33 (61.1)	21 (38.9)	1.000**
Dysuria	27 (49.1)	18 (66.7)	9 (33.3)	0.467*
Dyspareunia	26 (47.3)	18 (69.2)	80 (30.8)	0.284*

*Chi-Square Test (p<0,05). ** Fisher's Exact Test (p<0,05). Significant differences in bold.

All 55 women received empirical treatment. Of the antifungal agents, 44 patients received fluconazole and 5 were treated with clotrimazole. Only 26 (59.1%) of the women treated with fluconazole and eight (30.9%) who used other antifungals were culture positive for *Candida* spp. (Table 3).

Table 3. Empirical treatment used for women with clinical suspicion of VVC and positive culture for *Candida* sp.

Antifungal agents	Total n (%)	Culture	
		positive n (%)	negative n (%)
Fluconazole	41 (74.5)	23 (56.1)	18 (43.9)
Clotrimazole	5 (9.1)	3 (60.0)	2 (40.0)
Miconazole	2 (3.6)	2 (100)	-
Cetoconazole	1 (1.8)	1 (100)	-
Secnidazole	1 (1.8)	1 (100)	-
Nystatin	1 (1.8)	-	1 (100)
Fluconazole/Cetoconazole	1 (1.8)	1 (100)	-
Fluconazole/Miconazole	1 (1.8)	1 (100)	-
Fluconazole/Secnidazole	1 (1.8)	1 (100)	-
Itraconazole	1 (1.8)	1 (100)	-

The *in vitro* activity of the antifungals showed that *Candida* species presented high resistance to fluconazole (13 *C. albicans* and 3 *C. tropicalis*) and to itraconazole (17 *C. albicans* and 2 *C. tropicalis*). For clotrimazole, 63.8% of the isolates presented a very low MIC (>0.03 µg/mL), and the MIC_{90} was 0.5µg/mL. Furthermore, five strains showed clotrimazole MIC values equal to 16 µg/mL (four *C. albicans* and one *C. glabrata*). Among the polyenic drugs, all isolates were susceptible to amphotericin B, and MIC with values 8 µg/mL were verified for nystatin for 94.4% strains (Table 4).

Incubation of *Candida* spp. colonies for 7 days at 37°C on BSA agar showed that out of 36 *Candida* spp. strains obtained from women suffering from different vulvovaginal infections, 11 strains had very high protease activity ($Pz < 0.39$), 24 presented moderate activity ($0.69 > Pz < 0.40$), and 1 had low activity ($Pz > 0.69$) as shown in Table 5.

For phospholipase activity, out of the 36 tested strains, 9 (7 strains of *C. albicans* and 2 strains of *C. glabrata*) were moderately positive, 20 (19 strains of *C. albicans* and 1 of *C. glabrata*), had low activity, while 7 strains (one *C. albicans*, two *C. glabrata* and four *C. tropicalis*) were negative (Table 5).

Table 4. Antifungal susceptibility profile (µg/mL) of *Candida* species isolated from vulvovaginal candidiasis.

Species (No. de isolates)	Antifungal agent	MIC (µg/mL)		Susceptible	Resistant
		Range	GM	n (%)	n (%)
All species (36)	Fluconazole	0.12-64	4.75	22 (61.1)	14 (38.9)
	Itraconazole	0.12-16	1.88	17 (47.2)	19 (52.8)
	Clotrimazole	0.03-1.0	0.06	31 (86.1)	5 (13.9)
	Amphotericin B	0.12-2.0	0.64	36 (100)	-
	Nystatin	0.06-16	2.11	-	-
C. albicans (27)	Fluconazole	0.12-64	6.03	14 (51.9)	13 (48.1)
	Itraconazole	0.12-16	2.27	10 (37.0)	17 (63.0)
	Clotrimazole	0.03-1.0	0.06	23 (85.2)	4 (14.8)
	Amphotericin B	0.25-1.0	0.54	27 (100)	-
	Nystatin	0.06-8.0	1.70	-	-
C. glabrata (5)	Fluconazole	0.5-4.0	1.74	5 (100)	-
	Itraconazole	1.0-4.0	1.51	5 (100)	-
	Clotrimazole	0.03-0.5	0.07	4 (80.0)	1 (20.0)
	Amphotericin B	0.5-1.0	0.25	5 (100)	-
	Nystatin	1.0-16	2.29	-	-
C. tropicalis (4)	Fluconazole	1.0-32	3.36	3 (75.0)	1 (25.0)
	Itraconazole	0.5-1.0	0.70	2 (50.0)	2 (50.0)
	Clotrimazole	0.03-0.5	0.07	4(100)	-
	Amphotericin B	1.0-2.0	1.4	4 (100)	-
	Nystatin	4.0-16	8	-	-

MIC: Minimal inhibitory concentration; GM: Geometric mean

The study of the hemolytic activity of vaginal *Candida* spp. isolates showed that after 48h of incubation in sheep blood agar, all strains degraded hemoglobin by erythrocyte lysis. Two strains presented a very high hemolysin activity and 34 showed moderate activity (Table 5).

Table 5. Enzymatic activity of *Candida* species isolated from the vaginal mucosa of women attended at the Hospital das Clínicas in Goiânia-GO.

Enzymatic activity	*C. albicans* (n=27) n (%)	*C. glabrata* (n=5) n (%)	*C. tropicalis* (n=4) n (%)	Total (n=36) n (%)
Proteinase				
High	6 (22.2)	4 (80.0)	1 (25.0)	11 (30.6)
Moderate	21 (77.8)	-	3 (75.0)	24 (66.7)
Low	-	1 (20.0)	-	1 (2.8)
Nevative	-	-	-	
Phospholipase				
High	-	-	-	
Moderate	7 (25.9)	2 (40.0)	-	9 (25.0)
Low	19 (70.4)	1 (20.0)	-	20 (55.6)
Negative	1 (3.7)	2 (40.0)	4 (100.0)	7 (19.4)
Hemolysin				
High	1 (3.7)	1 (20.0)	-	2 (5.6)
Moderate	26 (96.3)	4 (80.0)	4 (100.0)	34 (94.4)
Low	-	-	-	-
Negative	-	-	-	-

Pz < 0.39 = high activity; Pz between 0.40 and 0.69 = moderate activity;
Pz between 0.70 and 0.99 = low activity; Pz=1.0 = negative.

DISCUSSION

Candidiasis is a major vulvovaginal infection that mainly affects women of reproductive age at least once in their life span. The present study revealed vaginal disorders in women in their second and third decades. This group is within the reproductive age, which is considered a risk factor for VVC due to sexual activity.

In the present work, we verified a PPV of 61.8% when observing such signs and symptoms as vaginal pruritus, leucorrhea, dysuria, dyspareunia, and burning presented by women in comparison with laboratory cultures, the diagnostic gold standard for VVC. Some authors have verified different PPV

values. Farhan et al. (2017), found a PPV of 76.2% in women with vaginal disorders seen in Egypt, Vijayalakshmi et al. (2016), in the city of Pune, India, verified a PPV of 88.9%, however in Brazil, Rosa and Rumel (2004), verified a PPV of 43%. The World Health Organization recommends empirical treatment for women with abnormal vaginal discharge. However, signs and symptoms noted for clinical diagnosis are nonspecific and may be related to other vaginal infections, therefore microbiological diagnosis is the best method and avoids many women receiving antifungal therapy unnecessarily. Although all 55 women included in this work received antifungal therapy, 21 (38.2%) were negative culture for *Candida* spp. Antifungal therapy is associated with renal and hepatic complications and hypersensitivity reactions, in addition to increasing rates of microbial resistance (Sobel, 2013).

Although azole derivatives have been used to treat and prevent VVC, *in vitro* studies have shown *Candida* spp. resistance to these drugs. In our work, high resistance to fluconazole and itraconazole was detected. Brandolt et al. (2017) in the State of Rio Grande do Sul, Brazil, in 2013, reported resistance in 42% of the isolates to fluconazole, and in 48% to itraconazole. Kandeel and Elmitwalli (2017), reported resistance to fluconazole in 17.3% of *Candida* spp. isolates in Saudi Arabia. The *in vitro* susceptibility of an infecting organism to the antifungal agent plays an important part in determining successful therapy, once high MIC values may be associated with resistance, indicating the need for different treatment (Pfaller & Diekema, 2012). In this study, many participants (32.7%) received fluconazole treatment despite having negative cultures. Thus, the high rates of resistance to these azole derivatives demonstrated in the present study are worrying. Unfortunately, we did not follow these patients.

Resistance to antifungals, especially azole derivatives, is associated with indiscriminate use. Antifungals are widely applied in agriculture, in antifouling coatings, in wood preservation, in addition to being routinely used in empirical therapy and human prophylaxis (Sanguinetti et al., 2015; Fisher et al., 2018).

C. albicans resistance to clotrimazole has not been previously well documented. In our study, the isolates presented high susceptibility to this drug, with five resistant isolates. In cases of fluconazole-resistant strains, topical clotrimazole is an effective therapeutic option. According to Kasper et al. (2015), clotrimazole ovule application may lead to a rise in vaginal lactate levels and may affect growth and membrane integrity of the infecting *C. albicans* cells.

Taking values of MIC > 2 μg/ml as resistant, no isolates were resistant to amphotericin B. This high susceptibility to Amphotericin B may be explained by the fact that this drug is not a choice in the empirical treatment for VVC. Resistance to polyenic agents such as amphotericin B is not seen as a clinical difficulty, few cases of species resistant to this antifungal are reported (Sanguinetti et al., 2015).

The susceptibility parameters of *Candida* species associated with VVC to nystatin are not established. According to Consolaro et al. (2012), nystatin has excellent *in vivo* activity against *C. albicans*, but low success rates against non-albicans species. In this study, we found low MIC values for nystatin for all *C. albicans* strains, but values of 16 µg/mL for two non-albicans species. This drug has been used as one of the main topical treatments of VVC in Brazil; it is available in the Brazilian Unified Health System (SUS) and is also a relatively safe option for treating pregnant or lactating patients (Consolaro et al., 2012; Brandolt et al., 2017).

The identification of *Candida* species is relevant not only to compose epidemiological data, but also to aid therapeutic response. Phenotypic methods are not always accurate, in addition to requiring a long time for species identification, while genotypic tests have high specificity and are less time consuming (ElFeky et al., 2016; Bonyadpour et al., 2016; Nurat et al, 2016; Devadas, 2017).

The production of hydrolytic enzymes is an important virulence factor for establishing infection. Proteinases assist in the invasion of host tissues and the protection of yeasts against antifungal action and the host's immune system (Rapala-Kozik et al., 2018), whereas phospholipases are related to membrane rupture at the time of host cell invasion, allowing the hyphal tip to enter the cytoplasm (Kantarciog & Yücel, 2002). The findings obtained in the present study demonstrated that all *Candida* spp. exhibited considerable proteinase activity. Similarly, Shirkhani et al. (2016) verified that the *Candida* spp. isolates from VVC exhibited high proteinase activity. In contrast, we found that 25% of *C. albicans* isolates had moderate phospholipase activity and no *C. tropicalis* isolates produced this enzyme. Shirkhani et al. (2016) demonstrated low phospholipase activities in *Candida* spp. isolates from VVC.

Although the hemolytic activity of *Candida* species has been poorly reported, the ability of these yeasts to use iron derived from hemoglobin from the production of erythrocyte lysis is associated with *Candida* spp. resistance (Figueiredo-Carvalho et al., 2017). In addition, iron depletion in *Candida albicans* enhances its sensitivity to several drugs, such as fluconazole, facilitating the drug's fluidity and diffusion, rendering fungal cells susceptible (Figueiredo-Carvalho et al., 2017). The knowledge of the role of these enzymes in establishing the infection is relevant as they present a promising target for treating fungal infections caused mainly by resistant species of *Candida* (Figueiredo-Carvalho et al., 2017; Rapala-Kozik et al., 2018).

This study presents limitations as the samples of vaginal secretion from women who complained of vaginal discharge and itching as their main symptoms were insufficient to establish the prevalence of candidiasis in the entire female patient population of the Gynecology and Obstetrics Outpatient Clinic. Although strains resistant to fluconazole were detected in the present study and this was the main antifungal agent used in treatment, we did not

perform the second collection to evaluate treatment progression. Each individual has peculiarities that interfere with therapy.

In conclusion, the results of this study showed *C. albicans* to be the most common species as the cause of VVC. The clinical diagnosis of VVC presented a moderate PPV, which highlights the need for laboratory confirmation by culture of *Candida* spp. isolates, which are important for the reliable diagnosis of VVC. The high resistance observed to azole derivative fluconazole and itraconazole indicates that the *in vitro* antifungal susceptibility test must be performed before treatment is prescribed.

CONFLICTS OF INTEREST

The authors have no conflicts of interest relative to this article.

REFERENCES

1. Aniebue UU, Nwankwo TO, Nwafor MI. Vulvovaginal candidiasis in reproductive age women in Enugu Nigeria, clinical versus laboratory-assisted diagnosis. *Niger J Clin Pract 21*: 1017-1022, 2018.
2. Bitew A, Abebaw Y. Vulvovaginal candidiasis: species distribution of *Candida* and their antifungal susceptibility pattern. *BMC Womens Health 18*: 94, 2018.
3. Bonyadpour B, Akbarzadeh M, Mohagheghzadeh A. A Descriptive Study on the Prevalence of Vulvovaginal Infections and Species-specific Distribution of Vulvovaginal Candidiasis in Married Women of the South of Iran. *J Midwifery Reprod Heal 4*: 741-747, 2016.
4. Brandolt TM, Klafke GB, Gonçalves CV, Bitencourt LR, Martinez AMB, Mendes JF, Meireles MCA, Xavier MO. Prevalence of *Candida* spp. in cervical-vaginal samples and the in vitro susceptibility of isolates. *Brazilian J Microbiol 48*: 145-150, 2017.
5. Buyukbayrak EE, Kars B, Karsidag AYK, Karadeniz BI, Kaymaz O, Gencer S, Pirimoglu ZM, Unal O, Turan MC. Diagnosis of vulvovaginitis: comparison of clinical and microbiological diagnosis. *Arch Gynecol Obstet 282*: 515-519, 2010.
6. Casali AK, Goulart L, Rosa e Silva LK, Ribeiro AM, Amaral AA, Alves SH, Schrank A, Meyer W, Vainstein MH. Molecular typing of clinical and environmental *Cryptococcus neoformans* isolates in the Brazilian state Rio Grande do Sul. *FEMS Yeast Res 3*: 405-415, 2003.
7. CLSI. *Reference method for broth dilution antifungal susceptibility testing of yeasts;* third edition; CLSI document M27-A3. Clinical and Laboratory Standards Institute: Wayne, 2008. 3-13p.
8. CLSI. *Epidemiological Cutoff Values for Antifungal Susceptibility Testing* This document includes the epidemiological cutoff value and SA, CLSI document M-59. Clinical and Laboratory Standards Institute: Wayne, 2016. 3-13p.
9. Consolaro M, Martins H, Silva M, Paiva L, Svidzinski T. Efficacy of Fluconazole and Nystatin in the Treatment of Vaginal *Candida* Species. *Acta Derm Venereol 92*: 78-82, 2012.
10. Devadas SM. Auxanographic Carbohydrate Assimilation Method for Large Scale Yeast Identification. *J Clin Diagnostic Res 11*: 10-12, 2017.
11. ElFeky DS, Gohar NM, El-Seidi EA, Ezzat MM, AboElew SH. Species identification and antifungal susceptibility pattern of *Candida* isolates in cases of vulvovaginal candidiasis. *Alexandria J Med 52:* 269-277, 2016.

12. Farhan A, Eldesouky E, Gaballah E, Soltan M. Comparison of visual, clinical, and microbiological diagnosis of symptomatic vaginal discharge in the reproductive age group. *Benha Med J 34*: 43, 2017.

13. Figueiredo-Carvalho MHG, Ramos LDS, Barbedo LS, Oliveira JCA, Santos ALS Dos, Almeida-Paes R, Zancopé-Oliveira RM. Relationship between the Antifungal Susceptibility Profile and the Production of Virulence-Related Hydrolytic Enzymes in Brazilian Clinical Strains of *Candida glabrata. Mediators Inflamm 2017*: 1-11, 2017.

14. Fisher MC, Hawkins NJ, Sanglard D, Gurr SJ. Health and Food Security - TC Local. *Science 742:* 739-742, 2018.

15. Foxman B, Muraglia RB, Dietz J-P, Sobel J, Wagner J. Prevalence of recurrent vulvovaginal candidiasis in 5 European countries and the United States: results from an internet panel survey. *J Low Genit Tract Dis 17:* 340-345, 2013.

16. Ghaddar N, Roz A El, Ghssein G, Ibrahim JN. Emergence of Vulvovaginal Candidiasis among Lebanese Pregnant Women: Prevalence, Risk Factors, and Species Distribution. *Infect Dis Obstet Gynecol 2019*: 1-8, 2019.

17. Kandeel A, Elmitwalli A. Prevalence, Risk Factors and Antifungal Susceptibility of Vulvovaginal Candadiasis among Saudi Females. *Egypt J Med Microbiol 26*: 47-52, 2017.

18. Kantarciog AS., Yücel A. Phospholipase and protease activities in clinical *Candida* isolates with reference to the sources of strains. *Mycoses 45*: 160-165, 2002.

19. Kasper L, Miramón P, Jablonowski N, Wisgott S, Wilson D, Brunke S, Hube B. Antifungal activity of clotrimazole against *Candida albicans* depends on carbon sources, growth phase and morphology. *J Med Microbiol 64*: 714-723, 2015.

20. Kurtzman CP, Fell JW, Boekhout T, Robert V. *Methods for Isolation, Phenotypic Characterization and Maintenance of Yeasts*. The Yeasts: Elsevier, 2011. p.87-110.

21. Manns JM, Mosser DM, Buckley HR. Production of a hemolytic factor by *Candida albicans. Infect Immun 62:* 5154-5156, 1994.

22. Martínez JMG, Gómez EV, Pemán J, Cantón E, García MG, Castillo Agudo L Del. Identification of pathogenic yeast species by polymerase chain reaction amplification of the RPS0 gene intron fragment. *J Appl Microbiol 108*: 1917-1927, 2010.

23. Mendling W. Guideline: Vulvovaginal Candidosis (AWMF 015/072), S2k (excluding chronic mucocutaneous candidosis). *Mycoses 58*: 1-15, 2015.

24. Ministério da Sáude. *Protocolo Clínico e Diretrizes Terapêuticas para Atenção Integral às Pessoas com Infecções Sexualmente Transmissíveis.* Brasilia-DF. 2015. Available in: http://bvsms.saude.gov.br/bvs/publicacoes/protocolo_clinico_diretrizes_terapeutica_atencao_integral_pessoas_infeccoes_sexualmente_transmissiveis.pdf/. Accessed in 13/08/2017.

25. Nurat AA, Ola BG, Olushola SM, Mikhail TA, Ayodeji AS. Molecular and phenotypic identification of *Candida* isolates from pregnant women in Ogbomoso, Southwestern Nigeria. *Int J Reprod Contraception Obstet Gynecol 5*: 317-322, 2016.

26. Pelletier R, Peter J, Antin C, Gonzalez C, Wood L, Walsh TJ. Emergence of resistance of *Candida albicans* to clotrimazole in human immunodeficiency virus-infected children: *in vitro* and clinical correlations. *J Clin Microbiol 38*: 1563-1568, 2000.

27. Pfaller MA, Diekema DJ. Progress in antifungal susceptibility testing of *Candida* spp. by use of Clinical and Laboratory Standards Institute broth microdilution methods, 2010 to 2012. *J Clin Microbiol 50:* 2846-2856, 2012.

28. Price MF, Wilkinson AND, Gentry LO. Plate method for detection of phospholipase activity in *Candida albicans. Sabouraudia 20*:7-14, 1982.

29. Ramos LS, Barbedo LS, Braga-Silva LA, Santos ALS, Pinto MR, Sgarbi DBG. Protease and phospholipase activities of *Candida* spp. isolated from cutaneous candidiasis. *Rev Iberoam Micol 32*: 122-125, 2015.

30. Rapala-Kozik M, Bochenska O, Zajac D, Karkowska-Kuleta J, Gogol M, Zawrotniak M, Kozik A. Extracellular proteinases of *Candida* species pathogenic yeasts. *Mol Oral Microbiol 33*: 113-124, 2018.

31. Rosa MID, Rumel D. Fatores associados à candidíase vulvovaginal: estudo exploratório. *Rev Bras Ginecol Obs 26*: 65-70, 2004.

32. Ruchel R, Tegeler R, Trost M. A comparison of secretory proteinases from different strains of *Candida albicans*. *Int Soc Hum Anim Mycol 20*: 233-244, 1982.

33. Sanguinetti M, Posteraro B, Lass-Flörl C. Antifungal drug resistance among *Candida* species: mechanisms and clinical impact. *Mycoses 58*: 2-13, 2015.

34. Shirkhani S, Sepahvand A, Mirzaee M, Anbari K. Phospholipase and proteinase activities of *Candida* spp . isolates from vulvovaginitis in Iran. *J Mycol Med 26*: 255-260, 2016.

35. Sobel JD. Factors involved in patient choice of oral or vaginal treatment for vulvovaginal candidiasis. *Patient Prefer Adherence 8*: 31-34, 2013.

36. Sobel JD, Sobel R. Current treatment options for vulvovaginal candidiasis caused by azole-resistant *Candida* species. *Expert Opin Pharmacother 19*: 971-977, 2018.

37. Sobel JD, Subramanian C, Foxman B, Fairfax M, Gygax SE. Mixed vaginitis - More than coinfection and with therapeutic implications. *Curr Infect Dis Rep 15*: 104-108, 2013.

38. Vijayalakshmi D, Sunil SP, Sambarey PW. Clinical And Microscopic Correlation of Vaginal Discharge. *Int J Contemp Med Res 3*: 1328-1331, 2016.

39. Wölber L, Prieske K, Mendling W, Schmalfeldt B, Tietz H-J, Jaeger A. Vulval pruritus-causes, diagnosis and therapeutic approach. *Dtsch Aerzteblatt Online 116*: 126-133, 2020.