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Candida species cause vulvovaginitis and resistance to antifungals used for treatment

Espécies de Candida causadoras de vulvovaginites e resistência aos antifúngicos utilizados no tratamento

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ABSTRACT
In this study were identified *Candida* species from vaginal secretion isolates, evaluated their *in vitro* antifungal susceptibilities, and correlated these features with antifungal agents prescribed for patients assisted in a primary care service. Species identification by Polymerase Chain Reaction showed that 36.5% of isolates were characterized as non-*C. albicans* species. In antifungal susceptibility tests most isolates were susceptible to ketoconazole, fluconazole, and itraconazole, although between 40% and 50% of isolates show resistance or dose-dependent susceptibility to miconazole and nystatin, respectively. Analysis of drugs prescribed to patients revealed that 34.2% of the isolates were considered resistant to agents used in treatment. Several *Candida* species can cause vulvovaginitis and exhibit different susceptibility profiles to antifungal drugs used in treatment. The identification of *Candida* species is relevant and useful to the epidemiological management of infections. The antifungal susceptibility test may also be useful for choosing most effective drug treatment for each patient.

Keywords: *Candida* spp. Microbial sensitivity tests. Polymerase chain reaction. Vulvovaginal candidiasis.

RESUMO
Neste estudo foram identificadas espécies de *Candida* em isolados de secreção vaginal, avaliados os perfis de suscetibilidade *in vitro* a antifúngicos e correlacionados com os antifúngicos prescritos para pacientes em um serviço de atenção primária. A identificação das espécies pela Reação em Cadeia da Polimerase mostrou que 36,5% dos isolados foram caracterizados como espécie não-*C.albicans*. Nos testes de sensibilidade a maioria dos isolados foi suscetível a cetoconazol, fluconazol e itraconazol, contudo cerca de 40% e 50% apresentaram resistência ou sensibilidade dose-dependente a miconazol e nistatina, respectivamente. A análise dos fármacos prescritos para as pacientes revelou que 34,2% dos isolados foram considerados resistentes aos agentes utilizados no tratamento. Diversas espécies de *Candida* podem causar vulvovaginite com variados perfis de suscetibilidade aos antifúngicos comumente utilizados no tratamento. A identificação das espécies de *Candida* é relevante para o gerenciamento epidemiológico das infecções, além de ser útil, assim como os testes de suscetibilidade, na escolha do tratamento farmacológico mais eficaz para a paciente.

Palavras-chave: *Candida spp.* Candidíase vulvovaginal. Reação em cadeia da polimerase. Testes de sensibilidade microbiana.

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INTRODUCTION

Vulvovaginal candidiasis (VVC) is a significant health problem, affecting nearly two-thirds of adult women during their lifetime, among whom approximately 50% experience further episodes [1,2]. Abnormal growth of *Candida* yeasts in the mucosa of the female genital tract causes pruritus, erythema, edema, and vaginal discharge[1]. Although this yeast is considered a member of vaginal microbiota, some conditions may upset the microbial balance in the genital tract and lead to an overgrowth of *Candida* species. Conditions such as Diabetes mellitus [3], use of oral contraceptives, and pregnancy [4] increase glycogen levels in vaginal mucosa, and the consequent drop in pH favors the development of site infection. Glycogen excess increases nutrient substrate and promotes increased adhesion ability of the pathogen [5]. Other risk factors for developing VVC include inadequate genital hygiene practice and the use of antibiotics, corticosteroids, tight-fitting clothing, and synthetic fabric underwear [6].

Prevalence studies of VVC indicate *Candida albicans* is the most frequent isolated species, accounting for 80 to 90% of all VVC cases [1,7]. However, episodes due to *non-C.albicans* species, with variable pathogenicity and antifungal susceptibility profile, have been increasing[1, 8, 9]. These VVC-causing *non-C.albicans* species include *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. parapsilosis*, indicating a trend for a change in the etiology of candidiasis after decades of *C. albicans* dominance [3,8,10]. Pharmacological treatment of patients with uncomplicated VVC involves the use of imidazole and triazole antifungal agents, including fluconazole, ketoconazole, miconazole, itraconazole, and clotrimazole, in addition to the polyene agent nystatin. These drugs are effective in controlling candidiasis from synthesizing ergosterol in the fungal cell membrane [7, 10]. However, the increased use of antifungal drugs and prolonged treatment without medical supervision are risk factors for the emergence of *Candida* species resistant to these drugs[1, 11].

Considering the increased diversity of *Candida* species related to VVC episodes and their various antifungal susceptibility profiles, in this study we identified, by species-specific Polymerase Chain Reaction (PCR), the *Candida* species distribution in vaginal secretions of women referred to a public health service in southern Brazil. Furthermore, we verified the *in vitro* susceptibility profile of vaginal isolates to antifungal agents generally used in VVC treatment, considering risk factors and drug prescriptions of the patients.

METHODOLOGY

POPULATION AND SAMPLING

The cross-sectional study was conducted with 180 patients assisted at the Municipal Laboratory of Clinical Analysis

and Environmental, Chapecó, SC, Brazil, from February to April 2012. The sample was obtained by spontaneous demand from patients referred by doctors or nurses in primary health care units to submit laboratory tests of vaginal content. All participants were informed about the study and signed an informed consent form. Patients were excluded from the study if they were younger than 18 years old, were not a resident in the county, and/or used any antimicrobial agent.

BIOLOGICAL SAMPLES AND DATA COLLECTION

Information was obtained from patients with a questionnaire that included open and closed questions covering socio-demographics, clinical, and epidemiological aspects and risk factors for VVC, such as contraceptive method, pregnancy, childbearing, post-defecation wiping practice, and use of tight clothing/synthetic underwear.

Vaginal fluid was collected using a vaginal swab that was immediately incubated in Stuart transport solution (Citotest Labware Manufacturing Co., Ltd., Haimen City, China) and sent to the laboratory. The isolates were plated on Sabouraud dextrose agar (Difco, Detroit, Mich, USA) supplemented with chloramphenicol and incubated at 35 ° C for 48 hours.

MOLECULAR IDENTIFICATION OF C*ANDIDA* SPECIES BY PCR

Genomic DNA was extracted from cultures according to the procedure described by Alves (2010). Genomic DNA was extracted from ATCC strains of *Candida* species analyzed in this study: *C. glabrata* (ATCC 2001), *C. krusei* (ATCC 6258), *C. tropicalis* (ATCC 750), *C. parapsilosis* (ATCC 90018), *C. albicans* (ATCC 24433), and *C. guilliermondii* (ATCC 90877).

The identification of the species *C. albicans, C. glabrata, C. tropicalis, C. krusei, C. parapsilosis,* and *C. guilliermondii* was performed by PCR with forward species-specific primer for the internal transcribed spacer (ITS) 1 and 2 and the reverse primer ITS4 as follows: ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'), CAL (*C. albicans*, 5'-TCAACT TGTCACACCAGATTATT-3') [12], CGL (*C. glabrata*, 5'-CAC GAC GCT ACA CTT TCT AAT T-3') [13], CTR (*C. tropicalis*, 5'-CAATCCTACCGCCAGAGGTTAT-3') [12], CKR (*C. krusei*, 5'-ACTACACTGCGTGAGCGG AA-3') [14], CPA (*C. parapsilosis*, 5'-TTG GTA GGC CTT CTA TAT GGG-3') [14] and CGU (*C. guilliermondii*, 5' - GTATTGGCATGGGTAGTACTG-3') [12]. PCR assays generated amplification products of 402 bp for *C. albicans*, 632 bp for *C. glabrata*, 434 bp for *C. tropicalis*, 464 bp for *C. krusei*, 424 bp for *C. parapsilosis*, and 185 bp for *C. guilliermondii*. PCR mixes were prepared in

a volume of 20 µL containing 20 ηg of genomic DNA, 0.2 mM dNTPs, 2.5 mM MgCl2, 10 pmol of each primer, 2.0 µL of 10x Reaction Buffer, and 1.0 U of Taq Platinum DNA polymerase (Invitrogen, Carlsbad, CA, USA). Positive controls consisted of genomic DNA extracted from ATCC strains of *Candida* species identified in this work. Negative controls were prepared by replacing DNA with water, and with DNA from different species of the species-specific primers used in the reaction. The reaction mixtures were incubated in MJ-96 thermocycler (Biocycler, Applied Biosystems, Foster City, USA) at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min (annealing), 72°C for 1 minute, and a final extension at 72°C for 5 min. The annealing temperature used for *C. parapsilosis* and *C. albicans* was 60°C. The amplified DNA fragments were analyzed on 2% agarose gel stained with 0.5 µg/mL ethidium bromide and visualized using a UV documentation system (Vilber Loumart, Marne-LaVallée, France).

EVALUATION OF *in vitro* SUSCEPTIBILITY TO ANTIFUNGAL AGENTS

Antifungal susceptibility of *Candida* spp. isolates was tested by disk diffusion method in Muller Hinton Agar, according to document M44-A [15] and following the manufacturer's instructions. Susceptibility tests were performed using discs of nystatin (100 IU), fluconazole (50 mg), miconazole (50 mg), and ketoconazole (50 mg), which were acquired from Bio-Rad Laboratories (Marnes La Coquette, France), and itraconazole (10 mg), which was acquired from CECON (Center for Diagnostics and Control Products, Sao Paulo, Brazil). Resistance, susceptibility, and dose-dependent susceptibility parameters were defined by measuring the inhibition zone around the discs. The criteria used for interpretation of the data obtained for fluconazole were those proposed by the CLSI, whereas for the other antifungals, for which there are no standardized methods, the criteria suggested by the manufacturers were used as described in table 1.

Table 1. Criteria for interpreting antifungigram results according to the M44-A manual and the disc manufacturer's manuals (Cecon, Bio-Rad Laboratories)

Antifungal	Susceptible	Susceptible dose-dependent	Resistant
Fluconazole (*)	>19 mm	14-19 mm	<14 mm
Ketoconazole (***)	>20 mm	10-20 mm	<10 mm
Itraconazole (**)	≥20 mm	12-19 mm	≤11 mm
Miconazole (***)	>20 mm	10-20 mm	<10 mm
Nystatin (**)	>10 mm	----	≤10 mm

(*) MA44-A; (**) Cecon; (***) Bio Rad

DRUG PRESCRIPTION ANALYSIS

Information regarding antifungal drugs prescribed to patients in this study was obtained from the database using the Winsaúde program. Information collection was permitted by the patient and authorized by the Municipal Health Service.

STATISTICAL ANALYSIS

Information obtained from the structured questionnaire was analyzed using Epi-info 3.5.2 (Center for Disease Control and Prevention, Atlanta, GA, USA), and the magnitude of the association between variables and the presence of VVC was estimated by odds ratio (OR) with a confidence interval (CI) of 95%. A p-value < 0.05 was considered statistically significant.

ETHICS

This study was approved by the Ethics Committee of the Universidade Comunitária da Região de Chapecó (protocol n° 140/11).

RESULTS

SOCIO-DEMOGRAPHIC CHARACTERISTICS, RISK FACTORS, AND SYMPTOMATOLOGY OF PATIENTS

Among the 180 samples of vaginal secretions obtained, 52 (28.9%) samples showed yeast growth in the selective culture medium and, thus, were used in further analyses. These 52 samples came from women between 18 and 52 years of age, with a modal and mean age of 18 and 27.25 years, respectively. The education level of most women was first grade and over sixty percent of them were married or cohabitating.

There were significant correlations between the presence of *Candida* spp. in vaginal culture and various risk factors (Table 2), including pregnancy, childbearing, inadequate post-defecation wiping practices, and the use of tight clothing/synthetic underwear ($p < 0.05$). However, no correlation was observed with the use of oral contraceptives and post-defecation washing practices.

Table 2. Distribution of patients with positive culture (n = 52) and their relationship to the risk factors under analysis

Risk factor	***Category***	**n**	**%**	**OR***	**CI****	***p value***
Pregnancy	Yes	18	34,6	1,98	0,97 - 4,03	0,04
	No	34	65,4			
Use of oral contraceptives	Yes	22	42,3	1,44	0,74 – 2,8	0,17
	No	30	57,7			
Childbearing age	Yes	50	96,2	3,31	0,73 – 9,78	0,03
	No	2	3,8			
Use of tight clothing/synthetic underwear	Yes	41	78,8	2,45	0,95 – 6,28	0,04
	No	11	21,2			
Post-defecation wiping practice	Anus-Vagina	18	34,4	1,87	0,94 – 3,72	0,04
	Vagina-Anus	34	65,4			
Post-defecation washing practice	Yes	19	36,5	1,25	0,63 – 2,44	0,31
	No	33	63,5			

*OR: odds ratio

**CI: confidence interval

The clinical symptoms that prevailed among patients were leucorrhea (71.2%), followed by pruritus (55.8%), dyspareunia (25.0%), dysuria (23.1%), erythema (23.1%) and edema (7.7%). Occurrence of vaginal discharge and itching were associated with positive culture results ($p < 0.05$), and patients who reported these symptoms showed 3.08 and 2.46, respectively, higher risk of having VVC. It should be emphasized, however, that the presence of signs and symptoms were reported by patients and that differences may arise if clinical examination were performed by a professional.

Candida SPECIES DISTRIBUTION OF VAGINAL ISOLATES

Identification of *Candida* species performed by species-specific PCR found *C. albicans* in 63.5% (33/52) of the isolates and non-*C. albicans* species were distributed as follows: 13.5% *C. glabrata* (7/52), 7.7% *C. tropicalis* (4/52), 7.7% *C. parapsilosis* (4/52), 5.7% *C. krusei* (3/52), and 1.9% *C. guilliermondii* (1/52). Only one vaginal sample tested positive for more than one species, *C. tropicalis* and *C. krusei*. Due to the inability to individually analyze each species, this sample was not analyzed in susceptibility tests.

In vitro ANTIFUNGAL SUSCEPTIBILITY

Among the antifungal agents analyzed, it was observed that most *Candida* isolates were sensitive to ketoconazole and more than 70% showed susceptibility to fluconazole, itraconazole, and miconazole (Figure 1). However, it was found in the miconazole and nystatin *in vitro* tests that 37% and about half of the isolates, respectively, showed resistance or dose-dependent susceptibility.

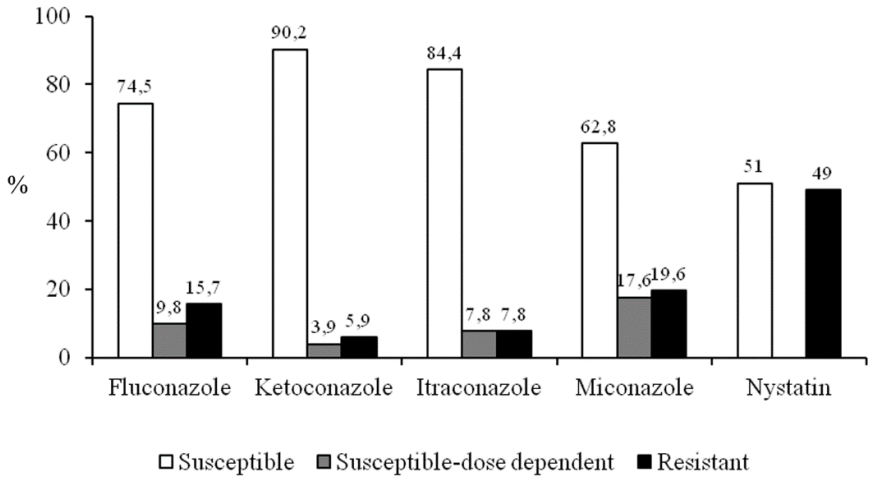

Figure 1. Susceptibility profile of *Candida* spp. isolates to antifungal agents. Samples were analyzed by CLSI M44-A disk diffusion and classified as susceptible (S), susceptible-dose dependent (SDD) or resistant (R) to every antifungal agent tested.

The percentage of *Candida* species isolates susceptible (S), susceptible-dose dependent (SDD), or resistant (R) compared to the antifungal agents tested is shown in Table 3. Most of the *C. albicans* species were susceptible to azole drugs, as follows: ketoconazole (94%), itraconazole (91%), fluconazole (84.8%), and miconazole (72.8%). Half of the isolates characterized as *C. krusei* showed resistance to ketoconazole, itraconazole, and miconazole, and all *C. krusei* isolates were resistant to fluconazole and nystatin. Considering all the antifungals tested, isolates showed the highest resistance to nystatin, as follows: *C. krusei* (100%), *C. guilliermondii* (100%), *C. tropicalis* (66.7%), *C. parapsilosis* (50.0%), *C. albicans* (45.5%), and *C. glabrata* (28.6%). Several isolates were also resistant to miconazole: *C. tropicalis* (66.7%), *C. krusei* (50%), *C. parapsilosis* (25%), *C. albicans* (15.1%), and *C. glabrata* (14.3%).

Table 3. *In vitro* susceptibilities of *Candida* species to antifungal agents

***Candida* species**	**Antifungal agent**	**Susceptibility *in vitro* profile***		
		S (%)	**SDD (%)**	**R (%)**
Candida albicans (n=33)	Fluconazole	84,8	9,1	6,1
	Ketoconazole	94,0	3,0	3,0
	Itraconazole	91,0	3,0	6,0
	Miconazole	72,8	12,1	15,1
	Nystatin	54,5	-	45,5
Candida tropicalis (n=3)	Fluconazole	66,7	-	33,3
	Ketoconazole	100,0	-	-
	Itraconazole	66,7	33,3	-
	Miconazole	33,3	-	66,7
	Nystatin	33,3	-	66,7
Candida glabrata (n=7)	Fluconazole	57,1	14,3	28,6
	Ketoconazole	71,4	14,3	14,3
	Itraconazole	71,4	14,3	14,3
	Miconazole	57,1	28,6	14,3
	Nystatin	71,4	-	28,6
Candida krusei (n=2)	Fluconazole	-	-	100,0
	Ketoconazole	50,0	-	50,0
	Itraconazole	-	50,0	50,0
	Miconazole	-	50,0	50,0
	Nystatin	-	-	100,0
Candida parapsilosis (n=4)	Fluconazole	100,0	-	-
	Ketoconazole	100,0	-	-
	Itraconazole	100,0	-	-
	Miconazole	50,0	25,0	25,0
	Nystatin	50,0	-	50,0
Candida guilliermondii (n=1)	Fluconazole	-	100,0	-
	Ketoconazole	100,0	-	-
	Itraconazole	100,0	-	-
	Miconazole	-	100,0	-
	Nystatin	-	-	100,0

*S: susceptible; SDD: susceptible-dose dependent; R: resistant

CORRELATION BETWEEN THE ANTIFUNGAL RESISTANCE PROFILE OF ISOLATES AND THE DRUG PRESCRIPTION OF PATIENTS

Of the 52 patients evaluated in this study, only 41 (78.8%) had received medication for vulvovaginitis treatment, and of these, 6 patients (14.6%) received combination therapy, namely a drug for oral use accompanied by a topical vaginal cream. Reviewing medication prescriptions, it was found that the antifungal drug most commonly prescribed was fluconazole (46.3%), followed by nystatin (29.3%), itraconazole (21.9%), miconazole (12.2%), and ketoconazole (4.9%). Of the 35 patients who received a single antifungal drug for the treatment of VVC, 60.0% (21/35) tested positive for *Candida* isolates susceptible to the drug prescribed, while 2.9% (1/35) were classified as susceptible-dose dependent and 37.1% (13/35) of patient isolates were considered resistant. Evaluating patients who received combination therapy (n = 6), 83.3% (5/6) tested positive for *Candida* isolates susceptible to both drugs prescribed, while 16.7% (1/6) of patients tested positive for isolates resistant to both drugs. In both groups it was observed that 34.2% (14/41) of patient isolates were resistant to the drug prescribed in the basic health services.

DISCUSSION

Approximately three-quarters of childbearing age women suffer from VVC with significant physical and psychological morbidity [1, 4]. In this study the frequency of *Candida* spp. vaginal isolates (28.9%) was close to that found other studies [16, 17], who reported frequency of this yeast in 33.2 and 24.7% of the isolates, respectively.

The assessment of risk factors indicated a relationship between VVC and pregnancy and childbearing age of patients, which may be explained by the higher level of sex hormones in these life phases, causing higher levels of glycogen in the vaginal mucosa, facilitating colonization and fungal germination [4, 18]. Regarding the hygienic habits of patients, the survey revealed that over 70% of isolates with *Candida* spp. were from patients who used tight fitting and/or synthetic fabric underwear. This type of clothing may result in poor aeration of the genitals and increased vaginal moisture, thus contributing to fungal growth. Furthermore, inadequate hygiene habits are also factors related to VVC episodes because they may lead to feces residues near the vagina [6].

Among the species identified in this study, it was found that *C. albicans* remains the most frequently isolated species, as has been observed in other studies [7, 11, 16, 17]. However, it should be noted that non-*C. albicans* species were found in approximately 35% of the samples, suggesting a rise in the prevalence of these

species as also observed by Bitew et al. [8]. It is speculated that the increase in non-*C. albicans* species may be a reflection of increased cultivation and identification being performed even before it is requested by the clinician [16]. However several studies have proposed the possibility of erroneous, inappropriate, or incomplete use of antifungals, which would allow for the elimination of the most sensitive *C. albicans* species, while promulgating the most resistant *non-C. albicans* species [1].

The problem with increased incidence of non-*C. albicans* species is that these species show resistance to the most commonly used drugs, making it difficult to control infection in these patients. Isolates of *C. glabrata* and *C. tropicalis* are often resistant to drugs or require larger doses of azoles to allow therapeutic success [10, 19]. The evaluation of antifungal susceptibility in this work revealed that the isolate of *C. guilliermondii* was resistant to nystatin, while all *C. krusei* isolates were resistant to both fluconazole and nystatin. A resistance of *C. krusei* to fluconazole has already been known, though the precise mechanism is not completely understood [10]. However a different result concerning the antifungal nystatin was reported [20] in that all analyzed isolates of *C. krusei* showed susceptibility or dose-dependent susceptibility.

Our study noted that among the azolic drugs, miconazole showed the worst results with *in vitro* susceptibility observed in only 62.8% (32/51) of *Candida* isolates. The rates of intermediate susceptibility (9,8%) and resistance (15,7%) to fluconazole were also relatively higher. Similar susceptibility profile was reported for 17.2% of samples from 87 vulvovaginal *Candida* isolates [8]. However other studies observed fluconazole resistance in less than 10% of the isolates analyzed [7, 21-23].

It is worth highlighting the results indicating the efficacy of nystatin against the most prevalent species of *Candida*, since it is one of the main options for treatment of VVC. Nystatin is widely used at a low cost in topical formulations with easy access to the target population. Among all drugs included in the survey, the polyene antifungal showed the worst performance with 49.0% (23/51) of isolates resistant to this drug. The low incidence of yeast sensitive to nystatin was also reported [24], which found 51.1% of isolates dose-dependently sensitive to nystatin. In contrast, Liu and Fan [7] described susceptibility of all isolates of *C. albicans* to this antifungal.

Comparing the results obtained in the *in vitro* susceptibility testing and antifungal drugs prescribed for patients, it was found that 34.2% (14/41) of patients had isolates resistant to medicines prescribed for treatment, which suggests that the drug selected has not been able to control the infection. It should be noted that the *in vitro* susceptibility test results do not always accurately reflect what occurs *in vivo* due to physiological variation for each individual, the characteristics of drugs, and the variable behavior of microorganisms in each individual. Another limitation of this study was the analyzed sample of patients

from a single health service, which added to the sample size, limits the generalization of the results obtained. In addition, the longer follow-up of patients and access to clinical data would allow a better assessment of the success or not of antifungal therapy. Nevertheless, studies like this contribute to the understanding of pharmacoepidemiology, since high values of resistance and dose-dependent susceptibility may indicate the need for adjustments in the dosage or in the medication prescribed for the treatment of candidiasis in some patients.

CONCLUSION

In general, for all *Candida* species identified in this study, the resistance rates found were higher than those reported by other authors, which indicates the need to perform antifungal susceptibility testing prior to drug prescription. This may contribute to the reduction in recurrent VVC cases caused by repeated exposure to drugs.

These results reinforce the importance of assessing the susceptibility of vaginal *Candida* infections to antifungal agents, especially in cases of recurrent VVC, since the use of some medications for treating previous infections may contribute to the selection of resistant *Candida* strains. In addition, the identification of the specific *Candida* species causing the infection can also assist in selecting the most effective drug, since some species may exhibit intrinsic resistance to some antimicrobials groups, as previously described in some studies and also demonstrated in this work. Thus, it would be possible to improve the management of patient's antifungal pharmacotherapy, preventing the recurrence of vulvovaginitis.

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