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Vulvovaginal candidiasis in a Flemish patient population

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ABSTRACT

Increased resistance to fluconazole has been reported in oral, oesophageal and urinary *Candida* isolates, but this has not been observed commonly in genital tract isolates. The rate of isolation of *Candida* spp. and their susceptibility to amphotericin B, flucytosine and azoles were determined in a number of clinical practices in the city of Ghent, Belgium. Patients with symptomatic vulvovaginal candidiasis (VVC) were treated with fluconazole, and the mycological and clinical outcomes were evaluated. Isolates were identified as *Candida albicans* (78.6%), *Candida guilliermondii* (17.3%), *Candida glabrata* (2.6%) and *Candida dubliniensis* (1.3%). The rates of mycological and clinical cures were 79.5% and 100%, respectively. Women with recurrent VVC were infected more frequently by non-*albicans Candida* spp., but no association was found between the use of antifungal agents and the presence of non-*albicans* spp. In-vitro resistance to fluconazole was not detected, even among subsequent *Candida* isolates from nine patients for whom mycological cure was not achieved.

Keywords Antifungal agents, *Candida*, fluconazole, susceptibility, vaginitis, vulvovaginal candidiasis

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INTRODUCTION

Vulvovaginal candidiasis (VVC) is a common mucosal infection among immunocompetent, healthy women, and is caused by members of the genus *Candida*, predominantly *Candida albicans* [1]. There is little evidence of a significant increase in the prevalence of fungal vaginitis caused by non-*albicans Candida* spp. [2,3]. In most VVC patients, oral treatment with antifungal agents, e.g., fluconazole, quickly relieves symptoms. Systemic antimycotic therapy could lead to the selection of non-*albicans Candida* strains [4], which are reportedly less sensitive to the antifungal agents used most frequently [5,6]. While the susceptibility of *Candida* blood isolates has remained essentially unchanged during recent years [7,8], increasing resistance to fluconazole has been reported in oral, oesophageal and urinary *Candida* isolates [9–12]. To evaluate the effect of the widespread use of antifungal agents on the occurrence of VVC, it is important to monitor the rate of isolation of vaginal *Candida* spp. and to study their susceptibilities to antimycotic agents.

In the present study, patients with symptomatic VVC from Ghent, Belgium, were treated with fluconazole, and their mycological and clinical outcomes were evaluated. The susceptibility of isolates was assessed using NCCLS, EUCAST and Etest methods in order to evaluate the correlation between in-vitro susceptibility results and outcome, and to ascertain the degree of antifungal resistance among vaginal *Candida* isolates.

MATERIALS AND METHODS

Patients

The study was conducted between September 2002 and September 2003, and included 77 women with a clinical diagnosis of VVC who visited outpatient clinics of the

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Departments of Gynaecology of four hospitals in Ghent, the Gynaecological Centre of Ghent, and a private physician's practice in Ghent. The study was approved by the Ethical Committee of Ghent University Hospital. Only women with clinical signs and symptoms of vaginitis (i.e., presence of vulvar pruritis and vaginal discharge, itching, burning pain or erythema) were included in the study. These patients were examined on two occasions. At the first visit, a vaginal swab was obtained and each patient was prescribed fluconazole 150 mg daily for 1 week. High-risk patients (e.g., those being treated with antibiotics, corticosteroids, immunosuppressive drugs or cytotoxic chemotherapeutic agents, diabetic patients, and patients with recurrent *Candida* infection) received 200 mg daily. Lactating and pregnant women, or women suspected of being pregnant, were excluded from the study. After 1 week, patients were assessed for clinical cure, defined as the resolution of signs and symptoms after treatment, and a second swab was taken. A mycological cure was recorded when the first swab was culture-positive and the second swab was culture-negative. Each physician completed a standardised questionnaire for each patient to determine the history of vulvovaginitis, use of antifungal agents during current or previous episodes of candidiasis, risk-factors (pregnancy, diabetes, immunosuppression, use of contraceptives, corticosteroids or antibiotics), symptoms and cure. None of the women in this study was diabetic or receiving immunosuppressive drugs.

Vaginal samples and identification methods

A sample of the lateral vaginal wall was taken using a sterile plain cotton-tipped swab. The swabs were placed in a transport gel and processed in the laboratory within 24 h. Each swab was extracted in 10 mL of buffered peptone by vortex mixing, and the extracts were then filtered through a 47-mm diameter, 0.45-μm pore size nylon membrane filter (Nylaflo; Gelman Sciences, Ann Arbor, MI, USA). The filters were then incubated on CHROMagar Candida (CHROMagar Co., Paris, France) for 48 h at 37°C. Phenotypic confirmatory tests included germ tube formation, API 20C AUX (bioMérieux Vitek, Durham, NC, USA), morphology on corn-meal agar containing Tween-80 0.5% v/v, latex agglutination (Krusei-Color; Fumouze, Levallois Perret, France), growth at 45°C on Emmons Modified Sabouraud dextrose agar, and trehalose assimilation.

The identity of all strains was also confirmed genotypically by PCR combined with an enzyme immunoassay, as described by Elie *et al.* [13]. Molecular identification of *Candida guilliermondii* was performed at the Centraal bureau voor Schimmelcultures (Utrecht, The Netherlands). DNA was isolated as described by O'Donnell *et al.* [14]. The internal spacer (ITS) region and D1/D2 domains were amplified using primers V9 (5′-TGCGTTGATTACGTCCCTGC) and RLR3R (5′-GGTCCGTGTTTCAAGAC) in 50-μL reaction volumes containing 150 nmol $MgCl_2$, 10 nmol each dNTP, 0.05 nmol each primer, 1 U of DNA polymerase and 1 μL of isolated genomic DNA. PCR conditions comprised 5 min at 94°C, followed by 35 cycles of 45 s at 94°C, 30 s at 52°C and 2 min at 72°C, and a final elongation step for 6 min at 72°C. Amplicons were purified using a GFX PCR DNA purification kit (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). PCR products (10–40 ng) were used in cycle sequencing reactions in a total volume of 10 μL, containing 1 μL of 5× sequencing buffer, 2 μL of BigDye terminator RR mix (PE Biosystems, Nieuwerkerk aan den IJsel, The Netherlands) and 400 nM sequencing primer. The primers used for the ITS1, 5.8S rDNA and ITS2 domains were ITS1 (5′-TCCGTAGGTGAACCTGCGG) and ITS4 (5′-TCCTCCGCTTATTGATATGC); the primers used for the LSU rDNA region were NL1 (5′-GCATATCAATAAGCGGAGGAAAAG) and RLR3R (5′-GGTCCGTGTTTCAAGAC). Purification of these amplicons was performed using the MultiScreen Filtration System (Millipore, Etten-Leur, The Netherlands) in combination with Sephadex G-50 Super fine (Amersham Pharmacia Biotech). Sequencing was performed on an ABI 3700 capillary sequencer (PE Biosystems), with analysis by the Lasergene software package (DNASTAR Inc., Madison, WI, USA).

The identity of *Candida dubliniensis* isolates was also confirmed at the Centro National de Microbiología (Majadahonda, Spain) by DNA sequencing of ITS regions of rDNA. DNA segments comprising the regions ITS1 and ITS2 were amplified with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG) and ITS4 (5′-TCCTCCGCTTATTGATATGC) [15]. Reaction mixtures contained 0.025 nmol each primer, 10 nmol each dNTP, 5 μL of 10× PCR buffer (Applied Biosystems, Madrid, Spain), 2.5 U of *Taq* DNA polymerase (Amplitaq; Applied Biosystems) and 5 ng of DNA, in a final volume of 50 μL. Samples were amplified in a GeneAmp PCR System 2400 (Applied Biosystems) for 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 45 s at 56°C and 2 min at 72°C, and a final extension for 5 min at 72°C. The reaction products were analysed in agarose 1.3% w/v gels. Sequencing was performed using 4 μL of BigDye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems), 1 μL of primers ITS1 and ITS4, and 5 μL of PCR product, in a final volume of 10 μL. Sequences were assembled and edited using the SeqMan II and EditsEq software packages (DNAstar, Inc.). The sequences obtained were analysed by comparison with the nucleotide sequences of *Candida* reference isolates obtained from GenBank (http://www.ncbi.nih.gov/GenBank/).

Susceptibility testing

Standard powders of amphotericin B and flucytosine (Sigma Aldrich Quimica, Madrid, Spain), fluconazole and voriconazole (Pfizer, Madrid, Spain) and itraconazole and ketoconazole (Janssen, Madrid, Spain) were used. MICs and endpoints were determined with the NCCLS and EUCAST reference procedures [16,17]. For amphotericin B, the endpoint with the EUCAST method was defined as the lowest drug concentration that resulted in a reduction in growth of ≥90% compared with that of a drug-free control well. Etests (AB Biodisk, Solna, Sweden) for amphotericin B, flucytosine, fluconazole, itraconazole and voriconazole were provided by Izasa (Madrid, Spain). Susceptibility testing, reading and interpretations of the results were performed in accordance with the manufacturer's instructions. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality control strains in each set of experiments.

Data analysis

Categorical data analysis of clinical and mycological data was carried out with SPSS v. 11.0 software (SPSS Inc., Chicago, IL, USA). Statistical analysis was performed on the patient group with VVC or recurrent VVC (RVVC); patients with a negative culture were not included. Patients were divided into two groups (VVC or RVVC). The associations between the type of

VVC and the use of antifungal agents, and the use of contraceptives and the presence of a non-*albicans Candida* sp., were analysed by Fisher's exact test in a two-way contingency table. The use of antifungal agents was considered to be a binary variable; no distinction was made between topical and systemic use of antifungal agents. Oral or mechanical contraceptives were not considered separately, and the use of contraceptives was included in the analysis as a binary variable. The associations between the use of antifungal agents and the presence of a non-*albicans Candida* sp., and between infection with an isolate with a fluconazole MIC > 1 mg/L at the first visit and a mycological cure, were analysed in two-way contingency tables.

Analysis of susceptibility results included both on- and off-scale MICs. The lower off-scale MICs were left unchanged, and the higher off-scale MICs were converted to the next highest concentration. The reproducibility between NCCLS, EUCAST and Etest results was calculated by determining the percentage of agreement between MICs. Agreement was defined as a discrepancy in MIC results of no more than two two-fold dilutions. Susceptibility testing results obtained by Etest were transformed to the nearest two-fold dilution, up or down, tested by the NCCLS reference method. In addition, the correlation between results was evaluated by using the intra-class correlation coefficient (ICC), which was expressed to a maximum value of 1 and with a 95% CI. In order to approximate a normal distribution, the MICs were transformed to $\log_2$ values, with $p < 0.05$ considered to be statistically significant. The ICC is a reverse measurement of the variability of the counting values. Apart from the agreement (concordance between MIC values), the ICC also evaluates the correlation between values offering statistical significance, since it takes into account the number of cases and the absolute value of the counting. The ICC is a scale analysis and exhibits the highest statistical power for reproducibility studies.

RESULTS

Patient population

On their first visit, 77.9% (60/77) of the women in the study had VVC and were culture-positive for *Candida*. Nine (15.5%) of these patients were considered to have RVVC, while, for two patients, the type of candidiasis was not specified. After treatment, 44 patients returned 1 week later; among these, cultures were still positive for 20.5% (9/44) at the second visit, giving a mycological cure rate of 79.5%. None of these patients suffered from RVVC. Sixteen of the original patients did not return within 1 week of treatment and no swab could be taken. The symptoms were reduced after treatment for all women with (R)VVC, with the exception of seven patients for whom the outcome was not specified on the questionnaire. The use of antimycotic agents during previous or current episodes of candidiasis, as well as current use of contraceptives or antibiotics, was assessed for the 58 patients with known VVC type. All patients with RVVC received antifungal treatment during the current or previous episodes, as opposed to 34.7% (17/49) of women with a non-recurrent type of VVC. Contraceptives were used by 33.3% (3/9) and 57.1% (26/49) of women with RVVC and non-recurrent VVC, respectively. Women with RVVC used significantly more antifungal agents (Fisher's exact test, $p < 0.05$), but there was no correlation between use of contraceptives and the frequency of RVVC. Only three patients had received antibiotics, and all three were reportedly suffering from their first episode of VVC.

Of all women with a clinical diagnosis of VVC, 22.1% (17/77) did not have a positive *Candida* culture at either their first or second visit. In these patients, the rate of clinical cure was 75% (12/16). The patients whose symptoms were not relieved received other therapies.

Species isolated

In total, 75 isolates of *Candida* spp. were obtained, i.e., 66 at the first visit (from 60 patients) and nine at the second visit (from nine patients). At the first visit, the isolates were identified as *C. albicans* (84.9%; 56/66), *C. guilliermondii* (10.6%; 7/66), *Candida glabrata* (3.0%; 2/66) and *C. dubliniensis* (1.5%; 1/66). Infection with non-*albicans Candida* spp. occurred in 44.5% of patients with RVVC. Thus, women with RVVC were significantly more likely to be infected by non-*albicans Candida* spp. than those with non-recurrent VVC (Fisher's exact test, $p < 0.05$). No association was found between the use of antifungal agents and the presence of non-*albicans Candida* spp. ($p < 0.05$). The species isolated at the second visit were identified as *C. guilliermondii* (66.7%; 6/9) and *C. albicans* (33.3%; 3/9).

Susceptibility testing results

Table 1 summarises the in-vitro susceptibilities of the 75 *Candida* isolates to the six antifungal agents, as determined with the EUCAST, NCCLS and Etest methods. *C. albicans* and *C. guilliermondii* differed most in susceptibility pattern among the azoles. For *C. guilliermondii*, the MIC ranges were broader and the geometric means were higher than those for *C. albicans*.

Table 1. Antifungal susceptibilities of 75 isolates of *Candida* spp., as determined by three methods

		MIC (mg/L) for each species (no of isolates)							
		C. albicans (*n* = 59)		*C. dubliniensis* (*n* = 1)		*C. glabrata* (*n* = 2)		*C. guilliermondii* (*n* = 13)	
Antifungal agent	**Method**	**Range**	**Geometric mean**	**Range**	**Geometric mean**	**Range**	**Geometric mean**	**Range**	**Geometric mean**
Amphotericin B	NCCLS	0.03–0.5	0.118	0.03–0.03	0.03	0.12–0.25	0.173	0.12–0.25	0.189
	EUCAST	0.03–0.25	0.125	0.12–0.12	0.12	0.25–0.25	0.25	0.03–0.25	0.143
	Etest 24 h	0.023–0.26	0.098	0.032–0.032	0.032	0.12–0.25	0.173	0.016–0.19	0.077
	Etest 48 h	0.012–0.5	0.162	0.094–0.094	0.094	0.25–0.38	0.308	0.094–0.38	0.199
Flucytosine	NCCLS	0.12–64	0.166	0.12–0.12	0.12	0.12–0.12	0.12	0.12–0.25	0.150
	EUCAST	0.12–64	0.222	0.12–0.12	0.12	0.25–0.25	0.173	0.03–0.25	0.134
	Etest 24 h	0.016–32	0.113	0.016–0.016	0.016	0.023–0.032	0.027	0.012–0.12	0.050
	Etest 48 h	0.032–32	0.207	0.023–0.023	0.023	0.047–0.094	0.066	0.12–0.38	0.214
Fluconazole	NCCLS	0.06–2	0.138	0.12–0.12	0.12	4–8	5.657	2–4	3.232
	EUCAST	0.06–64	0.199	0.25–0.25	0.25	2–4	2.828	2–8	4.450
	Etest 24 h	0.064–1	0.218	0.12–0.12	0.12	1.5–4	2.449	1–4	1.888
	Etest 48 h	0.094–0.75	0.231	0.12–0.12	0.12	2–12	4.899	1.5–6	2.881
Itraconazole	NCCLS	0.015–0.015	0.015	0.015–0.015	0.015	0.015–0.25	0.061	0.06–0.5	0.189
	EUCAST	0.015–8	0.020	0.015–0.015	0.015	0.25–0.5	0.354	0.12–0.5	0.277
	Etest 24 h	0.004–0.38	0.021	0.003–0.003	0.003	0.25–1.5	0.612	0.25–1	0.478
	Etest 48 h	0.004–0.25	0.031	0.003–0.003	0.003	1–1	1	0.5–2	1.051
Voriconazole	NCCLS	0.015–0.015	0.015	0.015–0.015	0.015	0.06–0.25	0.12	0.06–0.12	0.070
	EUCAST	0.015–8	0.0167	0.015–0.015	0.015	0.06–0.25	0.122	0.06–0.25	0.083
	Etest 24 h	0.003–0.047	0.0065	0.008–0.008	0.008	0.032–0.25	0.089	0.0032–0.064	0.030
	Etest 48 h	0.003–0.094	0.006	0.004–0.004	0.004	0.047–0.5	0.153	0.032–0.12	0.057
Ketoconazole[a]	NCCLS	0.015–0.03	0.015	0.015–0.015	0.015	0.06–0.25	0.122	0.03–0.25	0.063
	EUCAST	0.015–8	0.016	0.015–0.015	0.015	0.06–0.25	0.122	0.06–0.25	0.087

[a]Etest not performed.

Agreement was 97% between the NCCLS and EUCAST MICs, 93% between NCCLS and Etest data, and 92% between EUCAST and Etest data. The ICCs were 0.91 for NCCLS and EUCAST MICs, 0.88 for NCCLS and Etest data, and 0.89 for EUCAST and Etest susceptibility results. The ICC values were statistically significant ($p < 0.05$). The lowest percentages of agreement and ICCs, i.e., <80% and 0.70 ($p < 0.05$), respectively, were for itraconazole MICs for non-*albicans Candida* spp.

When the susceptibility data for the first visit and post-treatment isolates were compared, no difference was seen for fluconazole. However, microbiological cure was associated significantly ($p < 0.01$) with the isolation of *C. albicans* at the first visit. Isolates from patients without microbiological cure were largely *C. guilliermondii*, with significantly higher fluconazole MICs than for *C. albicans* ($p < 0.05$). Nevertheless, clinical cure was achieved in all women suffering from VVC. Despite the presence of strains with higher MICs for fluconazole, symptoms disappeared after fluconazole therapy.

DISCUSSION

Although monitoring of the azole susceptibilities of blood and oral isolates of *Candida* is now performed widely, limited information has been available regarding the susceptibilities of *Candida* spp. responsible for symptomatic vaginitis [2,4,18–21]. In the present study, as reported previously [3,4,19,22], *C. albicans* was identified as the predominant species (84.9%) isolated from patients with (R)VVC. Of the non-*albicans Candida* spp., *C. guilliermondii* (10.6%) was the most prevalent species, followed by *C. glabrata* (3%) and *C. dubliniensis* (1.5%). The unusually high isolation rate of *C. guilliermondii* in the present study was similar to that reported by Mendoza *et al.* [23], but contrasts with the findings of other workers. *C. guilliermondii* is associated rarely with infections in humans [24]. However, this species has been isolated from the human gastrointestinal and genitourinary tracts [25], and may be involved in cutaneous and subcutaneous infections [26]. It can cause fatal fungaemia [27], but has been reported only occasionally as a vaginal pathogen [21,24,28,29]. Remarkably, in the present study, all isolates originated from one practice, implying a pseudo-outbreak caused by cross-contamination. A pseudo-outbreak of *C. guilliermondii* fungaemia, resulting from contaminated heparin vials, was reported by Yagupsky *et al.* [24]. However, samples from the gynaecological practice concerned did not yield *C. guilliermondii*, and the *C. guilliermondii*-positive samples were not clustered in time.

The type of VVC correlated with the presence of non-*albicans Candida* spp. Women with RVVC were significantly more likely to be infected by non-*albicans Candida* spp. than were women with non-recurrent VVC. Similarly, Spinillo *et al.* [3] concluded that RVVC was an additional risk-factor for vaginitis caused by non-*albicans Candida* spp. As expected, women with RVVC used significantly more antifungal agents, which could lead to the selection of non-*albicans Candida* strains [4]; alternatively, the presence of an intrinsically less susceptible non-*albicans Candida* strain could account for RVVC. Because of the low number of cases, an association between the use of antifungal agents and the presence of non-*albicans Candida* spp., corrected for the type of VVC, could not be determined.

There was no correlation between use of contraceptives and the type of VVC. Oral contraceptives have been suspected of contributing to RVVC, but epidemiological data are conflicting and their role in this aetiology is still controversial [5,30]. Three patients who received antibiotics were suffering from their first episode of VVC. The use of broad-spectrum antibiotics has been suggested as a risk-factor for acute VVC [29,31].

In the present study, susceptibility testing of 75 vaginal *Candida* isolates was performed with the NCCLS, EUCAST and Etest procedures. Reproducibility and correlation indices were statistically significant ($p < 0.05$) and, unlike the results of Bauters *et al.* [18], no discrepancy was observed between fluconazole MICs obtained with the NCCLS and EUCAST methods. The lowest percentages of agreement and ICCs were obtained for itraconazole MICs of non-*albicans Candida* spp. Trailing growth, as well as non-homogeneous itraconazole solutions, may have contributed to this variability [32].

The overall sensitivity of vaginal *C. albicans* isolates to fluconazole, and the increasing resistance of *C. glabrata* [5,6], were also confirmed in the present study. Overall, *C. albicans* isolates were also sensitive to the other azoles tested, although Sojakova *et al.* [4] found that vaginal isolates of *C. albicans* were resistant *in vitro* to fluconazole and itraconazole in 14.1% and 16.6% of cases, respectively.

To date, there have been no reports on the in-vitro susceptibility patterns of vaginal *C. guilliermondii* isolates. With regard to *C. guilliermondii* bloodstream isolates, some studies suggest that they have moderate sensitivity to fluconazole and ketoconazole, and are susceptible to amphotericin B and flucytosine [25,33,34], while others suggest that they are highly susceptible to fluconazole, ketoconazole, itraconazole and amphotericin B [35,36], but resistant to flucytosine [35], with all tests having been performed according to NCCLS methodology. In the present study, vaginal *C. guilliermondii* isolates were susceptible *in vitro* to amphotericin B and flucytosine, but were only moderately susceptible to azole agents, regardless of the susceptibility testing procedure. MICs of fluconazole ranged from 1 to 8 mg/L.

Correlation of in-vitro susceptibility data with clinical outcome can be variable, depending on the test method. Clinical resistance to fluconazole of *Candida* isolates causing vulvovaginitis was demonstrated as in-vitro resistance in about one-third of the cases by Etest [21] or the NCCLS method [2]. However, Costa *et al.* [19] reported a positive correlation between Etest susceptibility results and clinical outcome in vaginal *Candida* infections. In the present study, a correlation could not be established because resistant isolates were detected only rarely *in vitro*. In addition, clinical cure was observed for all patients treated with fluconazole, irrespective of the fluconazole MIC. Isolates from patients for whom microbiological cure was not achieved were largely *C. guilliermondii*, with fluconazole MICs >2 mg/L. However, this finding could be biased because of the absence of a representative number of *C. albicans* isolates with fluconazole MICs >2 mg/L, as well as the low number of *C. guilliermondii* isolates with MICs <2 mg/L.

Antifungal susceptibility tests may be an important aid in treatment and the monitoring of outcome. The present study shows that use of fluconazole resulted in a clinical cure for all patients, even those with RVVC and VVC caused by non-*albicans Candida* spp. or isolates with higher in-vitro MICs for fluconazole. Resistance to fluconazole was not detected *in vitro* before or after treatment.

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