Proteinase detection, DNA typing and antimycotic susceptibility of *Candida* isolates from Colombian women with vulvovaginal candidiasis

Nora Cardona-Castro[1], Sanjay G. Revankar[2], Patricia Ortiz[1], Claudia Cuervo[1], William R. Kirkpatrick[2], Robert K. McAtee[2] & Thomas F. Patterson[2,3]

[1]Laboratorio de Microbiología, Instituto Colombiano de Medicina Tropical, Medellín-Colombia, [2]Department of Medicine, Division of Infectious Diseases, The University of Texas Health Science Center at San Antonio Texas, and [3]South Texas Veterans Health Care System, Audie L. Murphy Division, San Antonio, TX, USA

Summary

Forty non-pregnant Colombian women (ages 18 - 45) with vulvovaginal candidiasis diagnosis (VVC) were enrolled in a blinded study to compare the efficacy of Itraconazole (ITRA) 400 mg vs. Fluconazole (FLU) 150 mg. Sexual partners received similar therapy. Proteinase detection by the Staib method and minimal inhibitory concentration (MIC) for FLU and ITRA by Etest method were performed in all *Candida* isolates. Patients were followed one year to determine clinical evolution and recurrence of VVC (RVVC). The strain identity of the RVVC isolates was determined by contour-clamped homogeneous electric field (CHEF) gel electrophoresis karyotyping and restriction fragment length polymorphism (RFLP). Thirty patients (75%) had one or two episodes of VVC/year, 83% of these were due to *Candida albicans*, while ten patients (25%) developed RVVC (three or more episodes/year); seven of them were treated with FLU. Non-*C. albicans Candida* species were detected in five of 30 (17%) of the patients with VVC and in seven of ten (70%) patients with RVVC (p=0.003). Isolates from nineteen patients were proteinase positive. Proteinase production and type of treatment were not related to recurrence of VVC (p>0.05). DNA typing revealed that in this population RVVC might be due to the same strain, substrain shuffling or different strains and species.

Key words Vulvovaginal candidiasis, Proteinase, DNA typing, MIC

Detección de proteinasa, tipificación del ADN y sensibilidad a los antifúngicos de aislamientos de *Candida* de mujeres colombianas con candidiasis vulvovaginal

Resumen

Se realizó un estudio ciego para comparar la eficacia de itraconazol (ITRA) 400 mg y fluconazol (FLU) 150 mg, en 40 mujeres colombianas de 18 a 45 años, no embarazadas y con diagnóstico de vulvovaginitis candidiásica (VVC). Los compañeros sexuales recibieron el mismo tratamiento que las pacientes. En todas las cepas de *Candida* aisladas de las pacientes se detectó la producción de proteinasa por el método de Staib y se determinó la concentración mínima inhibitoria (CMI) por el método de Etest. Las pacientes fueron seguidas durante un año para evaluar la evolución clínica y la presencia de vulvovaginitis candidiásica recurrente (VVCR), definida como tres o más episodios en un año. La identidad de las cepas aisladas de pacientes con VVCR fue determinada realizando el estudio del cariotipo mediante electroforesis en campo pulsado y polimorfismo en la longitud de los fragmentos de restricción. Treinta pacientes (75%) tuvieron uno o dos episodios de VVC en el año, 83% de estos episodios fueron por *C. albicans*. Diez pacientes (25%) desarrollaron VVCR; siete de ellas tratadas con FLU. Especies de *Candida* no *C. albicans* fueron aisladas en cinco pacien-

Dirección para correspondencia:
Dra. Nora Cardona-Castro
Laboratorio de Microbiología,
Instituto Colombiano de Medicina Tropical
Cra. 43ª No. 52 Sur 99, Sabaneta,
Antioquia, Colombia AA 52162
Tel.: +574 3014300
Fax: + 574 3014258
E-mail: icmt@epm.net.co

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tes de 30 (17%) de las que desarrollaron VVC y en 7 de 10 (70%) de las pacientes que tuvieron VVCR (p=.003). Los aislamientos de 19 pacientes fueron proteinasa positiva. La producción de proteinasa y el tipo de tratamiento no se relacionaron con la recurrencia de VVC (p>0.05). La tipificación del DNA de las cepas de *Candida* reveló que en esta población VVCR puede ser causada por la misma cepa, por cepas que presentan variaciones mínimas en el patrón de bandas o por diferentes cepas y especies.

Palabras clave Candidiasis vulvovaginal, Proteinasa, Tipificación de ADN, CMI

Vulvovaginal candidiasis (VVC) is a mucosal infection caused by *Candida* spp. and *C. albicans* is the causative agent of VVC in 85 - 90% of the cases. *Candida glabrata*, *Candida tropicalis* and *Candida krusei* are the most frequent causative agents for most of the remaining cases [1,2]. After an initial episode of VVC, up to 5% of women will develop recurrent VVC (RVVC) that is defined as three or more episodes per year [3]. RVVC can be classified as primary or secondary depending upon underlying causes. Primary RVVC is idiopathic with no known cause; whereas secondary RVVC denotes infections that occur as sequella to uncontrolled diabetes mellitus, immunosuppression, exogenous hormone therapy, and AIDS [3-6].

Antifungal drug resistance does not appear to contribute to RVVC, however the general use of azoles for VVC treatment has been questioned in non-*C. albicans Candida* infection [1,3,7,8]. The prevalence of non-*C. albicans Candida* identified in cases of vaginitis, has more than doubled between the 1970s and the 1980s and several reports since 1980 have cited non-*C. albicans Candida* as causative species in more than 20% of cases of primary vaginal infection [8]. Minimal inhibitory concentrations (MICs) of fluconazole (FLU) are typically higher for *C. glabrata* and frankly resistant for *C. krusei*. Both *C. glabrata* and *C. krusei* have been associated with treatment failures and secondary resistance to FLU developed during the course of FLU therapy [1,7,8].

Some virulence properties of *Candida* spp. that could contribute to RVVC include pseudo-mycelium formation, antimycotic susceptibility, and production of proteolytic enzymes, among others [3,9,10]. The genetic evidence of a relationship between extracellular proteinase and the virulence of *C. albicans* has been demonstrated in experimental models [10-13]. Secretory proteinases are also found in *C. glabrata, Candida parapsilosis,* and *C. tropicalis* [9,13-16]. New evidence of the virulence role of *Candida* proteases, have been found with protease inhibitors in HIV patients. Gruber *et al.* [17] and Schaller et al [18] found that treatment of HIV patients with protease inhibitor also acts against the *Candida* protease, and thereby controls infections by proteolytic *Candida* in these patients.

Electrophoretic karyotyping has shown a high degree of variability among *Candida* species, making it a very good technique for epidemiological studies. Furthermore, this technique is reliable, reproducible and is a relatively simple method to perform [19]. Restriction fragment length polymorphism (RFLP) may allow further distinction of related microorganisms, including *Candida* species [20-22].

In this work we compared the efficiency of itraconazole (ITRA) vs. FLU in the treatment of VVC in 40 healthy Colombian women followed for one year. We determined the proteinase production and MICs for ITRA and FLU in *Candida* spp isolates to evaluate the relationship of these factors with RVVC. Also, the isolates from patients with RVVC were evaluated using contour-clamped homogeneous electric fields (CHEF) gel electrophoresis and RFLP for epidemiological issues such as persistence of the original strain of *Candida* or replacement of the original isolate in subsequent episodes of RVVC.

MATERIALS AND METHODS

Patients. Forty healthy female Colombian volunteers (ages 18-45), who were not pregnant and did not use hormones, who had symptomatic VVC (itching, burning, soreness, abnormal vaginal discharge, dyspareunia) with signs that included vaginal and vulvar erythema and edema [3], were enrolled in a blinded study to receive oral FLU 150 mg as a single dose or oral ITRA 200 mg every 12 h for one day. Sexual partners received treatment similar to that of the patient. Patients were followed for one year after initial diagnosis for evidence of clinical evolution and recurrence. The initial blinded treatment assigned for the first episode of VVC was repeated in each recurrent episode of VVC of the patient and her sexual partner.

The sample size of patients was calculated with an alpha error of 0.05, a beta error of 0.05, relative risk of 2.0, and with a confidence of 95%. Twenty patients for each antimycotic treatment arm were calculated and treatment was randomly administered.

Isolates. Yeasts were isolated from vaginal secretion swabs, plated on sabouraud dextrose and mycosel agar (Becton Dickinson, USA) and incubated overnight at 37 °C. Identification was performed by germ tube formation and carbohydrate assimilation tests (API 20-C, bioMérieux, France) [23].

Proteinase detection. The *Candida* proteinase was detected by the Staib method [24]. Briefly, *Candida* isolates were plated on BSA-medium (dextrose 2%, KH_2PO_4 0.1%, $MgSO_4$ 0.05%, agar 2%, pH 5, mixed after cooling to 50 °C with 1% bovine serum albumin solution) and incubated at 37 °C for five days. Before incubation the BSA-agar was slightly opaque. The plates were observed daily for proteolytic activity for five days. Staining with amidoblack was performed on the fifth day. Opaqueness of the agar, corresponding to a zone of proteolysis around the yeast colonies that could not be stained with amidoblack indicated degradation of the protein [24].

Antimycotic susceptibility. Minimal Inhibitory Concentrations were determined for ITRA and FLU using the Etest method (AB Biodisk, Sweden) [25] according to the manufacturer's recommendations. *C. krusei* ATCC 6258 was used as a control.

Molecular Typing. DNA typing of RVVC isolates was performed using previously described CHEF electrophoretic karyotype analysis and RFLP protocols using the restriction endonuclease *Sfi* 1 [22,26].

Table 1. Number of clinical episodes, isolates and proteinase production from patients with vulvovaginal candidiasis.

Patient	No. of clinical episodes	Species of isolate	Proteinase activity	Patient's status	Treatment
1	1	*C. albicans*	Positive	VVC	ITRA
2	1	*C. albicans*	Positive	VVC	FLU
3	1	*C. albicans*	Positive	RVVC	FLU
	7	*C. tropicalis*	Positive		
4	1	*C. krusei*	Negative	VVC	FLU
5	4	*C. krusei*	Negative	RVVC	FLU
6	1	*C. parapsilosis*	Negative	VVC	FLU
7	2	*C. albicans*	Positive	VVC	ITRA
8	1	*C. albicans*	Negative	VVC	ITRA
9	1	*C. albicans*	Negative	VVC	ITRA
10	3	*C. albicans*	Negative	RVVC	ITRA
11	2	*C. albicans*	Negative	VVC	FLU
12	1	*C. parapsilosis*	Positive	VVC	FLU
13	1	*C. albicans*	Negative	RVVC	ITRA
	3	*C. albicans*	Positive		
14	1	*C. parapsilosis*	Negative	VVC	FLU
15	4	*C. krusei*	Negative	RVVC	ITRA
16	1	*C. albicans*	Negative	VVC	ITRA
17	1	*C. albicans*	Positive	VVC	ITRA
18	1	*C. albicans*	Negative	VVC	FLU
19	1	*C. albicans*	Negative	VVC	FLU
20	2	*C. albicans*	Positive	VVC	ITRA
21	2	*C. albicans*	Positive	VVC	ITRA
22	3	*C. albicans*	Positive	RVVC	FLU
23	4	*C. albicans*	Positive	RVVC	FLU
	1	*C. glabrata*	Positive		
	3	*C. krusei*	Negative		
24	6	*C. krusei*	Negative	RVVC	FLU
25	4	*C. albicans*	Positive	RVVC	FLU
	1	*C. glabrata*	Positive		
26	1	*C. albicans*	Positive	VVC	FLU
27	1	*C. albicans*	Negative	VVC	ITRA
28	2	*C. albicans*	Positive	VVC	FLU
29	1	*C. albicans*	Positive	VVC	ITRA
30	1	*C. albicans*	Positive	VVC	FLU
31	2	*C. albicans*	Positive	VVC	ITRA
32	1	*C. albicans*	Positive	VVC	ITRA
33	1	*C. albicans*	Negative	VVC	ITRA
34	1	*C. albicans*	Negative	VVC	FLU
35	1	*C. albicans*	Negative	VVC	FLU
36	1	*C. albicans*	Negative	VVC	ITRA
37	2	*C. glabrata*	Negative	VVC	ITRA
38	1	*C. albicans*	Positive	VVC	ITRA
39	1	*C. albicans*	Negative	VVC	ITRA
40	4	*C. krusei*	Negative	RVVC	FLU

Karyotype analysis. Strains isolated from the same patient were considered to have different DNA types if there was a difference of 1 or more bands between them [27,28].

RFLP analysis. Strains isolated from the same patient were considered unrelated if there was a difference of 2 or more bands between them, related if a difference of only one band, and identical if no difference in the banding pattern was seen [27,28].

Definitions. Same or identical strain: was defined when the two typing methods (CHEF and RFLP) showed identical patterns [27,28]. *Minor genetic variation or substrain shuffling*: demonstrated when single band differences were minor or subtle, or one of the typing methods showed differences and the other typing method failed to show any difference [29]. *Different strains*: the 2 typing methods showed major differences between banding patterns [27].

RESULTS

Twenty three (57.5%) patients presented one VCC episode per year, seven (17.5%) patients experienced twoepisodes and 10 (25%) had three or more episodes of VVC per year. In the patients who had one or two episodes per year, 25 of 30 (83%) episodes were due to *C. albicans* alone. non-*C. albicans Candida* species were detected in five of 30 (17%) of the patients with VVC. Three patients had episodes with *C. parapsilosis*, one had *C. krusei*, and one had *C. glabrata*. Table 1 shows the number of clinical episodes, species isolated, proteinase production of isolates, and treatment arm to which each patient was randomly assigned. From the group of 30 VVC patients; 14 patients had proteinase producing strains and the other 16 patients had seemingly non-proteolytic strains. From the 10 RVVC patients, seven patients had recurrences by the same species and three patients had recurrences by different or multiple species. All strains isolated from three patients were proteinase producers; two patients had both proteinase producing and non-producing strains and all strains isolated from five patients were proteinase negative.

In 10 patients with RVVC, a mean of 4.9 episodes were documented during the year of follow-up. Of those patients, recurrent infection with *C. albicans* alone occurred in three patients. Infection due to non-*C. albicans Candida* species occurred in seven of 10 (70%) patients with RVVC, versus five of 30 (17%) patients with VVC whose infection was due to non-*C. albicans Candida* species ($p=0.003$). There were four RVVC patients whose infections were due to *C. krusei* alone. Recurrences with species other than *C. albicans* following initial isolation of *C. albicans* occurred in three patients; notably, species with reduced susceptibility to azole agents including *C. tropicalis* in one patient, *C. glabrata* and *C. krusei* in separate episodes in one patient, and recurrence with *C. glabrata* in another patient.

Karyotype analysis and RFLP with *Sfi* I digestion

Table 2. Strain identify from patients with RVVC using CHEF karyotyping of chromosomal DNA and *Sfi* I digested whole-cell DNA (RFLP) of *Candida* species.

Patient	Isolate	*Candida* species	Karyotype	RFLP	Comments
3	3.0	*C. albicans*	A	A	Different species, similar strain
	3.1	*C. tropicalis*	B	B	
	3.2	*C. tropicalis*	B	B	
	3.3	*C. tropicalis*	B	B	
	3.4	*C. tropicalis*	B	B	
	3.5	*C. tropicalis*	B	B	
	3.6	*C. tropicalis*	B	B	
	3.7	*C. tropicalis*	B	B	
5	5.0	*C. krusei*	C	C	Minor genetic changes
	5.1	*C. krusei*	D	C	
	5.2	*C. krusei*	C	C	
	5.3	*C. krusei*	D	C	
10	10.0	*C. albicans*	E	D	Minor genetic changes
	10.1	*C. albicans*	E	E	
	10.2	*C. albicans*	E	E	
13	13.0	*C. albicans*	F	F	Minor genetic changes
	13.1	*C. albicans*	F	G	
	13.2	*C. albicans*	F	G	
	13.3	*C. albicans*	F	G	
15	15.0	*C. krusei*	G	H	Similar strain
	15.1	*C. krusei*	G	H	
	15.2	*C. krusei*	G	H	
	15.3	*C. krusei*	G	H	
22	22.0	*C. albicans*	G	I	Different strains
	22.1	*C. albicans*	I	J	
	22.2	*C. albicans*	J	K	
23	23.0	*C. albicans*	K	L	Different species, similar strain
	23.1	*C. albicans*	K	L	
	23.2	*C. albicans*	K	L	
	23.3	*C. albicans*	K	L	
	23.4	*C. glabrata*	L	M	
	23.5	*C. krusei*	M	N	
	23.6	*C. krusei*	M	N	
	23.7	*C. krusei*	M	N	
24	24.0	*C. krusei*	N	O	Minor genetic changes
	24.1	*C. krusei*	N	O	
	24.2	*C. krusei*	O	O	
	24.3	*C. krusei*	N	O	
	24.4	*C. krusei*	P	O	
	24.5	*C. krusei*	P	O	
25	25.0	*C. albicans*	Q	P	Different species, similar strain
	25.1	*C. albicans*	Q	P	
	25.2	*C. albicans*	Q	P	
	25.3	*C. albicans*	Q	P	
	25.4	*C. glabrata*	R	Q	
40	40.0	*C. krusei*	S	R	Similar strain
	40.1	*C. krusei*	S	R	
	40.2	*C. krusei*	S	R	
	40.3	*C. krusei*	S	R	

from the isolates of the 10 patients with RVVC is shown in Table 2. Seven of the 10 RVVC patients (70%) had three or more episodes (range, 3-6) with the same yeast species. Four of these seven patients had multiple recurrences with *C. krusei*, two of them had episodes with the same strain as demonstrated by both karyotyping (patient 15; Figure 1) and RFLP (patient 15; Figure 2). The other two patients with recurrent *C. krusei* infection had strains with minor genetic variation of substrain shuffling (4), since karyotyping revealed differences between isolates and RFLP analysis showed similar patterns (patient 24; Figure 1 and Figure 2). In three patients, recurrent infection occurred with *C. albicans*. In one patient (patient 22) three different strains at separate recurrences were identified using both CHEF karyotyping and RFLP. However, in two patients (patients 10 and 13) identical karyotypes were demonstrated using CHEF while RFLP analysis showed differences between the isolates, suggesting minor genetic variation or substrain shuffling (29). In three patients (3, 23, and 25) the initial episode was due to *C. albicans* while subsequent episodes following azole therapy were due to other yeasts (Table 2).

MICs for ITRA and FLU by the Etest method were determined for the *Candida* isolates from these 40 patients. With few exceptions, *C. albicans* isolates were susceptible to both ITRA (MIC range 0.002 – ≥32, MIC_{90} = 0.28) and FLU (MIC range 0.25 – ≥256, MIC_{90} = 1.5). Within the group of 45 *C. albicans* isolates tested for susceptibility, only one isolate was resistant to both ITRA and FLU while three other isolates were resistant to ITRA, but remained susceptible to FLU. MICs for four *C. albicans* isolates were not determined. MICs for six *C. tropicalis* isolates revealed susceptibility to both ITRA (MIC range 0.032 – 0.38, MIC_{90} = 0.17) and FLU (MIC range 0.38 – 3, MIC_{90} = 0.77). One *C. tropicalis* isolate was not tested. There were three episodes where *C. parapsilosis* was isolated. One isolate was resistant to both ITRA (MIC ≥ 32) and FLU (MIC ≥256), one was resistant

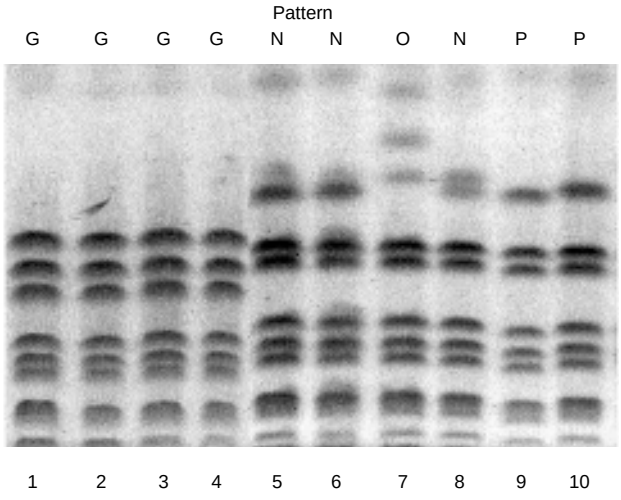

Figure 1. CHEF of chromosomal DNA from *Candida krusei* isolated from patients with recurrent VVC. Lanes 1-4 show identical karyotype patterns of isolates from patient 15: isolates 15.0-15.3. Lanes 5-10 show different karyotype patterns of isolates from patient 24: isolates 24.0-24.5 .

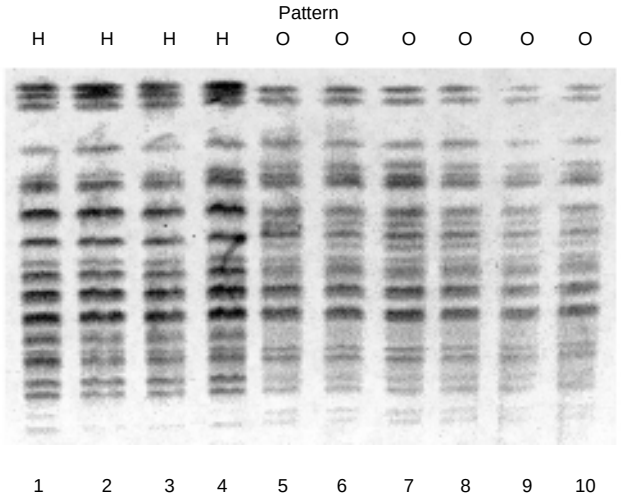

Figure 2. PFGE of *Sfi*1 digested whole-cell DNA from *Candida krusei* isolated from patients 15 and 24 with recurrent VVC. Lanes 1-4 show identical RFLP patterns of isolates from patient 15: isolates 15.0-15.3. Lanes 5-10 show identical RFLP patterns of isolates from patient 24: isolates 24.0-24.5.

to only ITRA (MIC 1.5), but not to FLU (MIC 4) and the third was susceptible to both ITRA (MIC 0.25) and FLU (MIC 0.5). MICs for the four isolates of *C. glabrata* ranged from 0.32 - ≥32 against ITRA and from 0.5 - ≥256 against FLU. Twenty (two not tested) *C. krusei* isolates had the highest MICs for both ITRA and FLU, and were considered resistant to these antifungal drugs. Regardless of MIC, all episodes vulvovaginitis in these 40 patients responded to ITRA or FLU.

DISCUSSION

Virulence in *Candida* is a complex issue; some attributes that are associated with virulence are mycelium formation, susceptibility to antimycotics, and efficient elaboration of proteolytic enzymes [2-4]. Secretory proteinases have been found in *C. albicans*, *C. parapsilosis*, *C. tropicalis* and *C. glabrata* [9,13-16]. In *C. albicans* the relationship between extracellular proteinase production and virulence has been demonstrated in experimental models [4,11]; however, in the current study, proteinase production, regardless of species, was not related to RVVC ($p<0.05$).

Other studies have shown that MICs of FLU for *C. glabrata* and *C. krusei* are generally higher than those for *C. albicans*, and that there is an association with treatment failures and secondary resistance to fluconazole developed during the course of FLU therapy for both *C. glabrata* and *C. krusei* [8]. In the present study, the treatment modality was not related with RVVC ($p<0.05$), however *C. glabrata* and *C. krusei* were less susceptible to either FLU or ITRA than *C. albicans* was.

While *C. albicans* is by far the most frequent pathogen in most VVC studies, other species such as *C. tropicalis*, *C. glabrata*, and *C. krusei* may be relatively common in certain areas [30,31]. In a study from Sudan, the most frequent isolate from patients with yeast vaginitis was *C. glabrata*, followed by *C. albicans* [30]. In this population of Colombian women, in contrast to women with VVC, RVVC was more often caused by non-*C. albicans* species ($p=0.003$), notably *C. krusei*. Recent reports of HIV infected women receiving weekly FLU describe a similar situation, with FLU treatment producing a decrease in *C. albicans* isolation and an increased recovery of non-*C. albicans* yeasts [32]. In our study, the first episode for three patients began with *C. albicans* and progressed to a different species with diminished susceptibility to azole agents, notably, *C. krusei* or *C. glabrata*. Most patients had recurrences due to the same strain, although some patients had strains with genetic variation causing recurrences, and one patient had two strains of *C. krusei* that were alternately isolated in serial episodes. This suggests that different patterns of recurrence may occur, sometimes in the same patient. The reason for some patients harboring strains with genetic variation or substrain shuffling that cause disease is unclear, however multiple and spontaneous chromosomal and phenotypic changes in *C. albicans* has been reported [19,20, 22,33-35].

Giraldo *et al.* [36] found that women with a history of RVVC have more easily detectable *Candida* in their vagina, even when asymptomatic, than do other women. They suggest that a relative inefficiency in regulating the proliferation of *Candida* in the vagina may increase susceptibility to periodic symptomatic recurrences.

Patients with RVVC may be more prone to develop infections due to non-*C. albicans* species than patients without recurrent infections [6,37]. However, some of this difference may be due to geographic variability in the distribution of *Candida* species.

The widespread use of azole antifungals in patients with VVC including those who suffered from infections by non-*C. albicans* species could be a reason for recurrent episodes due to the less susceptible or azole resistant yeasts [7,8].

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