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The Increasing Trend of Triazole-Resistant Candida from Vulvovaginal Candidiasis

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Purpose: Candida vaginitis is widely prevalent worldwide and is one of the common gynecological disorders. The aim of this study is to analyze the sensitivity of recurrent vulvovaginal (RVVC) candidiasis to antifungal drugs and its relationship with vaginal microbiota.

Patients and Methods: We Isolated and cultured Candida from RVVC patients, mass spectrometry and broth microdilution method were used to identify and determine MIC values of antifungal drugs. Clinical medical records and vaginal microbiota of RVVC patients were also collected.

Results: The main pathogens causing RVVC are predominantly *Candida albicans* (70.26%), but in recent years, there has been an increasing proportion of *Candida glabrata*(24.46%). However, only 15.70% of Candida albicans were sensitive to Voriconazole, 35.84% to Fluconazole and 25.60% to Itraconazole. No fluconazole-resistant *Candida glabrata* was found. Most *Candida krusei* strains were sensitive to voriconazole (81.80%). More important MIC values of triazoles were increased in Candida species, when exposed to clotrimazole. In addition, we found that the vaginal microecology of candida vaginitis and bacterial vaginitis was significantly different.

Conclusion: Triazoles resistant Candida species have emerged, leading to the failure of empirical anti-infective therapy. At the same time, the vaginal microecology of candida vaginitis and bacterial vaginitis was significantly different. In addition, a new breakpoint for Candida from RVVC needs to be established.

Keywords: triazole, Candida, vulvovaginal candidiasis, vaginal microbiota

Introduction

Vulvovaginal candidiasis (VVC) is a common gynecological condition that significantly impacts the quality of life and health of women. VVC is recognized as the second most prevalent cause of vaginal infections, affecting approximately 70–75% of women at some point during their lifetime.[1] Recurrent Vulvovaginal Candidiasis (RVVC), defined as experiencing symptomatic episodes of infection ≥3 times within a 12-month period, is a challenging-to-treat fungal infection that significantly affects the quality of life of many reproductive-aged women. It has been reported to have an annual global incidence rate of 3871 per 100,000 individuals.[2] The 25–34 years age group has the highest prevalence. It is reported that, in high-income countries, the economic burden from lost productivity could be up to US$14.39 billion annually.[2]

According to reports, individuals with HIV, diabetes, or other immune system disorders are more susceptible to RVVC.[3] Prolonged or frequent use of antibiotics may increase the risk of RVVC.[4] High estrogen levels, such as during pregnancy, the use of estrogen replacement therapy, or oral contraceptives, may increase the risk of RVVC.[5] Because, research had found that estrogen promotes the growth of Candida. Additionally, factors, such as sexual activity, vaginal douching, or the use of irritating cleansers, can alter the vaginal pH balance and microbial environment, thereby increasing the risk of RVVC.[6]

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For many years, microscopy and clinical suspicion have been the common methods used for diagnosing (VVC). However, culture has traditionally been considered the gold standard for diagnosing vaginal fungal infections. It is worth noting that fewer than half of the patients treated for VVC are diagnosed using objective assays. Microscopy and clinical diagnosis alone have limitations in terms of sensitivity, which can lead to misdiagnosis and inappropriate treatment, particularly in cases of RVVC.[7]

Fluconazole maintenance therapy is considered the first-line treatment for recurrent vulvovaginal candidiasis (RVVC). It has been demonstrated to significantly improve the quality of life in approximately 96% of women affected by RVVC.[8] However, studies have found that fluconazole largely does not cure RVVC, and the recurrence rate is higher than expected.[9] Indeed, certain Candida species, such as Candida glabrata, have been observed to exhibit a higher tendency to develop resistance to azole antifungal drugs.[10] Therefore, the treatment for VVC typically involves the use of topical intravaginal agents such as nystatin, boric acid, or 17% flucytosine cream either alone or in combination with 3% amphotericin B cream. This treatment regimen is typically administered for a duration of 14 days.[11]

There is a lack of comprehensive epidemiological studies that provide detailed information on the clinical and microbiological characteristics VVC and RVVC. In light of the aforementioned gap in knowledge, we undertook a retrospective cohort study to examine the pathogen distribution and drug sensitivity patterns of RVVC over the past five years. We also analyzed special cases of RVVC. Furthermore, we conducted an analysis of the vaginal microecology in cases of RVVC and bacterial vaginosis. The novelty of this study lies in its emphasis on fungal factors that can impact the effectiveness of therapeutic interventions. By exploring these aspects, we aim to provide valuable insights into the underlying mechanisms and potential therapeutic targets for managing RVVC.

Materials and Methods

Patients Collection

This retrospective study was performed in Chongqing Health Center for Women and Children in Southwest China from January, 2017 to January, 2022. Swab samples were obtained from patients presenting with symptoms, such as pruritus vulva, vaginal burning pain, dyspareunia, dysuria, edema, fissures, as well as vulvar and vaginal erythema. The diagnosis of VVC was confirmed through the detection of microscopic yeast structures and positive yeast/Candida cultures. These diagnostic methods were used to identify the presence of yeast/Candida organisms and their characteristic structures, providing evidence for the diagnosis of VVC. Antifungal treatment was inquired through the outpatient medical record system.

Physicians diagnosed RVVC based on the following criteria: patients with ≥3 episodes/year, failure to respond to initial antifungal therapy, and presence of Candida on microscopic examination and culture.

Physicians diagnosed vaginosis based on the following criteria: (1) Positive clue cells (defined as clue cells >20% of the total vaginal epithelial cells); (2) Positive amine test; (3) Vaginal pH >4.5; (4) Homogeneous, thin, grey-white vaginal discharge; with the presence of positive clue cells being a mandatory criterion. (5) Gram stain Nugent scoring system ≥7 points. Excluding mixed infections with other common vaginal inflammations.

The normal group is defined as comprising members without symptoms such as vaginal itching, with normal color and odor of vaginal discharge, dominated by lactobacilli, and excluding other common vaginal inflammations.

Isolation of Candida

This retrospective research was conducted at Women and Children's Hospital of Chongqing Medical University. A total of 417 Candida isolates were obtained in this study from January, 2017 to December, 2022. Candida was identified to species level using the MALDI-TOF/TOF (Bruker, Germany) automated system.

Antifungal Susceptibility Testing

Routine antifungal susceptibility testing was performed by using ATB Fungus (Merieux, America). Susceptibility breakpoints were referred to Clinical and Laboratory Standards Institute (CLSI2022) or European Committee on Antimicrobial

Susceptibility Testing (EUCAST). MIC values were interpreted according to CLSI-M60 version 2 and epidemiological community values (ECVs).

Vaginal Microecology Test

The detection of vaginal microecology is divided into two parts, one is the microscopic observation of vaginal secretion, and the other is the physicochemical properties of vaginal secretion. Physical and chemical properties of vaginal discharge include neuraminidase, β-glucuronidase, Acetylglucosaminidase and pH.

Statistical Analysis

All of the data analysis by SPSS25.0 software. Student's *t*-test was used to calculate continuous variables with a normal distribution, while the Wilcoxon rank-sum test was employed for non-normally distributed variables. Statistical significance was defined at a P-value of less than 0.05 for two-tailed tests in all calculations.

Ethics Statement

Our study complies with the Declaration of Helsinki. The data and samples analyzed in this study were collected following the standards and approval of Ethics Committee of Women and Children's Hospital of Chongqing Medical University. Informed consent and signed subject informed consent forms were obtained for all data from human subjects.

Results

Candida Species Distribution

A total of 417 non-repetitive Candida strains were collected from January, 2017 to December, 2022. Most of them were *C. albicans*, accounting for 70.26%, followed by *C. glabrata*, accounting for 24.46%. The third was *C. krusei* (2.64%). Other Candida species, such as *Saccharomyces cerevisiae* and *Candida tropicalis*, accounted for 2.64% (Figure 1a). In recent years, there has been an increasing proportion of *C. glabrata*, and the rate was as high as 30.43% in 2020 (Figure 1b). On the contrary, the proportion of *C. krusei* decreased year by year, and no C. krusei positive patients were found for three consecutive years from 2020.

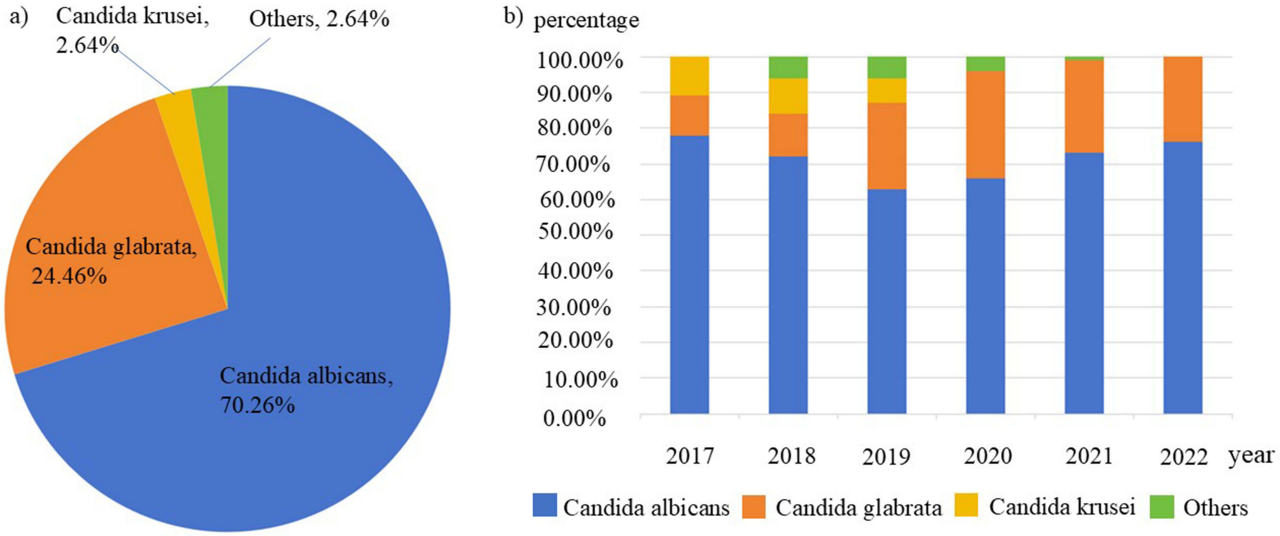

Figure 1 Distribution of Candida isolated from VVC patients from January, 2017 to December, 2022. (**a**) The proportion of different Candida species. (**b**) Changes in the proportion of Candida isolates in different years.

Antifungal Susceptibility of Candida Species

As shown in Table 1, the MIC_{50} of Fluconazole in *C. albicans* and *C. glabrata* is 4 mg/l. The MIC_{50} of Voriconazole in *C. albicans* is 0.5 mg/l, and 0.25 mg/l in *C. Glabrata* and *C. krusei*. AS for Itraconazole, the MIC_{50} is 0.25 mg/l in *C. albicans*, while 0.5 mg/l in *C. glabrata* and *C. krusei*.

At the same time, we found that with the increase of years, the proportion of high concentration MIC values of Fluconazole (≥4mg/l) and Itraconazole (≥0.5mg/l) increased in *C. albicans* (Figure 2a and c). There was no obvious annual trend in the MC value of voriconazole; however, in 2022, the MIC value of high concentration (≥1mg/l) was as high as 29.41% (Figure 2b) in *C. albicans*. As for Candida glabrata, increasingly higher MIC values of triazole antifungal agents appeared as the years progressed (Figure 3).

According to CLSI-M60 and EUCAST, about three-quarters of *C. albicans* were resistant to itraconazole, followed by voriconazole with a resistance rate of 39.93%, and fluconazole with a resistance rate of 32.08%. 48.04% of *C. glabrata* strains were non-wild type for voriconazole and only 0.98% for itraconazole. The resistance rate of *Candida glabrata* to Fluconazole was 4.90%. *C. krusi* was highly sensitive to Voriconazole, and 36.36% of *C. krusi* strains were non-wild-type to itraconazole. *C. krusi* is naturally resistant to fluconazole (Supplementary Table 1).

Treatment of Recurrent Candidiasis Vaginosis

We collected strains and their treatment regimens from 17 patients with RVVC. The MIC values of strains isolated from these patients to triazole antifungal drugs changed with the progression of treatment. Among them, Candida albicans was the most common, accounting for 52.94%, followed by Candida glabrata (35.29%) and Saccharomyces cerevisiae (5.88%). In addition, one patient was infected with *C. glabrata* and then *C. krusi*.

Although no fluconazole resistant *C. glabrata* was found in these patients, oral fluconazole did not eliminate *C. glabrata* when the MIC value of fluconazole was greater than 4mg/l (60.00%). Oral itraconazole may be a better treatment option.

The early vaginal use of clotrimazole ointment has been associated with an increased risk of fluconazole resistance in Candida albicans. However, when dealing with fluconazole-sensitive strains of C. albicans, the combination of clotrimazole with fluconazole has demonstrated improved efficacy (66.68%). This combination therapy approach can enhance treatment effectiveness and mitigate the risk of resistance development in susceptible strains. It is important to consider individual patient factors and conduct appropriate sensitivity testing when determining the most suitable treatment regimen for vulvovaginal candidiasis. The MIC values of Saccharomyces cerevisiae to triazole antifungal drugs were relatively high and showed poor response to fluconazole (Table 2).

The Vaginal Microecology of Candida Vaginitis and Bacterial Vaginitis

We compared the results of vaginal microecology between VVC patients and bacterial vaginitis patients (BV) and normal subjects. The mean age of VVC patients was younger than that of BV patients. The vaginal cleanliness of most VVC patients was grade II and III, and the diversity and density of the flora were +++. More than 90% of VVC patients are hydrogen peroxide positive, which contributes to the elevated vaginal pH in VVC patients (Table 3).

Table 1 Susceptibility of Different Candida Species to Antifungal Drugs

	Candida albicans (n=293)		Candida glabrata (n=102)		Candida krusei (n=11)	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Fluconazole	4	16	4	32	NA	NA
Voriconazole	0.5	2	0.25	2	0.25	1
Itraconazole	0.25	1	0.5	4	0.5	2

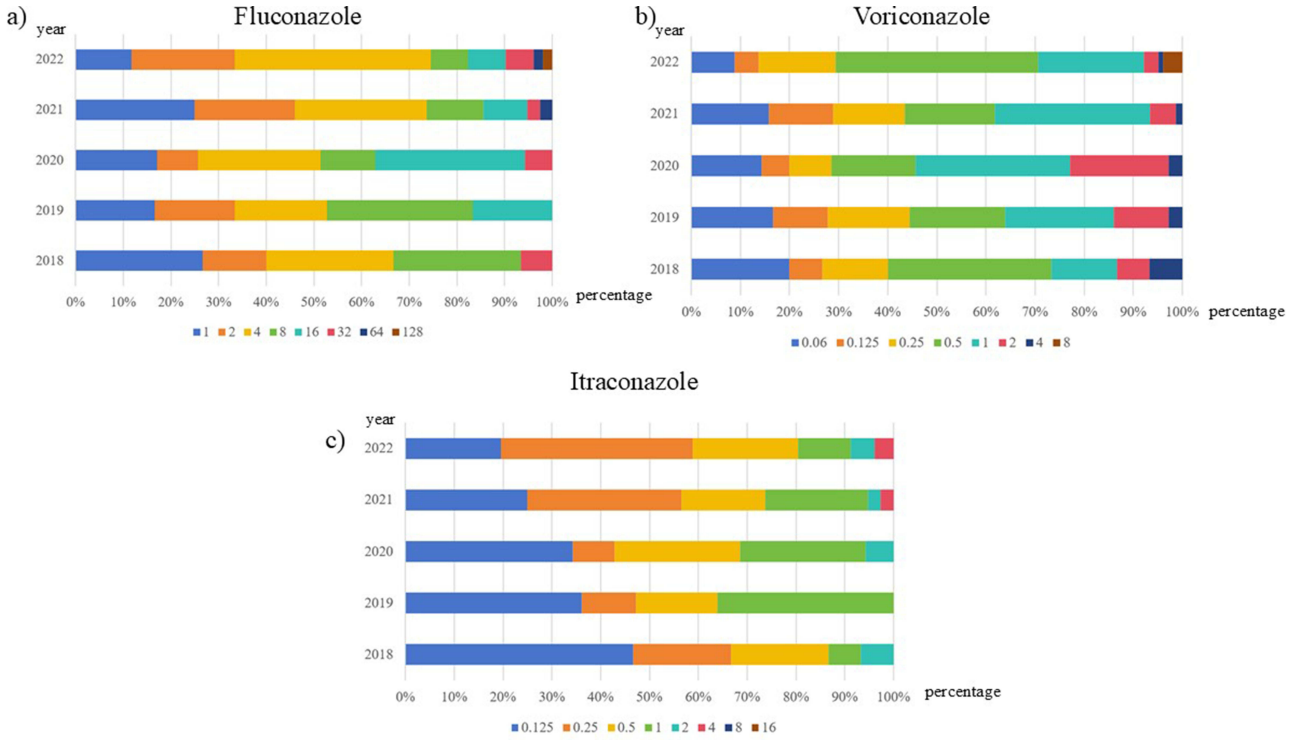

Figure 2 Changes of MIC values of *C. albicans* to azole antifungal drugs. (**a**) Changes of MIC values of *C. albicans* to fluconazole. (**b**) Changes in MIC values of voriconazole against *C. albicans* (**c**) Changes in MIC values of itraconazole against *C. albicans*.

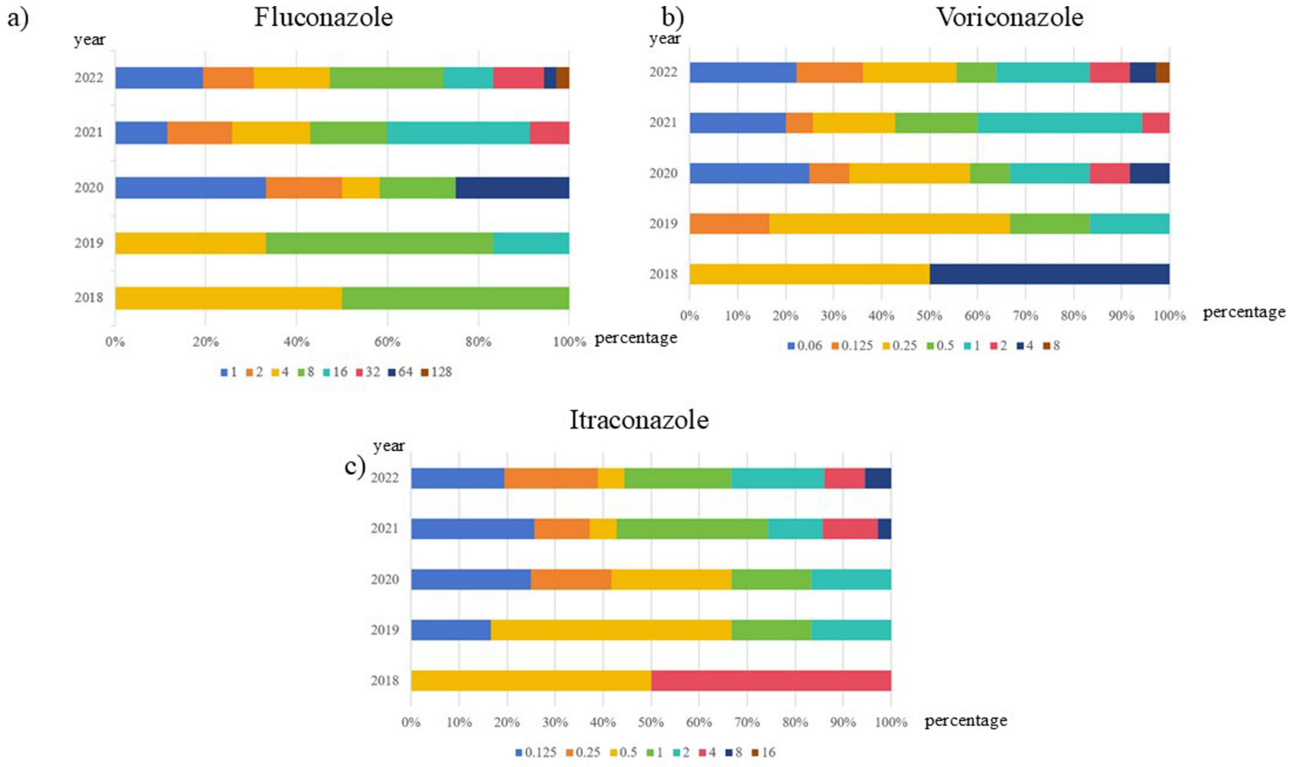

Figure 3 Changes of MIC values of *C. glabrata* to azole antifungal drugs. **(a)** Changes of MIC values of *C. glabrata* to fluconazole. (**b**) Changes in MIC values of voriconazole against *C. glabrata* (**c**) Changes in MIC values of itraconazole against *C. glabrata*.

Table 2 Cases of Recurrent Candida Vaginitis and Their Treatment Options

Cases	Ages	Species of Candida	Sensitivity of Antifungal Drugs			Therapy	Outcome
			Fluconazole	Voriconazole	Itraconazole		
Case1	42	Candida glabrata	1	0.06	0.5	Clotrimazole 5g qd+ Fluconazole 150mg q3d	Clear
		Candida glabrata	8	1	0.5	Caspofungin 50mg ivgtt	
Case8	26	Candida glabrata	8	0.5	1	Clotrimazole 5g qd+ Fluconazole 150mg q3d	Persistent
		Candida glabrata	16	1	1	Itraconazole 0.2g qd	
Case10	33	Candida glabrata	2	0.06	0.125	Fluconazole 150mg q3d	Persistent
		Candida glabrata	8	1	2	Itraconazole 0.2g qd	
Case12	30	Candida glabrata	8	0.5	1	Fluconazole 150mg q3d	Clear
		Candida glabrata	16	0.5	1	Itraconazole 0.2g qd	
Case16	46	Candida glabrata	1	0.06	0.5	Clotrimazole 5g qd	Persistent
		Candida glabrata	4	0.125	0.25	Fluconazole 150mg q3d	
Case18	26	Candida glabrata	1	0.06	0.5	Clotrimazole 5g qd	Clear
		Candida glabrata	2	0.06	0.5	Clotrimazole 5g qd+ Fluconazole 150mg q3d	
Case15	30	Candida albicans	1	0.25	0.125	Clotrimazole 5g qd	Clear
		Candida albicans	4	0.25	0.125	Clotrimazole 5g qd+ Fluconazole 150mg q3d	
Case7	33	Candida albicans	1	0.125	0.125	Clotrimazole 5g qd	Clear
		Candida albicans	4	0.06	0.125	Clotrimazole 5g qd+ Fluconazole 150mg q3d	
Case17	29	Candida albicans	2	0.25	0.125	Clotrimazole 5g qd	Persistent
		Candida albicans	4	0.25	0.125	Clotrimazole 5g qd+ Fluconazole 150mg q3d	
Case14	30	Candida albicans	2	1	0.25	Clotrimazole 5g qd	Persistent
		Candida albicans	16	8	4	Clotrimazole 5g qd+ Fluconazole 150mg q3d	
Case2	35	Candida albicans	4	0.25	0.5	Clotrimazole 5g qd+ Fluconazole 150mg q3d	Persistent
		Candida albicans	16	2	4	Itraconazole 0.2g qd	
Case6	27	Candida albicans	8	1	1	Clotrimazole 5g qd+ Fluconazole 150mg q3d	Persistent
		Candida albicans	16	1	0.25	Isoconazole nitrate 0.6g q3d + Fluconazole 150mg q3d	
Case4	35	Candida albicans	8	0.5	0.25	Clotrimazole 5g qd	Persistent
		Candida albicans	16	1	0.5	Isoconazole nitrate 0.6g q3d + Fluconazole 150mg q3d	
Case5	24	Candida albicans	8	1	0.5	Clotrimazole 5g qd+ Fluconazole 150mg q3d	Persistent
		Candida albicans	64	2	0.5	Isoconazole nitrate 0.6g q3d + Itraconazole 0.2g qd	

(*Continued*)

Table 2 (Continued).

Cases	Ages	Species of Candida	Sensitivity of Antifungal Drugs			Therapy	Outcome
			Fluconazole	Voriconazole	Itraconazole		
Case11	30	Candida albicans	16	4	1	Clotrimazole 5g qd+ Fluconazole 150mg q3d	Persistent
		Candida albicans	32	4	1	Clotrimazole 5g qd+ Fluconazole 150mg q3d	
Case9	34	Saccharomyces cerevisiae	128	32	32	Clotrimazole 5g qd+ Fluconazole 150mg q3d	Persistent
		Saccharomyces cerevisiae	128	32	32	Clotrimazole 5g qd+ Fluconazole 150mg q3d	
Case3	21	Candida glabrata	8	0.125	0.125	Itraconazole 0.2g qd	
		Candida krusei	NA	1	1	Itraconazole 0.2g qd	Clear

Notes: Clear: Symptom Relief: disappearance of symptoms in the patient, such as vaginal itching, burning, and abnormal discharge. Vaginal Secretions Testing: absence of Candida in vaginal secretions as determined by culture for three times. Persistent: Reappearance of symptoms after treatment cessation, accompanied by evidence of Candida infection, typically confirmed by positive wet mount or culture.

Discussion

Our results showed that *C. albicans* is still the main pathogen causing VVC, but only a small proportion of strains are susceptible to fluconazole, which may lead to treatment failure. Moreover, we analyzed the treatments of some RVVC patients and found that clotrimazole ointment alone may lead to the emergence of fluconazole-resistant strains. In addition, we also found that the vaginal microecology of VVC patients was different from BV and normal people, especially the pH increased significantly.

Table 3 The Vaginal Microecology of Candida Vaginitis and Bacterial Vaginosis

		VVC (n=6334)	BV (n=4827)	Normal Group (2478)	VVC VS BV	VVC VS Normal Group	BV VS Normal Group
					p	p	p
Ages		32.85±7.48	37.06±10.25	33.01±7.01	<0.001	0.06	<0.001
Degree of cleanliness	II III IV	49.79% 50.21% 0	0 21.07% 78.93%	69.53% 30.47% 0	<0.001	<0.001	<0.001
White blood cell (>10 /LB)		49.89%	34.08%	30.43%	<0.001	<0.001	0.02
The density of Vaginal Microbiota	+ ++ +++ +++ +	1.74% 32.16% 66.09% 0.02%	0.04% 3.44% 87.28% 9.24%	0 34.67% 65.05% 0.12%	<0.001	<0.001	<0.001
Diversity of Vaginal Microbiota	+ ++ +++ +++ +	1.93% 86.23% 11.84% 0	0 2.51% 96.19% 1.31%	1.09% 95.64% 3.11% 0	<0.001	<0.001	<0.001

(*Continued*)

Table 3 (Continued).

	VVC (n=6334)	BV (n=4827)	Normal Group (2478)	VVC VS BV	VVC VS Normal Group	BV VS Normal Group
				p	p	p
Dominant bacteria (Gram-positive large bacilli)	99.27%	0.21%	99.84%	<0.001	<0.001	<0.001
Hydrogen peroxide (positive)	92.80%	93.97%	0.24%	0.014	<0.001	<0.001
Neuraminidase (positive)	0.24%	81.42%	0	<0.001	<0.001	<0.001
β-glucuronidase (positive)	0	0.25%	0	<0.001	<0.001	<0.001
Acetylglucosaminidase (positive)	28.35%	27.78%	5.81%	0.54	<0.001	<0.001
Scores of Nugent	1.94±0.54	7.99±0.64	1.43±0.61	<0.001	<0.001	<0.001
Scores of AV	0.58±0.64	3.26±0.66	0.34±0.64	<0.001	<0.001	<0.001
pH	4.15±0.34	4.65±0.41	3.97±0.21	<0.001	<0.001	<0.001

Notes: The density of Vaginal Microbiota: +:oil lens (10× 100x magnification) to observe each vision.The average number of bacteria in the wild field was 1–9; ++: the average number of bacteria per field observed by oil microscope was 10–99; +++: the average number of bacteria in each field under the oil microscope was 100 or more. Under the light microscope, the bacteria covered the field of vision; ++++: the bacteria gathered into clusters or densely covered the mucosal epithelial cells under the oil microscope; Diversity of Vaginal Microbiota: +: 1 to 3 bacteria can be identified; ++: 4 to 6 species of bacteria were identified; +++: 7 to 9 kinds of bacteria can be identified; ++++:10 or more species of bacteria could be identified.

Abbreviations: VS, versus; AV, Aerobic vaginitis; BV, bacterial vaginosis; VVC, Vulvovaginal Candidiasis.

While recurrent vulvovaginal candidiasis (RVVC) was previously believed to affect a small percentage of women (<5%), a recent internet survey conducted in five European countries and the United States, involving over 6000 women, revealed a self-reported RVVC prevalence of 9%[12] with the highest prevalence in women 25 to 34 years old. Arastehfar A et al screened 300 women in Iran and approximately 53% were diagnosed with RVVC.[13] In our hospital, the morbidity of RVVC was similar to that reported, with more than half of VVC patients progressing to RVVC.

According to a global multicenter study conducted by Pfaller MA et al, it was found that less than 1% of invasive *C. albicans* isolates exhibited resistance to fluconazole. While, the results of a meta-analysis showed that the sensitivity of Candida albicans isolated from VVC patients to fluconazole was about 81.8%, and Itraconazole was about 79%.[14] A longitudinal study found that 50.00% Candida albicans resistant to fluconazole with MIC ≥8 μg/mL in RVVC.[15] *C. glabrata* isolates have intrinsically higher MIC values for fluconazole compared to other related specie.[16] Up to 10% of studied isolates were reported to be resistant to fluconazole.[17] However, in our study, only 35.84% of Candida albicans isolates were susceptible to fluconazole, which was much lower than other studies. This may be due to the long time span of the strains collected in this study. In fact, with the increase of years, the proportion of high concentration MIC values of Fluconazole increased in *C. albicans.*

Due to the perception of vulvovaginal candidiasis (VVC) as a superficial and readily treatable fungal infection, culture and susceptibility testing are not routinely performed by most gynecologists, but instead empirically apply topical clotrimazole and some vaginal cleans at the initial visit.[2] In addition, patients with VVC have poor compliance with medical advice and often withdraw their medication without an adequate course of treatment. Repeated exposure to azoles, such as fluconazole or over-the-counter (OTC) azoles, is the most likely cause of fluconazole resistance.[18] Our results also showed that the MIC values of triazoles were increased in Candida species, when exposed to clotrimazole.

Maintenance fluconazole is the first-line treatment for RVVC and has been shown to improve quality of life in 96% of women.[19] However, study showed that persistent infection occurred despite 63% of patients had completed maintenance therapy.[20] A randomized controlled trial conducted by Witt A found that monthly cycle-dependent itraconazole is more effective than classic homeopathy in the treatment of RVVC.[21] Our investigation found that itraconazole was relatively effective in the treatment of Candida glabrata. In addition, one patient self-administered carberfungin to treat RVVC and successfully cleared *C. glabrata.*

In addition, the susceptibility of Candida to antifungal agents was measured at a pH of 7, whereas we statistically found that the vaginal pH of VVC patients was about 4.15. The research findings indicated that all tested medications

(fluconazole, itraconazole, miconazole, clotrimazole, terconazole, and nystatin) exhibited higher MICs at pH 4 compared to pH 7.[22] The findings suggest that it is advisable to test the activity of antifungal drugs at a vaginal pH of 4 instead of the laboratory standard of pH 7. This approach is warranted due to the potential presence of clinically significant and unrecognized azole drug resistance, which could contribute to RVVC.

Conclusion

Our study showed a high resistance of Candida to triazole antifungal agents isolated from patients with RVVC. The early use of non-standard antifungal drugs could cause the resistance to fluconazole.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests.

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