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Research Article

The rate of antifungal resistance against Amphotericin B and Miconazole is increasing among *Candida* species isolated from women suffer from recurrent vulvovaginal candidiasis in Iran

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ABSTRACT

Vulvovaginal candidiasis affects at least one of two Females during their life and RVVC is characterized by 3 or more episode of VVC per year. The main causative agent of VVC is *Candida Albicans* with reminder caused by *C.glabrata*, *C.krusei*, *C.parapsilosis*, *C.tropicalis.* The purpose of this study was to isolate *Candida* from VVC patients, characterize it and test its susceptibility to two antifungal medications using the broth microdilution method. Swab samples of patients were obtained and VVC was confirmed by observation of budding yeast by providing direct smear by KOH 3% and also positive culture in Sabouraud dextrose agar. We identified species by phenotyping methods and molecular methods (21 plex PCR) and also assessed the rate of drug resistance by microdilution method against miconazole and Amphotericin B (AMB). Among 75 VVC and 43 RVVC patients, The most common species were *C.albicans* (73.7%), *C.glabrata* (16.9%), *C.krusei* (5.1%), *C. parapsilosis* (3.4%), *C.tropicalis* (0.8%). AMB was active against every isolate from VVC patients except for 18.6% resistance in RVVC patients. In total, only 10.7% of VVC patients were resistant to miconazole whereas 51% of RVVC patients were resistant to miconazole. Therefore, the identification of the causal agent, doing an antifungal susceptibility test (AFST), and genotypic identification will play a crucial role in the optimal selection of antifungal medication for the therapy in RVVC patients, especially those who lake any risk factors.

1. Introduction

Vulvovaginal candidiasis affects at least one of two Females during their human life and is the most frequent infection of the genital tract of them that adversely affect the quality of sexual activity, mental health and life (Campos, de Veerdonk et al., 2018).

RVVC is characterized by 3 or more episodes of VVC within of year; an increasing number of RVVC patients has been documented over the last decade (Gonçalves, Ferreira et al., 2016).

Uncontrolled Diabetes mellitus, endocrine disease, carbohydrate intake, antibiotic therapy, contraceptive use and immunosuppression

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disease are some predisposing factors that are associated with this disease. Opportunistic and commensal fungi, *Candida albicans* is the leading cause of this worldwide disease with reminder caused by *C.glabrata*, *C.krusei*, *C.tropicalis*, *C.parapsilosis* (SH, MR et al., 2008; Alizadeh, Kolecka et al., 2017).

Epidemiological investigations have estimated the prevalence of RVVC patients can be as high as 7-8% of women. Although some predisposing factors are related to RVVC but the main mechanism, underlying RVVC in the host remains undefined (Denning, Kneale et al., 2018; Ghods, Falahati et al., 2018).

Azoles specially Fluconazole and clotrimazole are the first therapeutic line for VVC and RVVC. However, azole resistance species increasing mainly in *Non-Albicans Candida species* (NACC). Therefore, identification of the causative species of *Candida* and investigation of other therapeutic lines for them is so important (Willems et al., 2020).

This study aimed to isolate *candida* species from women suffered from VVC, RVVC and molecular identification of *Candida species* by 21- plex PCR and also antifungal susceptibility testing against (miconazole, Amphotericin B) by broth microdilution method was carried out.

2. Materials and Methods

Patients with symptoms of VVC referred to gynecological centers in Tehran, Iran from 2020 to 2021 and confirmed by a gynecologist, included in this study. Swab samples of them were obtained and VVC was confirmed by observation of budding yeast by providing direct smear by KOH 3% and also positive culture in Sabouraud dextrose agar (SDA).

Antifungal treatment that is ordered by the gynecologist was topical clotrimazole 1% (for 10-12 days) with two fluconazole Tablet (100-150mg) for every 72 hours, if they had not been cured after 3 months, the gynecologist prescribe fluconazole 150 mg doses by weekly for 6 months.

In this study, we followed up the VVC and RVVC patients every 3 months for 1 year and identified species by phenotyping and molecular methods and assessed the rate of drug resistance by microdilution method against miconazole and amphotericin B (AMB)-antifungal drugs.

2.1. Specimen processing

Vaginal swabs from symptomatic patients were collected and transferred to a sterile falcon. After that, we checked the presence of budding yeast by performing a direct smear (KOH3%) and then culturing it on the SDA and Chrome agar *Candida* and incubating for 48 hours in 35°C.

2.2. Molecular method

Total DNA from *Candida species* extracted using puya gene Azuma kit (Tehran, Iran). After that purity and quantity of each DNA samples were investigated by nanodrop and electrophoretic separation on 1.5% agarose gel, respectively.

2.3. PCR condition

PCR was optimized in three multiplexes PCRs as performed previously (Arastehfar, Fang et al. 2019). The first multiplex assay identifies major pathogenic *candida* species (*Pichia kudriavzevii, Candida dubliniensis, Candida albicans, Candida parapsilosis, Candida auris, Candida glabrata, Candida tropicalis*).

The second multiplex assay identifies minor pathogenic *Candida* species *(Debiromyces hansenii, Diutina rugosa, Pichia norvegensis, C Lavispora Lusitaniae, Meyerozyma guilliermondii, Kluyveromyces marxianus, Yarrowia lipolytica)*. And the third multiplex assay recognizes non *Candida* pathogenic yeasts *(Trichsporon asahii, Cryptococcus neoformans, Geotrichum candidum, Cryptococcus deneoformance, Cryptococcus gattii, Rhodotorula mucilaginosa*).

The PCR was adjusted in an eventual volume 50 µL as supplant: 1 µL of DNA sample, 5 µL 10X buffer (No mgcl2, 10xNH4) 2.5 U of Taq enzyme (bio Taq DNA polymerase, Biolab) 1.5 mM Mgcl2, 0.2 mM of mix dNTP (dNTP mix, 100 Mm, biolab) and used D.W to optimized the volume to 50 µL. primers were thoroughly assessed using sightless tests performed in other researches. 50 bp ladder with PCR products were run for 75 min on 2% agarose gel (8 v/cm), indicated by gel red under ultra violet light.

Candida species detected by perception of each fragment band of the PCR products as

mentioned previously (Arastehfar, Fang et al., 2019).

2.4. Antifungal susceptibility test (AFST)

AFST was carried out by CLSI protocol, fourth edition. MIC values were predicted based on the CLSI-M60, second edition.

In RPMI 1640, antifungal drugs were dissolved after 24 hours, and MIC was calculated from plates that had been incubated at 35 c□. MICs were evaluated after 24 hours as AFST, which contained miconazole and AMB. *Candida albicans* (ATCC 10231) and *Candida krusei* (ATCC6258) and *Candida parapsilosis* (ATCC 22019) were employed for quality control objects. MICs of each species were: *C.albicans*, (mico≥16, AMB≥2), *C.krusei* (mico≥16, AMB≥2), *C.glabrata* (mico≥16, AMB≥2), *C.tropicalis* (mico≥1, AMB≥1), *C.parapsilosis* (mico≥0.0016, AMB≥1).

2.5. Statistical evaluations

All statistical calculations were performed with Microsoft SPSS version 22 software.

3. Results

3.1. Patients feature

Out of 300 cases of patients followed during 12 months in our study: 75 cases were classified as VVC and 43 cases were RVVC patients. The median age for patients with VVC was 37 (18-63) year old, whereas the average age for RVVC patients was 35 (20-68) year old. Demographic data of Patients shows in Table 2.

3.2. Molecular identification of Candida specie

In total, 118 yeast isolates were recovered from 75 patients with VVC and 43 patients with RVVC.

The most common species was *C.Albicans* (73.7%) followed by *C.Glabrata* (16.9%), *C.Krusei* (5.1%), *C.Parapsilosis* (3.4%), *C.Tropicalis* (0.8%) ,whereas in VVC patients *C.Albicans* stood the first level 69.3% followed by *C.Glabrata* 20%, *C.Krusei* 4%, *C.parapsilosis* 5.3%, *C.tropicalis* 1.3% and in RVVC 81.4% *C.albicans* followed by *C.glabrata* 11.62%, *C.krusei* 6.98%. As we mentioned above *C.Albicans* was the most causative agent among VVC patients (52 cases from 75 patients) and RVVC patients (35 cases from 43 patients). In addition, *C.Glabrata* was the second causative agent in both groups, while C. *tropicalis* and *C.parapsilosis* were isolated in VVC patients (Table 2).

3.3. Antifungal susceptibility testing

Drug susceptibility testing was performed following the clinical laboratory standards institute fourth edition (CLSI-M27/S3) against AMB and miconazole for all *Candida* spp.

AMB was active against every isolate from VVC patients except for 18.6% resistance in RVVC patients. In total, only 10.7% of VVC patients were resistant to miconazole whereas 51% of RVVC patients were resistant to miconazole (Table 3).

Table 1. Different predisposing factors among VVC and RVVC patients

Underlying disease	Thyroid disorder	Anemia	Diabetes	Fatty liver	Hypertension	Hysterectomy	Cholecystectomy	Ulcerative colitis	Benign breast mass
VVC	3.75=4%	1.75= 1.3%	-	1.75= 1.3%	1.75= 1.3%	1.75= 1.3%	1.75= 1.3%	1.75= 1.3%	2.75= 2.6%
RVVC	2.43= 4.6%	1.43= 2.3%	2.43= 4.6%	-	-	-	-	-	-
Total	5.118= 4.2%	2.118= 1.7%	2.118= 1.7%	1.118= 0.85%	1.118= 0.85%	1.118= 0.85%	1.118= 0.85%	1.118= 0.85%	2.118= 1.7%

Table 2. Total number of *Candida* species recovered from women with VVC and RVVC patients

Total number of patients			
Species	**Total**	**VVC**	**RVVC**
Candida albicans	n = 87 (73.7 %)	n = 52 (69.3%)	n = 35 (81.4%)
Candida glabrata	n = 20 (16.9%)	n = 15 (20%)	n = 5 (11.62%)
Candida krusei	n = 6 (5.1%)	n = 3 (4%)	n =3 (6.98%)
Candida parapsilosis	n = 4 (3.4%)	n = 4 (5.3%)	0
Candida tropicalis	n = 1 (0.8%)	n = 1(1.3%)	0
Total	n = 118 (100%)	n = 75 (100%)	n = 43 (100%)

Table 3. AFST for each *Candida* isolates from VVC and RVVC Patients resistance

Antifungal susceptibility profile of each *Candida* species				
Species	VVC (n=75)		RVVC (n=43)	
Drugs	Miconazol resistance %	AmB resistance %	Miconazol resistance %	AmB resistance%
C. albicans	2 (2.6%)	0	19 (44.2%)	6 (14%)
C. glabrata	2 (2.6%)	0	2 (4.7%)	1 (2.3%)
C. krusei	3 (4%)	0	1 (2.3%)	1 (2.3%)
C. parapsilosis	1 (1.3%)	0	0	0
C. tropicalis	0	0	0	0
Total	8 (10.7%)	0	22 (51.2%)	8 (18.6%)

4. Discussion

Vulvovaginal candidiasis is the most prevalent fungal infection in human that affect nearly 75% of women at least once in their lifetime. Whereas, in some cases, it changes to recurrent VVC (defined as 3 episodes per year) that infects approximately 8%of women (Campos, de Veerdonk et al., 2018; Lionakis and Levitz, 2018).

Risk factors for VVC and RVVC include immunosuppression, uncontrolled diabetes mellitus, antibiotic therapy, pregnancy, and genetic predispositions (Gonçalves, Ferreira et al., 2016).

Candida species are the causative agent for this common mucosal infection of the female reproductive tract, among them *Candida albicans* is the main cause of it whereas the none *C.albicans* species (*C.glabrata*, *C.krusei* and *C.tropicallis*) are becoming more prevalent, respectively (Lionakis and Levitz, 2018).

In this study of 118 total patients, 36/5% were RVVC patients that 74% of them affecting by *C.albicans*. The studies investigation of the yeast species that cause vaginitis in Iranian people found that *C. albicans* was the most prevalent causative agent. Our findings are in accordance with the previous data.

C.glabrata is typically the second-most frequent cause of VVC reported by several global studies that are the same as our study (20% affected by *C. glabrata*)

The AFST profile is different in several studies (Deorukhkar and Saini, 2013; Gandhi, Patel et al., 2015; Ghods Falahati et al., 2017; Arastehfar, Kargar et al., 2021). The assessment of miconazole and AMB susceptibility in *Candida* isolates from VVC and RVVC patients showed that 10/7% miconazole resistant in VVC patients and 51/2% miconazole resistant in RVVC patients whereas 74%of this resistance referred to *C. Albicans*. These results indicated the increasing prevalence of azole resistance species in Iran due to the elevation of OTC prescriptions and administering the azole by a gynecologist without doing MIC test that can be converted to an apprehensive subject for converting to a recurrent form.

Moreover, 18/6% of RVVC patients were AMB resistant and this is a serious problem for Iranian women with the appearance of azole-resistant species that would be due to continual prescription of azole and the high rate of consumption of OTC drugs by them.

In this study, all *Candida* species that cause VVC were sensitive to AMB which was consistent with other studies. As well as, in a study by Zaidi et all, 2018 that assessed AFST of *C.albicans* in human infections, a high percentage of fluconazole resistance and some azole resistance were reported

was consistent with our study (Zaidi, Mani et al., 2018).

In addition, because <2% of VVC and RVVC patients with predisposing factors, so, the main mechanism underlying RVVC in the host remains unknown. Recent studies have highlighted the role of the genetic basis of the monogenic defect and polymorphisms associated with disease and provided cellular and molecular insights into the host-fungus interaction and mechanism of host defense against fungus (Campos, de Veerdonk et al., 2018).

Several studies focused on the role of heritable factors perhaps the main component distinguishing susceptibility to RVVC.

The Presence of single nucleotide polymorphism (SNP) in the innate immune system against *Candida* was defined by several studies in the last decade. Such as, SNP in pattern recognition receptors (PRRs) on innate immune cells on the surface of *Candida* and SNPs in PAMPs of innate immune cells in the host like dectin1, TLR1, MBL, SNPs in CARD9, BCL10 intracellular signalling transducer molecules and finally SNPs in secreted molecules from the host immune cells such as PTX3, antimicrobial peptides (AMP)(Rosentul, Delsing et al., 2014, Parente, Doni et al., 2020).

Therefore, finding the main mechanism underlying RVVC in the host without any risk factors such as immunosuppression, uncontrolled diabetes mellitus, antibiotic therapy, cancer and etc. can help us to control the increase of RVVC.

Conclusion

This study gives details on the antifungal susceptibility and species pattern of *Candida* isolates from VVC and RVVC patients from some gynecological centers in Tehran, Iran.

As we told above, *C.albicans* was the primary cause of VVC with growing percentage of NACC levels. (*C.glabrata* 16.9%)

In this investigation, we used two significant antifungal drugs for AFST and we investigate the rate of resistance to these drugs in *Candida* species. Our results show that in RVVC patient's 18.6% AmB resistance and 51.2% miconazole resistance suggest that Iranian women are more likely to be prescribed OTC medications and common antifungals in the absence of species identifications and AFST.

Therefore, the identification of the causal agent, doing AFST, and genotypic identification will play a crucial role in the optimal selection of antifungal medication for the therapy in RVVC patients, especially those who lake any risk factors.

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Refereces

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