Genotyping and Drug Resistance Profile of Clinical Isolates of *Candida Albicans* From Vulvovaginal Candidiasis in The Eastern China

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Abstract

A total of 244 *Candida albicans* isolates recovered from VVC patients in Suzhou, Eastern China, were investigated. According to CLSI documents M27-A4 and M59-3ed / M60-2ed, the geometric mean MICs of nine antifungals in increasing order were micafungin(0.048mg/L), anidulafungin (0.132mg/L), caspofungin (0.19mg/L), itraconazole(0.23mg/L), posaconazole(0.25mg/L), voriconazole(0.28mg/L), 5-flucytosine (0.44mg/L), amphotericin B (0.49mg/L) and fluconazole (2.01 mg/L) respectively. Of note, 6.5% (16/244) *C. albicans* isolates showed resistance mainly to anidulafungin (mono-echinocandin resistance), while voriconazole had the lowest susceptibility rate of 34.8% (85/244), followed by fluconazole 59.4% (145/244), respectively. All isolates were genotyped by allelic combination of 3 microsatellite markers (CEF3, CAIII and LOC4). A total of 129 different allelic genotypes were identified, in which seven different clades were recognized with a discriminatory power of 0.96. Genotype A-D were present in 35% of the isolates. In conclusion, decrease of antifungal drug susceptibility to *C. albicans* isolates from VVC is alarming. Our findings revealed the genetic diversity of *C. albicans* isolates among VVC patients and provided insights into the molecular epidemiology of *Candida* infections in China.

Introduction

Vulvovaginal candidiasis (VVC) is one of the most common vaginitis that caused by *Candida albicans*, which accounts for 80–95% of all episodes of VVC worldwide, though the number of non-*albicans* species (such as *C. glabrata*) are increasing as etiological agents of VVC[1, 2]. An increasing prevalence of fungal resistance is also reported in antifungal surveillance studies globally[3–5]. Antifungal susceptibility testing of *C. albicans* isolates therefore plays a crucial role for appropriate and effective management strategies of VVC [5]. In addition, it is important to look into the population genetic characteristics of *C. albicans* infections owing to increasing reports indicating dynamic antifungal susceptibility profile with different genotype and geographic origin[6]. Thus, understanding the genetic diversity of *C. albicans* could help clinicians to implement appropriate diagnostic, therapeutic and preventive strategies[7, 8]. Microsatellite marker is highly reproducible with strong discriminatory potential and is widely applied in molecular typing [9]. In previous studies, microsatellite analysis has proved itself to be a powerful tool in investigating the relationship between genetic diversity and antifungal susceptibility of *C. albicans* isolates [10, 11]. However, population genetic and antifungal susceptibility profile of isolates from VVC patients in China still remain fragmented and limited. Therefore, in this study, we investigated the antifungal susceptibility profile based on M27-A4 and M59/M60 documents approved in 2020 for interpretive breakpoints and ECV, and performed molecular typing utilizing three microsatellite loci (CAIII, CEF3, and LOC) on a hundreds of *C. albicans* isolates from VVC patients in eastern China (Suzhou area).

Materials And Methods

Isolates and Identification

A total of 244 vaginal *C. albicans* isolates were recovered from patients with Vulvovaginal candidiasis in the People's hospital of Suzhou New District during 2018 in this study. The patients and case definition, vaginal samples collecting information have been reported in previous publication[5]. All isolates were identified to the species level by sequencing 26S ribosomal DNA gene D1/D2 domains as described previously[5]. Isolates information and GenBank accession numbers of D1/D2 sequences are listed in Supplementary table1.

Microsatellite analysis

Microsatellite genotyping was performed on all 244 *C. albicans* isolates, with a panel of three different short-nucleotide repeat fragments, using fluorescently labeled primers CAIII (5-Tamra -TTGGAATCACTTCACCAGGA-3, 5-TTTCCGTGGCATCAGTATCA-3); CEF3 (5- Hex-TTTCCTCTTCCTTTCATATAGAA-3, 5- GGATTCACTAGCAGCAGACA-3); LOC4 (5- FAM –GTAATGATTACGGCAATGAC-3, 5-AGAACGACGTGTACTATTGG-3) [11]. A multiplex polymerase chain reaction (PCR) was performed in 10μl reaction volumes containing 5μl of Qiagen Multiplex PCR (2x, Lot 148031955), 0.25μl of each primer (F+R), 3μl of ddH2O, and 1μl of genomic DNA. PCR amplifications were performed in a thermocycler (BOECO, TC-Pro, Germany) operated with a temperature-cycling program that consisted of an initial denaturing step at 95◦C for 15 min, followed by 35 cycles of 30 s at 94◦C, 90 s at 57◦C, and 60 s at 72◦C. The final extension step was for 10 min at 72◦C. The sizes of the fragments were determined by addition of the GeneScan LIZ [500] marker and subsequent analysis of the fragments on the Applied Biosystems 3730 DNA analyzer. Assignment of repeat numbers in each marker was determined from the GeneScan data by using the Peak GeneMapper5.0 software (Applied Biosystems, Foster City, CA, USA). The sizes of the fragments were determined based on the LIZ500 marker, Allele-sharing distance matrices were generated from the tandem repeat numbers and were used as input for UPGMA clustering analysis. The UPGMA clustering of the 244 *C. albicans* isolates was performed using R package phangorn. The UPGMA tree was then plotted using R (version 3.4.4). The discrimination power (DP) of the microsatellite genotyping method used in this study, which calculates the probability of any pair of isolates to have different genotypes, was calculated by the online calculator created by the university of the basque country (http://insilico.ehu.es/mini_tools/discriminatory_power).

Antifungal Susceptibility Testing

All isolates were tested for *in vitro* antifungal susceptibility to 9 antifungal agents according to the CLSI reference guideline M27-A4[12]. Antifungal drugs tested were anidulafungin (ANF), caspofungin (CAS), micafungin (MFG), amphotericin B (AmB), 5-flucytosine (5-FC), fluconazole (FLC), itraconazole (ITC), voriconazole (VRC), posaconazole(POS). Anidulafungin, and voriconazole were purchased from Toronto Research Chemicals Inc,Canada, micafungin was provided by Astellas Pharma,Japan, and remaining antifungals were obtained from Sigma-Aldrich. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as control strains in all experiments. All isolates were sub-cultured onto Sabouraud Dextrose Agar at 35 °C for 24h for viability and purity. Colonies were suspended in sterile saline, and the final inoculum concentration of the suspension was adjusted to 0.5-2.5 x 10^3 CFU mL^{-1} with RPMI1640

broth medium. The 96-well plates were incubated for 24 or 48 h at 35°C, and minimum inhibitory concentrations (MIC) were determined visually. Drug concentration ranges, time of MIC reading and interpretive breakpoints used for 9 antifungal agents are listed in Supplementary table 2.

Interpretation of MIC results

Interpretation of susceptibility was performed by applying the clinical breakpoints (CBPs) defined by the document M60-2ed[13]. In the absence of CBPs, isolates were defined as having a wild-type (WT) or a non-wild-type (NWT) drug susceptibility phenotype (to amphotericin B, posaconazole, itraconazole and 5-flucytosine) according to the epidemiological cutoff values (ECV)defined by the document M59-3ed[14], as shown in Supplementary table 2.

Ethical statement

Ethical approval and patient consensus were not considered necessary due to the descriptive nature of the study that implied only the samples obtained during routine laboratory activity.

Results

Table 1 summarized the antifungal susceptibility profile of 244 *C. albicans* isolates to 9 antifungals. The Geometric Mean MIC of the antifungals across all isolates were the following (in increasing order): micafungin(0.048mg/L), anidulafungin(0.132mg/L), caspofungin(0.19mg/L), itraconazole(0.23mg/L), posaconazole(0.25mg/L), voriconazole(0.28mg/L), 5-flucytosine (0.44mg/L), amphotericine B (0.49mg/L) and fluconazole (2.01mg/L).

Table 1 Antifungal susceptibility profile of 244 C. albicans isolates from VVC to 9 antifungal agents.

Drug	Range	MIC_{50}/ MIC_{90}	GM	S(%)	I (%)	SDD (%)	R (%)	NWT (%)
MFG	0.016-1	0.0313/0.25	0.048	227/93.0%	15/6.2%	NA	2/0.8%	NA
ANF	0.016-4	0.125/0.5	0.132	210/86.1%	18/7.4%	NA	16/6.5%	NA
CAS	0.0313-1	0.25/0.25	0.191	222/91.0%	21/8.6%	NA	1/0.4%	NA
FLC	0.125-64	2/16	2.011	145/59.4%	NA	36/14.8%	63/25.8%	NA
VRC	0.125-4	0.25/1	0.278	85/34.8%	126/51.6%	NA	33/13.5%	NA
POS	0.0313-4	0.25/1	0.251	NA[a]	NA	NA	NA	221 / 90.5%
ITR	0.0313-16	0.25/1	0.232	NA	NA	NA	NA	NA
AmB	0.25-1	0.5/0.5	0.485	NA	NA	NA	NA	0/0%
5-FC	0.125-64	0.25/2	0.436	NA	NA	NA	NA	NA

Note: a: not applicable; GM: geometric mean values; S%: susceptible rate; I%: intermediate susceptible rate; SDD%: susceptible dose dependent rate; R%: resistant rate; NWT%: rate of non-wild type.

Table 2 summarized MIC distribution, R% and NWT% of 244 *C. albicans* isolates from VVC to 9 antifungal agents. Of the 244 *C. albicans* isolates, 86% (210/244) - 93% (227/224) isolates were susceptible to the three echinocandins tested, and 6.5% of the *C. albicans* isolates were resistant to anidulafungin which was much higher than those of two other echinocandins tested (0.4% and 0.8%, respectively).

Table 2. MIC distribution, R%, NWT% of 244 *C. albicans* isolates from VVC to 9 antifungal agents.

Drug	0.016	0.0313	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	≥64	R%	NWT%
MFG	16.4	61.5[a]	80.7	87.3	93.0[b]	99.2	100.0	100.0	100.0	100.0	100.0	100.0	100.0	0.8%	38.50%
ANF	3.3	8.2	37.3	67.6	86.1	93.4	97.1	99.2	100.0	100.0	100.0	100.0	100.0	6.5%	32.4%
CAS	0.0	2.9	10.7	34.4	91.0	99.2	100.0	100.0	100.0	100.0	100.0	100.0	100.0	0.4%	NA
FLC	0.0	0.0	0.0	5.7	13.9	25.4	42.6	59.4	74.2	86.1	92.2	99.6	100.0	25.8%	74.6%
VRC	0.0	0.0	0.0	34.8	66.0	86.5	98.0	99.2	100.0	100.0	100.0	100.0	100.0	13.5%	100%
POS	0.0	5.3	23.0	42.2	59.4	81.6	90.2	97.5	100.0	100.0	100.0	100.0	100.0	NA[c]	90.5%
ITR	0.0	14.8	24.2	39.8	60.2	84.4	93.0	95.9	98.8	99.6	100.0	100.0	100.0	NA	NA
AmB	0.0	0.0	0.0	0.0	11.5	93.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	NA	0
5-FC	0.0	0.0	0.0	35.7	51.6	66.0	76.2	95.5	98.0	98.8	99.2	98.2	100.0	NA	NA

Note: a: percentage of isolates with MIC≦ECV; b: percentage of isolates with MIC≦susceptible CBP; c: not applicable; R%: resistant rate; NWT%: rate of non-wild type

The *in vitro* activity of triazoles against 244 isolates of *C. albicans* was variable; Fluconazole was active against 59.4% (145/244) isolates tested, and 25.8% (63/244) of the *C. albicans* isolates were fluconazole resistant. However, 14.8% (36/244) of the isolates were fluconazole SDD. Voriconazole had reduced activity to approximately more than half of the isolates with susceptibility rate 34.8% (85/244), whereas 51.6% (126/244) isolates were intermediate susceptibility and13.5% (33/244) were resistant to voriconazole. Resistance to both fluconazole and voriconazole were found in 23 isolate of *C. albicans*. However, 90.5% isolates of the *C. albicans* showed relatively higher MICs than epidemiological cutoff value (ECV 0.06 mg/L) to posaconazole, when testing the susceptibility to itraconazole, 60.2% isolates had MICs value >0.125 mg/L, and 15.6% isolates had MICs value>0.5mg/L.

As expected, all *C. albicans* isolates tested revealed lower MICs than ECV (2 mg/L) to amphotericin B, while 98.0% of isolates showed lower MIC than 4mg/L to 5-flucytosine.

The genotypic relationship of 244 C. albicans isolates from VVC patients was determined based on UPGMA analysis of 3 microsatellite markers CEF3, CAIII, LOC4 . Seven different clades were recognized (Figure 1). Microsatellite genotyping of three loci showed considerable diversity among 244 *C. albicans* isolates, and 129 different allelic combinations were identified among 244 unrelated *C. albicans* isolates with 108 singleton genotypes. The combined discriminatory power (DP) of the 3-loci (CAIII, CEF3, and LOC4) typing method was 0.96. The most frequent genotype was genotype A (34/244, 13.9%) followed by genotype B (25/244, 10%) and C (18/244, 7%), respectively. The other remaining genotypes had a frequency less than 5% Supplementary table 1.

Discussion

This study investigated the antifungal susceptibility profile and genetic diversity of 244 *C. albicans* isolates from VVC patients in Suzhou, eastern China.

The majority of *C. albicans* isolates in this study showed good antifungal activity to the three echinocandins. Micafungin (MIC_{50}/MIC_{90}: 0.031/0.25mg/L) showed slightly higher potency than caspofungin (MIC_{50}/MIC_{90}: 0.25/0.25 mg/L) and anidulafungin (MIC_{50}/MIC_{90}: 0.125/0.5 mg/L) which were consistent with previous studies[5, 15]. Of note, less than 10% *C. albicans* isolates was found to have intermediate susceptibility to three echinocandins tested, whereas 16 in 244 (6.5%) *C. albicans* isolates showed resistant mainly to anidulafungin(mono-echinocandin resistance), furthermore, of the 16 isolates of *C. albicans* with high-MIC anidulafungin phenotype(MICs≧1 mg/L), only one isolate had MICs≧1 mg/L for micafungin, and another one isolate had MICs≧1 mg/L for caspofungin, which is not in agreement with those reported previously that *C. albicans* resistant to echinocandins accounted for less than <1% [6, 16] and the mono-echinocandin resistance phenotype was rare[17, 18]. However, our previous study reported one isolate with MIC 1mg/L for anidulafungin among 207 *C. albicans* isolates from VVC patients in western China[5]. Pfaller et al. presented similar results on anidulafungin against *C. albicans* causing invasive infections [19]. Lindberg et al.[20] determined the *in vitro* susceptibility of *Candida* isolates from the blood samples of patients with candidemia at a Swedish hospital and found that 17% *C. albicans* isolates were not susceptible to anidulafungin by applying the EUCAST CBPs, however, when the CLSI CBPs were applied, all the isolates exhibited susceptibility to anidulafungin. Thus, isolates with this high anidulafungin MIC warrant further study.

All triazoles tested had reduced activity against *C. albicans* isolates from VVC patients. Voriconazole had the lowest susceptibility rate of 34.8% (85/244), followed by fluconazole 59.4% (145/244), respectively. Of note, about half of *C. albicans* isolates (51.6%) tested were classified as intermediate susceptibility to voriconazole, whereas 25.8%, 13.5% of the isolates were resistant to fluconazole and voriconazole, respectively. Compared to our previous study in western China[5], fluconazole resistance of *C. albicans* isolates from VVC significantly increased in eastern China (8.2% Resistaant rate in western China versus 25.8% Resistaant rate in Suzhou, eastern China). Our results were also compatible with the most Chinese reports which presented approximately half isolates of *C. albicans* causing VVC were susceptible to fluconazole [21-23]. Notably, percentage of isolates on resistance and I/SDD to fluconazole and voriconazole in Suzhou are much higher than those in previous reports from Dota KFD et al.[15], Ying C et al.[21] and Shi et al.[23].

The MIC values for posaconazole in present study were overall high with 90.5% NWT isolates which was higher than 60% NWT isolates found in our previous study in west area when the CLSI ECV (0.06mg/L) was applied. Our findings is contrary to those from northern America[24] and Kuwait[25] which showed good activity of posaconazole against *C. albicans* isolates from VVC (MIC_{90}: 0.03mg/L and 0.064 mg/L, respectively). However, 37.6% NWT isolates to posaconazole were reported from invasive candidiasis[26]. Although no interpretive breakpoint for itraconazole to *C. albicans* based on the newly described CLSI breakpoints[13, 14], it must be recognized as distinctly unusual, given that 60% isolates had MICs value >0.125 mg/L, and 15.6% (38/244)isolates had MICs value≧1mg/L(Table 2). Overall, resistance to triazoles among *C. albicans* isolates was found to have increasing significantly with time. It could be associated with relatively high frequency use of those azoles in clinical settings in Suzhou area[27], Therefore, continued surveillance on changes of antifungal susceptibility are necessary to guide treatment of VVC.

Microsatellite genotyping of three loci showed considerably high diversity, and 129 different allelic combinations were identified among 244 isolates of *C. albicans* recovered from patients with VVC. We observed that the dominant genotype A-C (77/244) accounted for 31.5 % of the isolates, however, a total of 108 isolates were shown to represent unique molecular type which account for 44% isolates tested, indicating high genetic diversity within the isolates in this study (Supplementary table 1). Seven clades were recognizable based on a categorical analysis of CEF3, CAIII, LOC4 microsatellite markers in combination with UPGMA clustering (Fig.1). Our results confirmed high genetic diversity among *C. albicans* isolates which is consistent with previous studies[10, 11]. The relatively high genetic variability among C. albicans samples may be related to high dynamism of the *C. albicans* genome with frequent translocations, deletions, and duplications.

Conclusions

Drug resistances of *C. albicans* presents significant challenges to implement appropriate therapies and treatment for VVC. Therefore, antifungal susceptibility testing of *Candida* isolates plays a crucial role in the management of *Candida* infections. The microsatellite data of *C. albicans*, confirmed that this medically important yeast has maintained high levels of genetic diversity in Eastern China.

Declarations

Author Contributions: Conceptualization, N.H., N.Y. & SW.D.; Methodology, Y.L., L.Y, H.Z., R.F., DY.H., G.W. & XF. C.; data analysis, N.H. & H.C.; writing—original draft preparation, SW.D., N.H. & N.Y.; writing—review and editing, SW.D. & H.C.; visualization, D.H. & H.C; supervision, WQ.L. & SW.D. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Sequence data used in this study was available in Genbank under accession number MZ172462-MZ172702 and MZ226435-MZ226437.

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Figures

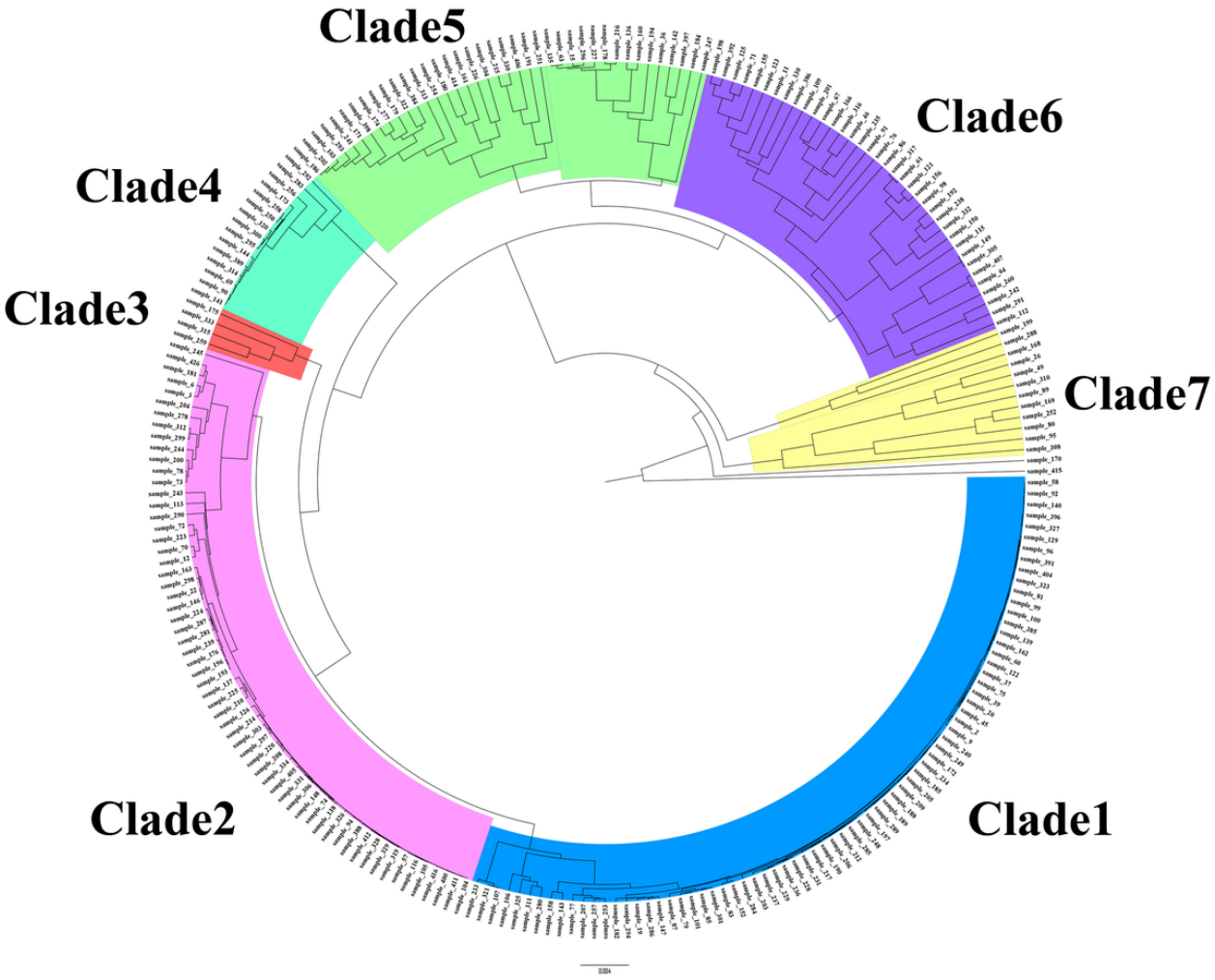

Figure 1

UPGMA clustering dendrogram of the 244 C. albicans isolates from VVC patients based on the sequences of CEF3,CAIII and LOC4 microsatellite makers

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarytable1.docx
- Supplementarytable2.docx