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ORIGINAL PAPER

Comparative Study of the Effects of Fluconazole and Voriconazole on *Candida glabrata*, *Candida parapsilosis* and *Candida rugosa* Biofilms

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Abstract Infections by non-*albicans Candida* species are a life-threatening condition, and formation of biofilms can lead to treatment failure in a clinical setting. This study was aimed to demonstrate the in vitro antibiofilm activity of fluconazole (FLU) and voriconazole (VOR) against *C. glabrata*, *C. parapsilosis* and *C. rugosa* with diverse antifungal susceptibilities to FLU and VOR. The antibiofilm activities of FLU and VOR in the form of suspension as well as pre-coatings were assessed by XTT [2,3-*bis*-(2-methoxy-4-nitro-5-sulfophenyl)-*2H*-tetrazolium-5-carboxanilide] reduction assay. Morphological and intracellular changes exerted by the antifungal drugs on *Candida* cells were examined by scanning electron microscope (SEM) and transmission electron microscope (TEM). The results of the antibiofilm activities showed that FLU drug suspension was capable of killing *C. parapsilosis* and *C. rugosa* at minimum inhibitory concentrations (MICs) of 4× MIC FLU and 256× MIC FLU, respectively. While VOR MICs ranging from 2× to 32× were capable of killing the biofilms of all *Candida* spp tested. The antibiofilm activities of pre-coated FLU were able to kill the biofilms at ¼× MIC FLU and ½× MIC FLU for *C. parapsilosis* and *C. rugosa* strains, respectively. While pre-coated VOR was able to kill the biofilms, all three *Candida* sp at ½× MIC VOR. SEM and TEM examinations showed that FLU and VOR treatments

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exerted significant impact on *Candida* cell with various degrees of morphological changes. In conclusion, a fourfold reduction in MIC_{50} of FLU and VOR towards ATCC strains of *C. glabrata*, *C. rugosa* and *C. rugosa* clinical strain was observed in this study.

Keywords Biofilms · Non-*albicans Candida* · Fluconazole · Voriconazole · XTT assay · Electron microscopy

Introduction

There is an increasing incidence in invasive fungal infections over the last decade due to the rise in the population of immunocompromised patients. Most opportunistic fungal infections are caused by the genus *Candida*, which includes superficial infections like vaginal and oral infections, and deep-seated systemic infections of the bloodstream and internal organs. They are present as normal microbiota on the skin, mouth, large intestines, urinary and reproductive systems [1]. They can cause diseases in humans when the physiological balance of the body is upset or the host's defence is in a compromised state. One of the notorious virulence factors of *Candida* is its ability to adapt to various habitats and form surface-attached communities known as biofilms. Total parenteral nutrition, increased usage of broad-spectrum antibiotics, cytotoxic chemotherapies and the use of intravenous catheters are among the factors that contribute to increased *Candida* infections [2]. Indwelling medical devices such as catheters, implants, heart valves, ocular lenses, artificial joints and shunts act as substrates for biofilm formation of *C. albicans* which routes to candidiasis in the patients [3–5]. The ability to form biofilms differs from one species to another [6]. However, the growth confluence within strains of the same species may also vary. Many in vitro models using plastic materials to form biofilms that mimic the medical devices used in patients have been studied. *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis* are able to form biofilms on any biomaterials implanted in patients and were reported to exhibited high resistance to the antifungals. In addition to implant devices, contact lenses and retinal implants using glass materials may also lead to *Candida* biofilm infections [7].

Biofilms tend to cause recurrent and invasive candidiasis which is categorized as difficult-to-treat infections. Sessile cells of *C. albicans* biofilms are less susceptible to antibiotics as compared to their planktonic counterparts [8]. All these factors pose a serious threat to the health care system within which medical costs and duration of hospitalization will be increased [9]. Despite the infections caused by *C. albicans*, infections due to non-*albicans Candida* were also constantly being reported from various parts of the world. Of several non-*albicans Candida* spp known, *C. glabrata* which is intrinsically more resistant to FLU [10], *C. parapsilosis* which has emerged as a significant nosocomial pathogen [11–13], *C. rugosa* and *C. guilliermondii* which were very uncommon/infrequent but have been isolated from patients with candidiasis [10, 14, 15] are of much clinical significance. Co-existence of *C. albicans* and *C. rugosa* biofilms was reported in denture liners [16], especially from intrauterine devices (IUD) [17]. The increase in drug-resistant strains and the prevalence of non-*albicans Candida* spp trigger a necessity to search for contemporary methods to prevent biofilm formation by *Candida* sp.

One way of controlling biofilms in hospital settings is by employing antifungal lock therapy (AfLT) strategies with various antifungal agents which can effectively prevent *C. albicans* biofilms [18–20]. The idea of AfLT involves coating of intravascular devices with appropriate antibiotics prior to inserting them into patients. This method is implemented to minimize or prevent the formation of biofilms and its associated infections [21]. Promising results have been reported with the use of antifungal agents such as ethanol [20], EDTA + amphotericin B complex [22] and echinocandins [21].

Furthermore, the use of electron microscopy to study the morphological changes in a cell is an inclusionary approach to study the effects of FLU and VOR on *Candida* cells. Immunoelectron microscopy, ultra-high resolution field emission scanning electron microscope (FESEM), focused ion beam coupled with SEM were also found to be ideal to study various properties in a yeast model.

Triazoles like FLU are known to be less effective against *C. glabrata* and *C. krusei* [23]. Although *C. krusei* is intrinsically resistant to FLU, in *C. glabrata* various strategies are used to evade being destroyed by FLU [24]. Besides, majority of the clinical *C. glabrata*

causing bloodstream infections (BSIs) were resistant to FLU and VOR [25]. *C. parapsilosis* is another species of *Candida* that has been increasingly associated with contaminated devices or total parenteral nutrition [26]. This particular species is known to colonize the hands of health care workers which may lead to nosocomial infections. In the present study, antifungal susceptibility test was performed prior to select three categories of strains which correspond to the CLSI guidelines, namely susceptible, susceptible dose-dependent (SDD) and resistant to FLU/VOR. The effect of FLU and VOR on the biofilm formation and cellular morphology of FLU-resistant strain of *C. glabrata*, FLU-SDD strain of *C. rugosa* and a FLU-susceptible strain of *C. parapsilosis* were assessed. A scoring system was designed to determine the changes that occurred to the cellular morphology of the selected *Candida* species under SEM. We also report the intracellular changes exerted by different concentrations of FLU and VOR.

Materials and Methods

Strains and Antifungal Drugs Used

Clinical isolates of *C. glabrata*, *C. parapsilosis* and *C. rugosa* that were obtained from University Malaya Medical Centre (UMMC) and Gleneagles Intan Medical Centre (GIMC) in Kuala Lumpur, Malaysia, were used in this study. *C. glabrata* was selected because of its FLU resistance, *C. parapsilosis* being the second most common cause of *Candida* infections worldwide and *C. rugosa* as it is a rare/infrequent clinical isolate. *C. glabrata* ATCC 14053, *C. parapsilosis* ATCC 22019 and *C. rugosa* ATCC 10571 were used as reference strains. FLU (Sigma-Aldrich, USA) and VOR (Glenmark Generics Limited, Gujarat, India) antibiotics were received in powder form. Test antifungal agents were reconstituted in dimethyl sulfoxide (DMSO) and subsequently diluted in RPMI-1640 medium (Sigma-Aldrich, USA) to obtain a high concentration of 64 and 16 μg mL^{-1}. The concentrations used for FLU were 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32 and 64 μg mL^{-1} and for VOR were 0.03125, 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8 and 16 μg mL^{-1}. These concentrations adhere to the guidelines recommended by CLSI to perform broth microdilution method [27] and the MIC breakpoints recommended by CLSI guidelines were followed.

Antifungal Susceptibility Tests

For metabolic activity experiments using FLU and VOR, MICs of test antifungals for planktonic cells were determined prior to biofilm experiments. MICs were determined by micro-broth dilution method following the guidelines recommended by CLSI in Document M27-A3 [27] with slight modifications in the inoculum size. An inoculum size of 1×10^6 cells mL^{-1} was used which could be compared with the biofilm results. From the results obtained, 3 clinical strains with varying azole susceptibilities were selected for biofilm studies.

Biofilm Metabolic Assay

Biofilms of *C. glabrata*, *C. parapsilosis* and *C. rugosa* were grown in vitro according to the previously described methods [28, 29]. Briefly, a volume of 100 μL of standardized inoculum size (1×10^6 cells mL^{-1}) was pipetted into the wells of 96-well round-bottom plates (Greiner bio-one, Germany). Between 2 and 4 replicates were used for each concentration of the drugs. The plates were incubated at 37 °C for 48 h without shaking. The supernatant was aspirated out carefully using a multichannel pipette and washed with PBS. The metabolic activities of FLU and VOR were quantified by XTT reduction assay. Using a multichannel pipette, a volume of 100 μL of the XTT/menadione solution was added to each well and the plates were covered with aluminium foil and incubated at 37 °C for 2–3 h in dark. Approximately 75–80 μL of the resulting supernatant from each well (coloured orange) was transferred into a fresh microtitre plate and measured at 490 nm in a microplate reader. Simultaneously, another set of experiment was performed by coating of FLU and VOR to the microtitre plates at the same concentration used in the metabolic assay above [18]. The plates were sealed and incubated at 4 °C for 24 h without shaking. The wells were washed with sterile PBS followed by seeding of fungal inoculum. Biofilms were allowed to over 24 h. After incubation, planktonic cells were removed by aspiration and washed with sterile PBS. The biofilm inhibitory effects of FLU and VOR were expressed as the percentage of the

optical density of FLU/VOR-treated wells compared to control wells (no drug).

Ultrastructure Microscopical Analysis

Preparation of Antifungal-Treated Candida Cells

To study the ultrastructural changes exerted by FLU and VOR on test *Candida* strains, cells were treated with 10× MIC_{80} of FLU and 1× MIC_{80} of VOR. In terms of MIC_{80} treatments with FLU and VOR, *C. parapsilosis*, *C. glabrata* and *C. rugosa* were treated with FLU at concentrations of 10, 960 and 160 μg mL^{-1}, while *C. parapsilosis*, *C. glabrata* and *C. rugosa* were treated with VOR at concentrations of 1, 2 and 1 μg mL^{-1}, respectively (Table 1). Yeast samples for electron microscopy were prepared by following the previously described methods [30, 31]. One mL of the drug concentration was added to 1 mL of standardized inoculum (1×10^6 cells mL^{-1}), according to the CLSI guidelines. The cultures were incubated at 35 °C for 48 h, and aliquots of 5 mL were placed onto each well of a 6-well polystyrene tissue culture plate. Samples were treated with FLU and VOR for 4 h, and the cells were harvested by centrifugation and washed twice with 0.1 M PBS (pH 7.2) before proceeding for SEM and TEM analysis. *Candida* cells without any drugs were included as controls.

SEM

Yeast cells were fixed with glutaraldehyde (4%) for 12–24 h, washed thrice with 0.1 M sodium cacodylate buffer and post-fixed in 0.1 M Osmium tetroxide (OsO_4) for 2 h at 4 °C. Following fixation, samples were dehydrated in a graded acetone series (35–100%), mounted using double-sided tape and subjected to critical point drying (CPD 030, Bal-TEC, Switzerland) and gold coating in a sputter coating unit (E5100 Polaron, UK). The specimens were examined in a SEM (JEOL JSM-6400, Japan) at 15 kV, and the images were recorded with a SemAfore Programme.

TEM

For TEM analysis, yeast cells were washed with PBS and fixed in 5% bovine serum for 2 h prior to the experiment. Samples were fixed with glutaraldehyde and post-fixed similar to SEM sample preparation as described in "SEM" section. Samples were dehydrated with a series of acetone grade (35, 50, 75, 95 and 100%) for 10–15 min each and infiltrated with increasing concentrations of acetone/resin mixture. Epoxy resin-embedded samples were subjected to ultramicrotome, and the ultrathin sections (70–90 nm) were double-stained with uranyl acetate and lead citrate. The specimens were examined under a Leo 912ab TEM.

Morphology Scoring and Analysis

In this study, a scoring system was developed to evaluate the morphologies of FLU and VOR-treated *Candida* cells (Table 5). Approximately 35–40 cells were observed under 10,000× magnification from at least 5 randomly selected fields. The cell morphologies were assessed using a scoring system obtained by a total grade based on the following three parameters:

S1 No changes in the cell (oval-shaped cells or elongated pseudohypha with smooth surface and intact cell membrane).

Table 1 Concentrations of FLU and VOR used for electron microscopy studies

Clinical *Candida* species and strain susceptibility	FLU concentrations (μg mL^{-1})	VOR concentrations (μg mL^{-1})
C. parapsilosis (FLU-susceptible)	$MIC_{80} = 1$	$MIC_{80} = 1$
	10× $MIC_{80} = 10$	
C. glabrata (FLU-resistant)	$MIC_{80} = 96$	$MIC_{80} = 2$
	10× $MIC_{80} = 960$	
C. rugosa (FLU-SDD)	$MIC_{80} = 16$	$MIC_{80} = 1$
	10× $MIC_{80} = 160$	

MIC minimum inhibitory concentration, *FLU* fluconazole, *FLU-SDD* fluconazole-susceptible dose-dependent, *VOR* voriconazole

S2 Mild changes (mild wrinkles and/or dimples on cell surface).
S3 Overt changes (raisin-like appearance, detachment of cell membrane and/or membrane ruffling).

When morphology was intermediate, a higher score was assigned. Results were expressed as mean in percentages (± SD).

Statistical Analyses

The inhibitory effects of FLU and VOR on *Candida* biofilms were expressed as the percentage of the optical density of FLU/VOR-treated wells compared to the control wells (no drug) for the XTT assay. Statistical analysis was performed by one-way ANOVA, with Tukey's multiple comparison post-hoc tests for this part. A P value of ≤ 0.05 was considered significant. Statistical significance ($p < 0.01$) of the results between the scores for the electron microscopy was determined by the Bonferroni post-test. The analysis was performed using Prism version 7.00 for Windows (GraphPad Software, San Diego, California).

Results

FLU and VOR MICs for Planktonic Cells

The MIC_{50} and MIC_{80} of FLU and VOR are shown in Table 2. Reference strains of *C. glabrata* ATCC 14053, *C. parapsilosis* ATCC 22019 and *C. rugosa* ATCC 10571 were highly susceptible to both azoles, while clinical isolate *C. glabrata* 5 was resistant to FLU, but susceptible to VOR, and *C. rugosa* 2745 was FLU-SDD. *C. parapsilosis* was susceptible to both azoles.

Anti-metabolic Activity of FLU and VOR Against *C. glabrata*, *C. parapsilosis* and *C. rugosa* Biofilms

The MIC_{50} and MIC_{80} of FLU and VOR against *Candida* biofilms are shown in Table 3. The activity of FLU on *C. parapsilosis* and *C. rugosa* biofilms was moderate with higher MIC_{50} and MIC_{80}, when compared to its activity against planktonic cells. The activity of VOR on *C. glabrata* biofilms was similar to planktonic cells, and complete killing of biofilms was achieved only at 2× MIC_{50} VOR and 4× MIC_{80} VOR. The activity of FLU against *C. glabrata* biofilm was similar to planktonic cells. However, the activity of FLU against *C. parapsilosis* biofilms (ATCC and clinical isolate) was slightly poor, with 256× MIC_{50} FLU and 32×– 64× MIC_{80} FLU. The MIC_{50} and MIC_{80} of VOR on *C. parapsilosis* ATCC strain were 4×– 8× higher compared to its activity against clinical isolate (2× and 4× MIC_{50} and MIC_{80}, respectively). The MIC_{50} and MIC_{80} of FLU against *C. rugosa* ATCC strain were 64×/> 64× higher than their planktonic counterparts. The biofilms of clinical strain were susceptible at 4× MIC_{50} FLU and > 4× MIC_{80} FLU, respectively, while *C. rugosa* biofilms were inhibited at 8× and 4× MIC_{50} FLU, and 32× and 8× MIC_{80} FLU. From the above results, it is evident that there is a twofold increase in the MICs of azoles towards *Candida* biofilms. This clearly underlines the current resistance levels of *C. glabrata*, *C. parapsilosis* and *C. rugosa* biofilms towards FLU and VOR.

The Effect of FLU and VOR AfLTs on *C. glabrata*, *C. parapsilosis* and *C. rugosa* Biofilms

The MIC_{50} and MIC_{80} of pre-coated FLU and VOR against *Candida* biofilms are shown in Table 4. In this study, the AfLT was employed to coat the wells of the

Table 2 MIC_{50} and MIC_{80} of FLU and VOR on planktonic cells of *Candida* species

Species	FLU (μg mL^{-1})/(% inhibition)		VOR (μg mL^{-1})/(% inhibition)	
	MIC_{50}	MIC_{80}	MIC_{50}	MIC_{80}
C. glabrata ATCC 14053	0.5 (55)	4 (81)	0.125 (49)	2 (79)
C. glabrata 5	> 64	> 64	0.125 (48)	2 (80)
C. parapsilosis ATCC 22019	0.25 (55)	2 (82)	0.625 (50)	0.5 (79)
C. parapsilosis 6	0.25 (51)	1 (76)	0.125 (50)	1 (82)
C. rugosa ATCC 10571	0.25 (49)	1 (82)	0.625 (52)	1 (82)
C. rugosa 2745	4 (48)	16 (78)	0.625 (53)	1 (82)

MIC minimum inhibitory concentration, *FLU* fluconazole, *VOR* voriconazole

Table 3 MIC_{50} and MIC_{80} of FLU and VOR on biofilms of *Candida* species

Species	FLU (μg mL^{-1})/(% inhibition)		VOR (μg mL^{-1})/(% inhibition)	
	MIC_{50}	MIC_{80}	MIC_{50}	MIC_{80}
C. glabrata ATCC 14053	0.5 (50)	2 (80)	0.25 (50)	2 (78)
C. glabrata 5	> 64	> 64	0.25 (51)	8 (78)
C. parapsilosis ATCC 22019	> 64 (52)	> 64	0.25 (48)	4 (80)
C. parapsilosis 6	> 64	> 64	0.25 (51)	4 (78)
C. rugosa ATCC 10571	16 (48)	> 64	0.5 (50)	4 (78)
C. rugosa 2745	16 (56)	> 64	2 (55)	8 (79)

MIC minimum inhibitory concentration, *FLU* fluconazole, *VOR* voriconazole

microtiter plates with FLU and VOR for 24 h at 4 °C prior to biofilm formation. The MIC_{50} of pre-coated FLU was 256× lesser for *C. parapsilosis* ATCC 22019, 2× lesser for *C. rugosa* and 128× higher for *C. parapsilosis* clinical isolate. No change in the MIC_{50}/MIC_{80} was observed for *C. glabrata*. Pre-coating of the FLU and VOR for their antibiofilm potential revealed that the activity of VOR was stronger than that of FLU. The biofilms of *C. glabrata*, *C. parapsilosis* and *C. rugosa* were inhibited at ¼×–½× MIC_{50} VOR.

FLU and VOR Treatments Caused Extracellular and Intracellular Damage to *Candida* Cells

SEM

Based on the scoring system, intact cells without any alterations in their morphology were assigned as grade S1 (Fig. 1a, b). Grade S1 was observed in non-treated cells (control) which appeared to be intact oval shaped with smooth surface (Fig. 1a). Budding was observed in few cells undergoing reproduction (Fig. 1b). Cells with mild changes were assigned as grade S2 (Fig. 1c, d). Mild wrinkles (Fig. 1c) and depletion of the outer cell surface (Fig. 1d) were observed in cells treated with 1× MIC FLU. Cells with overt changes, i.e., grade S3 were shown in Fig. 1e, f. At 10× MIC FLU, overt shrinkage of cells and dimple-like structures on cell surface was observed as shown in Fig. 1f. Damages in cell wall and cell membrane were evidenced by a bumpy and/or a more bumpy appearance as observed in *C. glabrata* treated with 10× MIC FLU (Fig. 1g). At 1× MIC VOR treatments, ruffled membranes were observed as shown in Fig. 1h. The mean percentage for each grade using this scoring system is shown in Table 5. The changes in the percentage of grade S1 morphology was not significant, since the control was not treated with drugs, and therefore 96% of the cells appeared to be normal. The highest score, i.e., grade S3, was observed in FLU-resistant *C. glabrata* treated with 10× MIC FLU (60%) and 1× MIC VOR (63.75%). Grade S3 was also observed in FLU-susceptible *C. parapsilosis* treated with 10× MIC FLU (52.5%) and 1× MIC VOR (55%). Grade S3 was also observed in FLU-SDD *C.*

Table 4 MIC_{50} and MIC_{80} of FLU and VOR on biofilms by AfLT

Species	FLU (μg mL^{-1})/(% inhibition)		VOR (μg mL^{-1})/(% inhibition)	
	MIC_{50}	MIC_{80}	MIC_{50}	MIC_{80}
C. glabrata ATCC 14053	0.5 (55)	2 (80)	0.625 (50)	1 (81)
C. glabrata 5	64	> 64	0.125 (48)	4 (80)
C. parapsilosis ATCC 22019	0.25 (57)	64 (80)	0.125 (50)	1 (77)
C. parapsilosis 6	0.5 (53)	> 64	0.125 (48)	2 (79)
C. rugosa ATCC 10571	32 (46)	> 64	0.125 (51)	1 (79)
C. rugosa 2745	8 (57)	> 64	0.5 (46)	2 (79)

MIC minimum inhibitory concentration, *AfLT* antifungal lock therapy, *FLU* fluconazole, *VOR* voriconazole

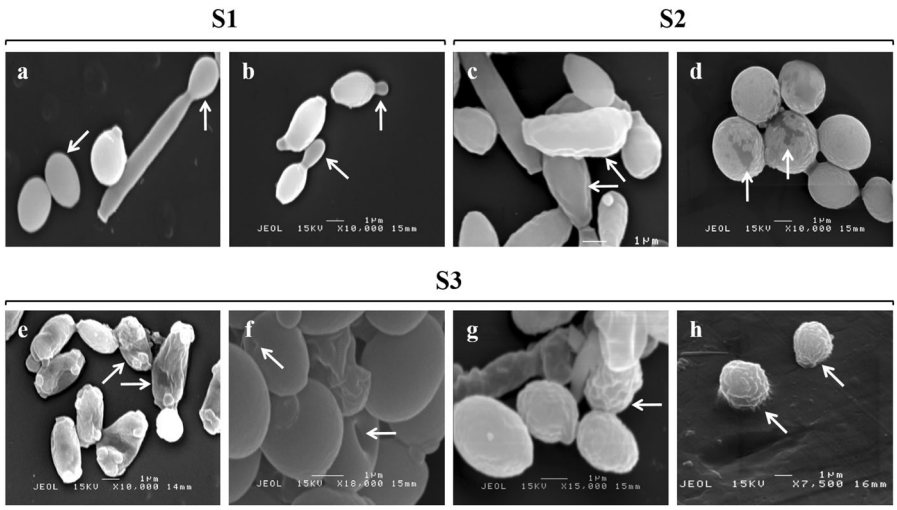

Fig. 1 Representative micrographs of SEM showing grade S1 morphology in white arrows **a** normal oval-shaped cells and **b** budding cells; grade S2 morphology of *Candida* cells showing **c** wrinkled cells and **d** depleted cell surface; grade S3 morphology of *Candida* cells under various magnifications showing **e** wrinkled cells, **f** dimples on cell surface, **g** raisin-like appearance and **h** ruffled membrane. Magnification ×10,000

Table 5 Morphology scoring for *C. glabrata*, *C. parapsilosis* and *C. rugosa* from SEM observation

Drug concentrations	S1		S2		S3	
	Mean (%)	SEM	Mean (%)	SEM	Mean (%)	SEM
C. glabrata						
Control	96.2	1.20	3.8[a]	1.2	0[a]	0
1× MIC FLU	28.75	3.75	42.5[a]	0	28.75	3.75
10× MIC FLU	5	0	35[a]	2.5	60[a]	2.5
1× MIC VOR	3.75	1.25	32.5[a]	2.5	63.75[a]	1.25
C. parapsilosis						
Control	96.1	1.40	2.6[a]	2.6	1.25[a]	1.25
1× MIC FLU	12.6	2.4	38[a]	3	49.35[a]	0.65
10× MIC FLU	2.5	0	45[a]	2.5	52.5[a]	2.5
1× MIC VOR	2.5	0	45[a]	0	55[a]	2.5
C. rugosa						
Control	96.05	1.05	3.95[a]	1.05	0[a]	0
1× MIC FLU	11.75	3.25	42.7[a]	0.20	38.75[a]	1.25
10× MIC FLU	3.75	1.25	35[a]	5	61.25[a]	3.75
1× MIC VOR	5.35	0.35	40.2[a]	2.70	54.45[a]	3.05

S1, S2 and S3 indicate the grades used in this scoring system. The results are based on the mean of duplicates ± standard error of mean

MIC minimum inhibitory concentration, *FLU* fluconazole, *VOR* voriconazole

[a]Indicates significant value compared to S1 at $p < 0.01$

rugosa strain treated with 10× MIC FLU (61.25%) and 1× MIC VOR (54.45%). Based on the above scoring system, it is apparent that 10× MIC FLU and 1× MIC VOR were able to induce a grade S3 morphology on *Candida* cells.

TEM

Analysis of TEM micrographs revealed that all the control samples (Fig. 2a–c) were found to be normal without any cell damage. Intact cell wall with uniform thickness was observed in non-treated samples (Fig. 2a–c). However, marked alterations in the cell wall, such as cell wall thickening and increased lacuna (increase in space/gap between the cell wall and plasma membrane) were observed in FLU and VOR-treated *Candida* cells. At 1× and 10× MIC FLU and 1× MIC VOR, the cell membranes were disrupted in all FLU- and VOR-treated samples. FLU-treated *Candida* cells showed disruption of cell membrane which resulted in invagination of the cell membrane (Fig. 2d, e). The presence of vacuoles was also

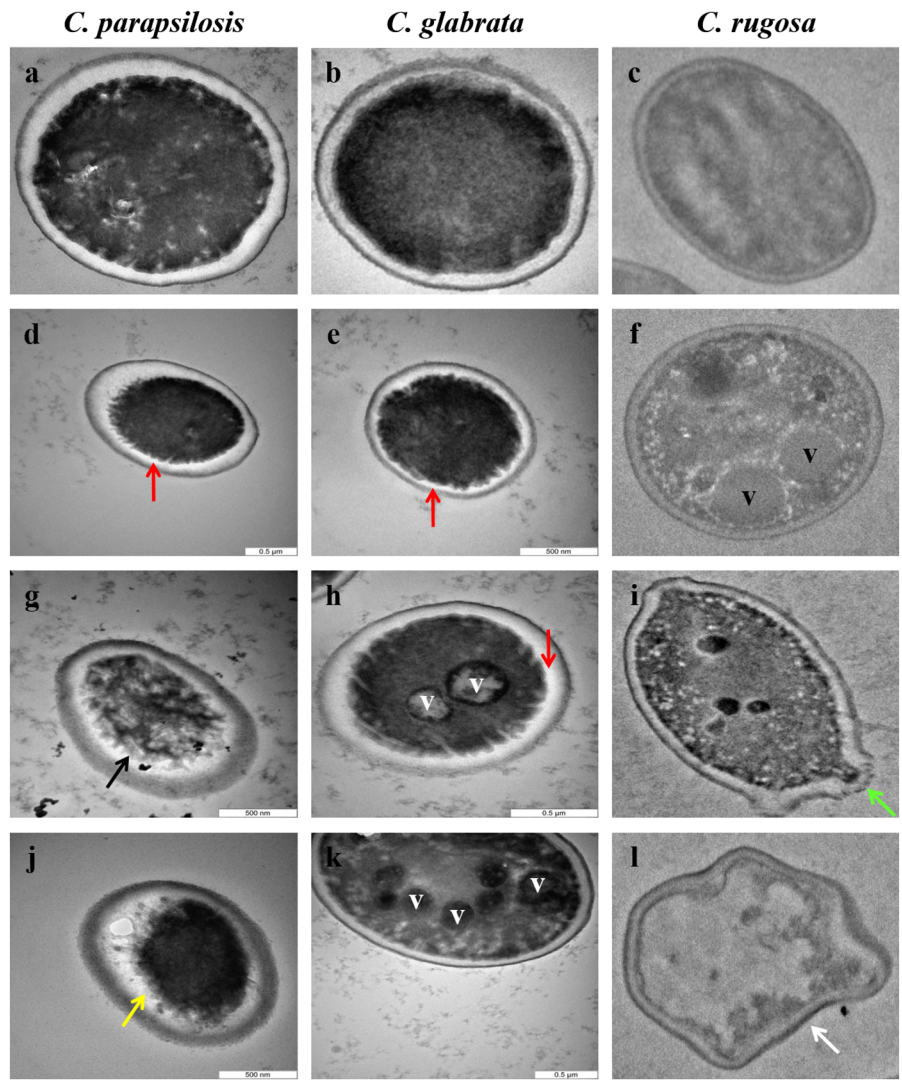

Fig. 2 Representative micrographs of TEM showing non-treated cells of **a** *C. parapsilosis*, **b** *C. glabrata*, **c** *C. rugosa*, **d** 1× MIC FLU-treated *C. parapsilosis*, **e** 1× MIC FLU-treated *C. glabrata*, **f** 1× MIC FLU-treated *C. rugosa*, **g** 10× MIC FLU-treated *C. parapsilosis*, **h** 10× MIC FLU-treated *C. glabrata*, **i** 10× MIC FLU-treated *C. rugosa*. The activity of 1× MIC VOR on **j** *C. parapsilosis*, **k** *C. glabrata* and **l** *C. rugosa*. Red arrows indicate invaginated cell membrane; black arrow indicates short fibrils; green arrow indicates disruption in budding; cytoplasmic shrinkage is shown in yellow arrow, and white arrow indicates completely distorted cell. **V** represents vacuoles. Magnification ×60,000

evident, particularly in *C. rugosa* cells (Fig. 2f). At 10× MIC FLU, more intense cell damage like disruption of cellular components was observed. *C. parapsilosis* treated with 10× MIC FLU resulted in the formation of short discontinuous fibrillar like structures (Fig. 2g). This could be due to the partial digestion of fibrillar network (glucan) in FLU-treated samples. No such structures were observed in non-treated samples. The cell membrane of FLU-treated *C. glabrata* was deeply invaginated (Fig. 2h). Budding was interrupted/disrupted in FLU-treated *C. rugosa* cells (Fig. 2i). Cytoplasmic shrinkage was noticed in *C. glabrata* cells treated with 1× MIC VOR (Fig. 2j). VOR treatments further resulted in the formation of vacuoles (Fig. 2k) and complete distortion of *C. rugosa* cells (Fig. 2l).

Discussion

Various factors affect biofilm formation in *Candida* species, of which growth media used is one factor which affects the density of biofilms. Supplementation of 8% glucose in the medium allows fast biofilm formation [32], and this condition is similar to the patients receiving parenteral nutrition rich in glucose. However, this factor is contradictory based on the reports by other researchers [33, 34]. The type of *Candida* strain and species also contribute to biofilm

development. For instance, *C. albicans* biofilm tends to grow more when compared to the biofilms of non-albicans *Candida*. Next to *C. albicans*, non-*albicans Candida* spp., such as *C. dubliniensis* [32, 34], *C. glabrata* [35] and *C. krusei* [36], are strong biofilm producers. *C. glabrata* biofilms tend to grow slow however, and the biofilm formation of *C. glabrata* observed in this study was much similar to the biofilms of *C. parapsilosis* and *C. rugosa* at 48 h of incubation. Clinical isolates of *C. krusei* and *C. glabrata* recovered from patients with IUD were reported to be strong biofilm producers [17]. Regarding the activity of FLU and VOR against biofilms, FLU MICs were 64–1000-fold higher than the planktonic MICs. This is in agreement with a recent study, where biofilms of non-*albicans Candida* strains isolated from BSIs were highly resistant to flucytosine and fluconazole [37]. Mostly, the biofilm architecture of *C. albicans* has a basal blastopore layer with a dense overlying matrix comprising of exopolysaccharides and hyphae. In view of this, the 3 species studied here had clumped blastospores with hyphae in between, which is in agreement with previous reports [33]. Phenotypic switching is very common in *C. parapsilosis* and has been adequately reported [38, 39]. Despite the common phenotypic morphologies like crepe, concentric, snowball, rough and smooth [40], biofilms of *C. parapsilosis* also constitute to the formation of crater [40, 41]. Therefore, phenotype switching was considered to affect biofilm formation by *C. parapsilosis* and further investigation is needed to understand its underlying mechanism. Literatures on the biofilm formation by *C. rugosa* are limited in number, and hence it is difficult to compare the biofilm-forming ability of *C. rugosa* with previous works. In a study conducted using Turkish Anatolian buffalos with mastitis, *C. rugosa* isolates (72.7%) were strong biofilm producers. However, the description of biofilm production by *C. rugosa* was not reported [42]. The biofilm-forming ability of *C. rugosa* observed in this study was similar to *C. glabrata* and *C. parapsilosis*.

Cells in a biofilm have to be in contact with the surface material to initiate biofilm formation, and a biofilm architecture is dependent on surface-induced gene expression [8, 43]. In this study, only blastospores were visible within 6 h which could be due to the difficulty in the adherence of biofilm to smooth glass surfaces. The first adhesion is known to be mediated by hydrophobic and electrostatic forces, between the cells and substratum [44]. This is when the blastospores adhere to any non-specific surface, followed by the expression of specific adhesion molecules that are expressed to facilitate better adhesion like cell-surface glycoproteins encoded by the ALS (agglutinin-like sequence) gene family [45]. The blastospores then divide to form cell aggregates that enhance the growth of a complex three-dimensional structure [46]. Therefore, an in vitro system using a static state or agitation could also account for the differences in the biofilm formation.

The ability of *Candida* species to form drug-resistant biofilms remains as an important virulence factor in the survival of *Candida*. Biofilms forms of *C. albicans* and *C. parapsilosis* express high-level resistance to lipid formulations of amphotericin B and echinocandins [47]. On the other hand, *C. glabrata* biofilms were reported to be 8–500 times resistant to ketoconazole [48]. Our results are also in agreement with these reports in which *C. glabrata* biofilms exhibited 2- and 64-fold higher MIC_{50} and MIC_{80} to FLU and VOR as compared to planktonic MICs [46, 49]. Several underlying factors, such as the presence of extracellular matrix, expression of resistance genes, presence of persister cells and altered metabolic rate of biofilm cells, directly contribute to drug resistance. Extracellular matrix plays a vital role in reducing the drug penetration into the cells and induces drug efflux activities [50]. Secondly, cell density also contributes to antibiotic resistance of biofilms [51, 52]. Since the inoculum size used in this study was the same for biofilms and planktonic cells, the above factor did not affect the high MICs accomplished by the biofilms. Cellular ageing of cultures, growth media and incubation time play very minimal effects or did not affect the high MICs of biofilms [52]. There are few proposed mechanisms of antifungal resistance by *C. albicans* biofilms [53]; however, the exact mechanism of biofilm resistance to such antifungals is yet to be explored. Further studies through genomic and proteomic approach could help us to elucidate key factors that contribute to the antifungal resistance of biofilms.

In this study, AfLT with VOR markedly reduced the biofilms of *C. glabrata*, *C. parapsilosis* and *C. rugosa*. The mean value of the drug was notably high (16 mg L^{-1}) to inhibit 50% of *C. glabrata* and *C. parapsilosis* biofilms [54]. AfLT with FLU reduced the MIC_{50} for *C. parapsilosis* ATCC strain and

clinical isolates of *C. glabrata*, *C. parapsilosis* and *C. rugosa*. AfLT using VOR significantly reduced the MIC_{50} and MIC_{80} for all the strains. More clinical strains with different azole susceptibilities could be studied in vitro and in vivo to confirm the effect of coating the wells with azoles prior to biofilm formation.

It has been proposed that the mode-of-action of VOR is by inhibiting cytochrome P-450-dependent 14α-demethylase, a key enzyme in the ergosterol biosynthesis [55]. In an earlier investigation, VOR at concentrations ranging from 0.003 to 4 μg mL^{-1} was reported to completely inhibit the ergosterol synthesis and accumulation of its biosynthetic precursors in FLU-susceptible *C. albicans*, FLU-resistant *C. albicans* and *C. krusei* [36]. Upon treatment with VOR, several pathways intermediates of *C. albicans* (obtusifoliol and lanosterol) [56] and *C. krusei* (squalene, 4,14-dimethylzymosterol and 24-methylene dihydrolanosterol) were reported to be inhibited [36], while, in VOR-treated *C. glabrata*, the accumulation of methylated sterols such as lanosterol, 4,14-dimethyl zymosterol and squalene has been reported [56]. These results collectively indicate the significant impact of VOR on cytochrome P-450-dependent 14α-demethylase resulting in the accumulation of different intermediates in *Candida*. It is noteworthy that VOR exhibits a dose-dependent activity on ergosterol biosynthesis which results in the reduction in ergosterol synthesis to 46% at 1/16× MIC VOR, 89% at 1/8× MIC VOR and 100% at 1/2× MIC VOR. Moreover, VOR at 1/16× MIC is sufficient enough to completely block obtusifoliol synthesis. Both FLU and VOR inhibit ergosterol synthesis by 12 and 75%, respectively [36]. The above findings act as an effective indicator to use VOR in controlling *C. krusei* infections. Similar effects were observed in the FLU-resistant *C. glabrata* strain in this study.

With regard to electron microscopy studies, the presence of large vacuoles in the cytoplasm of *C. glabrata* cells treated with 10× MIC FLU and 1× MIC VOR in an interesting finding in this study. Similar cellular damages in *C. glabrata* treated with 4× MIC FLU resulted in a damaged outer envelope, cell wall degradation and cell shrinkage [57]. The marked separation observed in FLU and VOR-treated *Candida* cells has also been reported in *C. albicans*, *C. krusei* and *C. glabrata* treated with VOR [55, 57]. The primary septum formation involves chitin which is known to be a determining factor in fungal morphogenesis and disruptions in the septum formation will affect chitin synthesis [58]. Therefore, regions with thick cell walls can be attributed that budding would have been disrupted by sterol biosynthesis inhibition [36]. Indirect effect on the protein synthesis could also result in cell wall thickening [56]. The activity of FLU and VOR in terms of causing morphological changes observed in this study is in agreement with a recent study conducted using fluconazole, voriconazole and amphotericin B [58].

With regard to drug penetration, both FLU and VOR are hydrophilic in nature and therefore penetrates very well into body fluids and tissues, including biofilm matrices. This successively allows the possibility to treat less susceptible fungi with higher doses of FLU and lower doses of VOR respective to their MICs towards the clinical isolate. Moreover, FLU and VOR therapies are generally well tolerated even at high doses in surgical or intensive care patients with proven efficacy and tolerability [59, 60]. In the present study, VOR was more effective than FLU in altering the yeast structure. With 10× MIC FLU, similar effects were observed in *C. glabrata* and *C. rugosa* treated with 1× MIC VOR. For FLU-susceptible *C. parapsilosis*, 1× MIC FLU was sufficient to alter its cellular morphology. Therefore, we can conclude that VOR exhibits a wide spectrum of activity, particularly against FLU-susceptible, FLU-SDD and FLU-resistant *Candida* sp. The present study has several limitations. First, we do not use additional control, i.e., diluent control using DMSO in order to confirm whether DMSO has any effect on planktonic/biofilms of *Candida*. Next, the pre-coating of antifungals was not washed prior to adding the yeast inoculum. The mechanism of antifungals (suspension and pre-coatings) in terms of plastic interactions is not known, and further investigation is warranted to test whether the interaction of the antifungals is stable or material dependent. Also, it is very difficult to correlate the electron microscopy results with the antibiofilm activity of FLU, therefore considered only as indicative.

Conclusions

Our results demonstrated that treatment of *Candida* biofilms with FLU and VOR resulted in significant

damage to the vitality and integrity of *Candida* cells. The effect of 1× MIC VOR and 10× MIC FLU was found to be effective against biofilms of *C. glabrata*, *C. parapsilosis* and *C. rugosa*, including the FLU-resistant and FLU-SDD strains. Compared to FLU and VOR as suspensions, pre-coatings of FLU and VOR showed more potency and efficacy, in terms of drug concentration and antibiofilm activity which is a major significance of this study. Nevertheless, SEM and TEM analyses of the biofilms samples from FLU and VOR treatments (suspensions and AfLTs) could be a more effective approach to understand how these drug formulations interact with the biofilms. The reactive oxygen accumulation, DNA fragmentation, other intracellular changes together with the molecular mechanisms on biofilms are in line with this work.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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