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Bifonazole in vitro activity and its azole-parallel resistance in clinical yeast isolates

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ABSTRACT

The susceptibility/resistance profile of bifonazole (BFZ) in 170 dermatophyte strains including azole parallel-resistance in 324 clinical yeast isolates was determined, additionally with impact on patient-relevant factors. Overall susceptibility to four azoles tested in parallel was 70%, with differences to both, the azoles, and species-specific for isolates from patients with superficial or invasive/systemic infections. 86% of the *C. glabrata* (n=166) isolates were susceptible to bifonazole, 76% were BFZ-susceptible to fluconazole-resistant *C. glabrata* (n=184) isolates, whereas 45% of the bifonazole-resistant strains (n=82) were susceptible to FLC. However, compared to voriconazole most of the other non-*C. albicans Candida, and* non-*Candida* species were less susceptible (< 50%) to bifonazole. As the other azoles tested, BFZ showed bi-modular MIC-distribution. Susceptibility pattern analysis (SPA) demonstrated that isolates from antifungal agent pre-treated patients had zero to significant less complete susceptible isolates (SP: SSSS) compared to non-treated patients. Furthermore, SPA revealed zero to fourfold parallel-resistance, species-specifically distributed, most prominently in *C. glabrata* and *C. parapsilosis*. Evaluation of azole susceptibility and two-way hierarchical clustering revealed a high grade of diversity and heterogeneity among the clinical *C. glabrata* isolates. A modified MIC assessment system was introduced to achieve a more realistic, well-arranged, and therapy oriented reporting of MIC *in vitro* data.

KEYWORDS

azoles, bifonazole, yeasts, *C. glabrata*, cross-resistance, MAR indexing

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INTRODUCTION

Bifonazole ($C_{22}H_{18}N_2$), a 1:1-mixture of (R)-1-(4-Phenylbenzhydryl)imidazole and (S)-1-(4-Phenylbenzhydryl)imidazole) exhibits fungistatic properties, and interacts with the enzyme lanosterol demethylase, which is involved in the synthesis of ergosterol, an important component of the cell membrane of fungi [1, 2]. Currently this lipophilic antifungal agent is mainly used as topical agent to treat superficial skin and nail infections, e.g. Athlete's foot (tinea pedis), fungal sweet rush, jock itch, ringworm of the body, and other skin infections caused by fungi and yeasts (e.g. skin infections which may be associated with nappy-rash, otomycoses, erythrasma, sebopsoriasis, seborrhoeic dermatitis and rosacea) [3,4]. Already in 1983, the *in vitro* and *in vivo* activity of bifonazole towards fungi was described by Plempel et al. [5]. Several clinical studies demonstrated the antifungal efficacy of the drug [6]. New investigations found that antifungal medications like bifonazole exhibited aromatase-inhibiting properties, which indicates that such antimicrobial agents may have the potential to treat oestrogen-sensitive breast cancers [7]. Bifonazole was among 19 standard antifungal drugs (SAD) of which in three different assays, its antifungal/anti-*Candida* activity has been confirmed by Stylianou et al. [8].

Candida species patterns in infected patients are changing [9, 10]. Aside of the prevailing *C. albicans* isolates, *Candida parapsilosis* and *Meyerozyma guilliermondii* are involved as emerging pathogens in onychomycosis [11], and *C. glabrata* and *C. tropicalis* are steadily rising in oral [12, 13], invasive (INV), superficial (SFI), and vulvovaginal (VVI) infections, respectively in all major types of candidiasis [14-18]. *C. tropicalis* is reported as second or third most common opportunistic etiological agent in candidemia in Europe and North America and this species is more commonly found in Brazil. *C. glabrata* ranged country-specific as second or third most common pathogen in hospital infections in Europe and North America, whereas it is less commonly isolated in Latin America [10, 18]. In addition, geographical divergence in the incidence of voriconazole resistance has been observed for other NCS (non/not-*Candida* species) members, e.g. *Issatchenkia orientalis* [20].

Candida glabrata possesses both, intrinsic and acquired resistance against antifungal drugs, has the ability to modify ergosterol biosynthesis, mitochondrial function, and/or activation of efflux systems. These resistance factors may allow *C. glabrata* for overgrowth over other susceptible species and contribute to the recent emergence in common mucosal, cutaneous, oral, and vaginal infections [21, 22]. The pathogenic behaviour of the opportunistic *Candida* species includes the expression of certain virulence factors like, formation of adhesins, biofilms, germ tubes, hydrolytic enzymes, and phenotypic switching [23-26]. NCA pathogens are now more often isolated in patients showing some of about 16 particular risk factors [27], like pre-therapy treatment/prophylaxis with antibacterial and antifungal agents, prolonged antibiotic use, IV-catheterisation, steroid use, diabetes, HIV-infection, malignant diseases, ICU stay >2 days, immunosuppressed-, elderly-, or neonate patients, or other factors [28-30]. In contrast to *C. albicans,* risk factors for *C. glabrata* vaginitis include a higher age of patients, vaginal douching, and underlying medical conditions like diabetes [29]. Infections with *C. glabrata* are also more frequently associated with recurrent vulvovaginal mycosis. However, unlike *C. albicans* the inflammatory reaction in vaginitis caused by *C. glabrata* is less pronounced [31].

Historically, both, *C. glabrata* and *C. tropicalis,* accounted for approximately 5-8% of the isolates recovered from patients suffering from vaginal mycosis. Although the infectious *C. albicans* profile did not change, this species is still worldwide the most often identified yeast pathogen [9, 10]. However, the portion of *C. glabrata* infections has increased remarkably in the last decades [32, 33]. For example, in a prospective survey of 931 patients with culture-confirmed symptomatic vulvovaginal candidiasis [34], where 77 patients (10%) had a history of chronic recurrent vulvovaginal candidosis (RCVV), *C. albicans* was the predominant species (77.1%), followed by *C. glabrata* (14.6%) and *I. orientalis* (4.0%). It has been demonstrated that from 5,802 consecutive vaginal swabs, 1,221 (21%) yeasts could be cultured, of which 129 (10.6%) were non-*Candida albicans Candida* (NAC), respectively, 89 (7.3%) *C. glabrata* isolates [35]. In another study *Candida* spp. were isolated in 44.9% (n=160) of 356 women with abnormal discharge, of which 43.1% were identified as *C. albicans*, 32.5% as *C. glabrata* and 8.1% as *C. tropicalis* [36]. A retrospective study of 1,263 patients with symptomatic yeast vaginitis confirmed by culture, examined the prevalence of NAC-vaginitis and found that its incidence increased significantly from 9.9% (10/101) in 1988 to 17.2% (36/209) in 1995 (p = 0.002) [37]. Recent studies from 2013/2014 show significant higher amounts of the NCA-isolates, e.g. for *C. glabrata* 21.6%-37.8%/27.9%/29.8% [12, 16, 37], and for *C. tropicalis* 4.7%-10.5%/30.9%/3.2% [13, 17, 38]. *C. glabrata* (20.3%) and *C. tropicalis* (16.9%) were the most frequent isolates obtained from 148 oropharyngeal lesions of HIV infected patients [39]. In India, *C. tropicalis* is reported to be the most common NAC isolate to cause nosocomial candidemia [40, 41] accompanied by an emerging resistance to fluconazole [42, 43].

Several clinical studies have documented a selection of *C. glabrata* in patients treated for prolonged periods with fluconazole, ketoconazole or itraconazole, or even after short-term treatment with fluconazole [32, 43-47]. Additionally, in some of these fluconazole-resistant strains cross-resistance to other azoles has been observed [32, 48]. The ploidy level and the degree of dominance are essential factors in the development of antifungal drug resistance [49]. Therefore, *C. glabrata* as whole-genome-replicating and haploid organism, like *C. tropicalis*, *Clavispora lusitaniae* or *Meyerozyma guilliermondii* is more prone to mutations than a diploid yeast like *C. albicans*, *C. parapsilosis* or *Issatchenkia orientalis,* which means that changes by mutation will become more visible [43, 50]. In clinical yeast isolates azole resistance to *Candida* and NCS strains is mostly due to changes in drug efflux [51-54], which tends to result in parallel-resistance of azoles [55-58], in cross-resistance of azoles and echinocandins [48, 59-62], and of azoles with amphotericin B [63, 64].

Despite its frequent use as topical antifungal agent, and as counted among the SADs, actual minimum inhibitory concentrations (MICs) and comparable, quantitative cross-resistance data of bifonazole in clinically relevant yeast species are scarce or up today still not available. Instead of the proper product containing clotrimazole, by accident, 8 of 10 patients reported the cure of vaginal mycoses by bifonazole, bearing the same basic trade name, however, which is only approved for patients with Tinea pedis and Tinea corporis. Aim of this study was therefore to compare bifonazole (BFZ) to

relevant antifungal agents for the therapy of systemic and superficial mycoses, i.e. fluconazole (FLC), itraconazole (ITC), and voriconazole with the focus on *Candida glabrata* for a potential extended *in vitro* spectrum. In this respect, the distribution, frequency and intensity of azole parallel-(cross)-resistance in clinical yeast isolates was determined by susceptibility pattern analysis (SPA).

1 Material and Methods

1.1 Organisms

The 324 clinical yeast isolates of this collaborative study (S-324) were from patients who all had reports on treatment with different antifungal agents or were under therapy with fluconazole (Table 1), thereof, 166 *C. glabrata* strains (51.2%). In addition, 170 dermatophyte isolates had been tested (Table 4). All isolates were investigated for their susceptibility to fluconazole (FLC), itraconazole (ITC), voriconazole (VRC), and bifonazole (BFZ), the dermatophytes additionally to ciclopiroxolamine (CIC), griseofulvin (GRF), and terbinafine (TER). The isolates were derived from the university hospitals of Berlin, and Munich, and the FLC-therapy strains from a special dermatology ward of the Charité in Berlin. For comparison purposes *C. glabrata* isolates from other recent collaborative *in vitro* studies with specimens from mainly sterile body sites, which had been tested at least to FLC, ITC, VRC were taken. Study S-2029 comprised 2029 clinical yeast isolates (data not published), thereof 345 *C. glabrata* strains. Study S-60 included 60 pheno- and genotyped *C. glabrata* strains [65], and S-4860 covered 889 *C. glabrata* isolates (data not published). The species distributions are listed in Table 1. Species identification was performed as already described [66]. Serotyping was performed with commercially available Iatron® anti-sera (Iatron Laboratories, Tokyo). Species differentiation was done by FT-IR. IR spectra of freshly prepared samples were recorded at a wavelength from 4,000 cm^{-1} to 600 cm^{-1} using a Bruker IFS 28/B spectrophotometer with OPUS® 2.2 software for IR analysis, data processing and cluster analysis (Ward's algorithm; average linkage).

Throughout this text the currently valid nomenclature [67] according to SpeciesFungorum [68], respectively, MycoBank [69] is applied.

1.2 Susceptibility testing

The isolates were tested against the antifungal azole agents (AFA) bifonazole and fluconazole (MIC range: 0.0625 mg/l–128 mg/l), itraconazole and voriconazole (0,008mg/l–32 mg/l) by microdilution with an adapted EUCAST [70] method (inoculum 2-5x10^4 cfu/ml, and visual endpoint determination instead of the photometrical 50% endpoint reading). Itraconazole, ciclopiroxolamine, griseofulvin, and terbinafine were purchased at Sigma-Aldrich Chemie GmbH (Munich, Germany), fluconazole and voriconazole, and bifonazole were obtained free of charge by Pfizer GmbH (Berlin, Germany), and Bayer AG (Germany), respectively. The endpoint determinations (MIC) were performed after 24 h incubation at 36°C ± 1°C, with a second verification after 48h. All MIC values had been read visually against the growth control and recorded as the lowest concentration of the AFA that caused no growth or at least a significant reduction of the growth (≥80%). The testing of dermatophytes was submitted elsewhere for publication [139].

1.3 Breakpoints and MIC assessment

Due to the lack of appropriate breakpoints for bifonazole, and as only partly EUCAST breakpoints for *Candida* are available [71], for comparison purposes in this study, the MIC-assessments were: for bifonazole: S ≤ 0.5 mg/l, and R >1 mg/l; for FLC: S ≤ 2, R > 4, for ITC: S ≤ 0.125, R > 0.25, and for VRC S ≤ 0.25, R > 0.5.

The epidemiological cut-off value (ECV) was calculated according to Arendrup et al. [72], with the median MIC as basis. In addition, parallel-resistance (defined as resistance among members of the same drug-class) and cross-resistance (resistance of members among different drug-classes) was determined after the assessment of the MIC as susceptible (S), intermediate (I) or resistant (R), called here as “3-leg system” (3-LS), and the “2-leg” system (2-LS) according to Grimm [73], with only S* and R*. For the 2-LS (if present) the percentages of intermediate (I) assessed strains are split, and 40% were added to the susceptible (S) fraction (S* = S + 40% I), and 60% to the resistant (R) category (R* = R + 60% I; 100% = S* + R*) – (depending for other purposes, e.g. species with high mutation rates, the ratio may be changed by adding 25% of “I” to “S” and 75% of “I” to “R”)..

1.4 Susceptibility pattern analysis and MAR indexing

Susceptibility patterns (SPs) were evaluated by susceptibility pattern analysis [74, 75]. The SP was defined as the artificial sequence of the assessed MIC of each AFA as “S”, ”I”, or “R”, in a default sequential arrangement (SP-profile, e.g., SP: R_{ITR}-R_{FLC}-R_{BFZ}-R_{VRC}), where as appropriate, “R” may be replaced by “S” or “I”.

The method of "multiple antibiotic resistance indexing" (MAR) described by Krumperman [76] was used to group the multi-resistant isolates. MAR_{index} = a/c; where "a" represents the number of AFA to which the isolate is resistant, and “c” is the total number of AFAs to which the isolate was exposed.

1.5 Statistical analyses

All calculations and statistical analyses were performed with log_2-MIC values, and with *SAS*® software (*SAS*® Institute, Cary, USA – Heidelberg, Germany). The antilog of the calculations is displayed as MIC. If not otherwise indicated, and for a better overview, percentage-values are given in round figures.

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2 RESULTS AND DISCUSSION

2.1 Clinical isolates and patient related factors

The 324 yeast strains for this study were obtained from the routine isolates derived from different clinic specialities/wards (CSW), partly from patients with genital, vaginal, mucous and other superficial infections (SFI), and from patients with systemic/invasive infections (IVI). It turned out that all patients with superficial mycoses (132 patients, 41%) underwent fluconazole treatment for 3 weeks with 400mg fluconazole per day. The distribution of these species per CSW, specimen type, patient-risk factor (where at the least one risk factor [26] for candidiasis had been reported) are given in Table 1. In Table 2 the distributions of the isolates from the diverse clinic specialities and specimens thereof are species-specific displayed. In Table 3 the isolates with their association to AFA-(pre)-treatment, patient risk factor, gender, and age are shown species-specific. In addition, these species distributions are given according to the severity profile of the infections, for both, the SFI (n=132, 41%) and IVI (192, 59%) patients. They show partly quantitatively and qualitatively quite different species profiles, which are also reflected in the demographic factors (Table 3). *C. glabrata* (n=62, 48.1%), *C. tropicalis* (n=26, 20.2%), and the *C. parapsilosis* complex (n=11, 8.5%) were the most frequent clinical isolates. When the current valid nomenclature is taken into account [67-69], aside of *Candida albicans* (n=2, 2%) only one further (true) *Candida* species (*C. magnolia,* n=1) and the NCA isolates had been isolated from these samples (total *Candida* spp. n=91, 70.5%). The not-*Candida* species (NCS) and former NAC isolates (n=38, 29.5%) were: *Clavispora lusitaniae* (*C. lusitaniae*), *Debaryomyces hansenii* (*C. famata*), *Issatchenkia orientalis* (*C. krusei*), *Kluyveromyces marxianus* (*C. kefyr*), and *Meyerozyma guilliermondii* (*C. guilliermondii*).

Table 1. Species distribution from patients with reported superficial (SFI) and/or invasive (IVI) yeast infections in this, and from two compared studies.

Study:	**Present**						**For comparisons**			
Study No.:	**S-324**						**S-60**		**S-2029**	
Infection type:	**All**		**IVI**		**SFI**		**IVI**		**IVI**	
Genus / species	N	%	N	%	N	%	N	%	N	%
	324	100	192	59.3	132	40.7	60	100	2,029	100
Candida albicans	24	7.4	19	9.9	5	3.8			1,045	51.4
C. glabrata	166	51.2	100	52.1	66	50.0	60	100	362*****	17.8
C. humicola	0								1	0.1
C. inconspicua	0								8	0.4
C. magnoliae	2	0.6	2	100.0	0	0			0	0.0
C. parapsilosis	24	7.4	13	6.8	11	8.3			84	4.1
C. tropicalis	44	13.6	18	9.4	26	19.7			185	9.1
Clavispora lusitaniae	2	0.6	0	0	2	1.5			45	2.2
Debaryomyces hansenii	10	3.1	5	2.6	5	3.8			11	0.5
Geotrichum candidum	0								1	0.1
Issatchenkia orientalis	33	10.2	27	14.1	6	4.6			147	7.2
Kluyveromyces marxianus	6	1.9	2	1.0	4	3.0			21	1.0
Meyerozyma guilliermondii	4	1.2	2	1.0	2	1.5			36	1.8
Pichia fermentans	0								1	0.1
Pichia norvegensis	0								1	0.1
Saccharomyces cerevisiae	9	2.8	4	2.1	5	3.8			17	0.8
Yarrowia lipolytica	0								1	0.1
Cryptococcus laurentii	0								1	0.1
Cryptococcus neoformans	0								56	2.8
Exophiala dermatitidis	0								2	0.1
Trichosporon cutaneum	0								5	0.2

***** Including 4 clinical control strains, therefore only 358 clinical strains were taken for comparisons

Except of *C. albicans*, which is still the most prominent pathogen in IVI and SIF infections, the species profile of this evaluation matches the distribution profile of SFI- and IVI-isolates in a recent study [9], and is in concordance with those reported in the literature [26-28, 77, 78]. Therefore isolate-populations from a parallel ongoing study with predominantly IVI-patients tested also for FLC, ITC and VRC, were compared. Multiple risk factors have been reported for the SFI-patients (Table 3). The fact that these are associated with fungal infections in the critically ill patients and candidemia in ICUs were mainly due to NAC species [79-82], is documented in Tables 1 and. 2. Parallel to the literature reports, a changed species spectrum was encountered. This may be boosted by nomenclature changes [9, 67] and the outcome of genotype studies [31, 83-87]. However, the incidence of nosocomial candidemia in Germany [81] and Spain [82] has not changed over the last decade. However, the greater amount of, and the lower susceptibilities of *C. glabrata* to FLC and to other commonly used azoles may indicate that species with less azole-susceptibility have been replaced in the patients under azole therapy.

This could also be the reason that *C. glabrata* in this study is the most prominent pathogen isolated at all CSW, and is therefore being associated to almost all different specimens, gender and age-range. This is supported by the species ranking, where the less azole susceptible species *C. tropicalis*, *C. parapsilosis,* and *I. orientalis* follow. Distribution differences were also seen within the age pattern. Despite the fact that 19 direct surface contact cultures (patient age not known) were identified, and about 9% more samples were derived from male patients, species-specific isolation differences were seen in female (n=47, 43%) and male (n=63, 52%) patients, and within the age pattern. Considering the NCS isolates, 2% *C. glabrata*, 11% *C. tropicalis*, and 2% *I. orientalis* were more isolated, however, 3% *C. parapsilosis* strains less in male patients compared to females (Table 3). *C. glabrata* does not normally penetrate tissues [88], however efficiently immunocompromised and is more often found in elderly patients [89]. C. glabrata was isolated in all age groups, however was most prominent in the age-range from 61-80, whereas *C. tropicalis* was only found in the age-ranges from 41y to ≥ 81y. All other species were differently distributed within the age pattern (Table 3). Within the age distributions, the patients between 61y to 70y (total patients, N=110, 31%) and 71y to 80y (33%) were the most significant groups. In the patients of the 71y to 80y group an equal amount of *C. tropicalis*, however 4% more *C. glabrata*, and 6% more *C. parapsilosis* isolates were encountered compared to the age range of 61y to 70y (Table 3). Whereas *C. glabrata* and *I. orientalis* are rather infrequent in older paediatric patients [61], *C. parapsilosis* was the most frequent species in Spanish children < 15y [90]. The fact that *C. glabrata* is more frequently, and geographically differently isolated in the elderly (>60y), and in paediatric (<3y) patients has been reported by several authors [18, 40, 91-95]. Interestingly, in younger patients with CRVV, however, otherwise symptomless, *C. glabrata* was the most important pathogen and was permanently traceable in most cases. This may be due to the fact thast e lderly patients are more easily colonized by pathogenic fungi and have an increased incidence of *C. glabrata* fungemia, which has higher mortality rates as well as higher rates of resistance to fluconazole, especially after exposure to the drug [91]. Additionally, where SFI and IVI patients could be distinguished species-specific differences can be seen in the different patient groups

2.2 Azole - bifonazole – susceptibility

In vivo and *in vitro* studies on the antifungal activity of bifonazole are very scarce since its discovery in 1969, respectively, the description of its efficacy by Plempel et al. in 1983 [5]. These authors described already the sequential mode of action of BFZ, the inhibition of cytochrome P450-dependent C14-demethylation of sterols and direct inhibition of HMG-CoA-reductase [96]. An inhibitory effect of BFZ at the adhesion of *C. albicans* to vaginal epithelial cells has been described by Wächter et al. [97]. BFZ also demonstrates a strongly pH-dependent efficacy when tested in vitro [98-99]. The action of BFZ in seborrhoeic dermatitis [100], which was similar to ketoconazole, has been shown by Zienicke et al. [101], respectively, the susceptibility to *Malassezia* has been reported by van Gerven and Odds [102]. Activity against *Corynebacterium minutissimum* was demonstrated by Nenoff et al. [103]. The antifungal action in comparison to ciclopiroxolamine was demonstrated by Hanel et al. [104]. Whereas bifonazole was unable to kill *Trichophyton rubrum* in an in vitro model described by Schaller et al. [105], BFZ was clinically effective in SFIs [106], and in onychomycosis [107]. The antifungal action of BFZ to different hyphomycetes [108] and to *C. albicans* in a new topical drug delivery system [4, 109] was reported. Previous findings have demonstrated that in contrast to miconazole the action of BFZ is not fungicidal [108].

A comparison of the in vitro and in vivo activity of bifonazole versus terbinafine to the most common aetiological agents of Tinea pedis was given by Korting et al. [110]. The report covered also the most frequent and clinically relevant dermatophytes. Therefore, and to complete the BFZ update, exemplarily the MICs for some *Microsporum canis* and *Trichophyton* species are here displayed (Table 4). Of the dermatophytes derived from human or animal sources there was no statistically significant difference in characteristic MIC-values detected. The MIC results determined were similar to those reported by Korting et al. [110], with voriconazole and terbinafine as the most effective antifungal agents against all dermatophyte species tested. That bifonazole acts fungicidal in concentrations ≥ 5 mg/l to *T mentagrophytes* and *T. rubrum* has been reported [5]. Additionally, their findings that BFZ inhibits the growth of the majority of the dermatophytes in concentrations below 2.5 mg/l [5, 11, 110-111] could be confirmed by the dermatophyte species tested (Table 4).

The overall susceptibilities to *C. albicans*, *C. glabrata, and K. marxianus* were in the range of 79% to 86%, however, to other *Candida* and non-*Candida* species the *in vitro* activity was much lower (21% to 67%; Tables 5 and 6). The isolates from SFI (n=132) and IVI patients (n=192) demonstrated overall equal susceptibility levels (70%), however demonstrated differing individual azole-profiles (Tables 5, 6). 76% of the fluconazole-resistant (FLC^r) isolates (n=184, 57% of total strains) were susceptible to bifonazole, 9% to ITC, and 97% to VRC, whereas 45% of the bifonazole-resistant strains (BFZ^r) were susceptible to FLC, 4% to ITC and 94% to VRC. The corresponding values for FLC^r - IVI (n=115) and SFI (n=67), respectively, BFZ^r - IVI (n=49) and SFI (n=33) values are given in Table 5. The MIC distributions with normal and non-parametric distribution curves of the azoles tested are displayed in Figures 1 to 4.

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Table 2. Species distribution (N=324) per of clinic speciality and specimen type, associated to the initial patient infection-type (IVI ≙ invasive/systemic infections; SFI ≙ superficial infections). The differences in species distribution for IVI and SFI patients are highlighted by different colouring of the appropriate species percentages.

Parameter Clinic Speciality / Specimen Type (*UGT = uro-genital tract)		Frequency (N=324)	Infection type	Frequency (N)n	Incidence per species *C. glabrata*	*C. albicans*	*C. tropicalis*	*C. parapsilosis*	*I. orientalis*	*D. hansenii*	*S. cerevisiae*	*K. marxianus*	*M. guilliermondii*	*Cl. lusitaniae*	*C. magnoliae*
			IVI	192	100	19	18	13	27	5	4	2	2	0	2
			SFI	132	66	5	26	11	6	5	5	4	2	2	0
Clinic speciality	Dermatology	37	IVI	23	**15**	**3**			**4**			**1**			
			SFI	14	**14**										
	Gynaecology	3	IVI	0											
			SFI	3	**1**						**1**	**1**			
	ICU	185	IVI	111	**67**	**7**	**11**	**7**	**14**	**1**	**1**	**1**	**2**		
			SFI	74	**34**	**5**	**19**	**8**	**1**	**1**	**2**	**3**		**1**	
	Internal Medicine	67	IVI	36	**11**	**9**	**4**	**2**	**6**	**2**	**2**				
			SFI	31	**12**		**6**	**2**	**4**	**2**	**2**		**2**	**1**	
	Neurology	1	IVI	1	**1**										
			SFI	0											
	Paediatrics	7	IVI	4						**2**					**2**
			SFI	4	**1**			**1**		**2**					
	Surgery	18	IVI	16	**5**		**3**	**4**	**3**		**1**				
			SFI	2	**2**										
	TNE	2	IVI	0											
			SFI	2	**1**		**1**								
	Transplantation	1	IVI	0											
			SFI	1	**1**										
	Urology	2	IVI	1	**1**										
			SFI	1	**1**										
Specimen type	Aspirate	26	IVI	1	**1**										
			SFI	25	**8**		**9**	**5**	**2**			**1**	**1**		
	Blood	16	IVI	6	**4**	**2**									
			SFI	10	**2**	**4**	**2**			**1**				**1**	
	Catheter	4	IVI	0											
			SFI	4	**2**						**2**				
	Fluid (non sterile)	13	IVI	1											**1**
			SFI	12	**4**		**3**	**1**	**1**	**3**					
	Stool	16	IVI	15	**9**	**9**	**1**		**2**		**2**				
			SFI	1	**1**										
	Swab	138	IVI	102	**47**	**12**	**14**	**5**	**16**	**4**	**2**	**1**			**1**
			SFI	36	**23**		**9**		**2**		**2**				
	UGT* specimen	37	IVI	24	**14**	**2**	**2**		**5**			**1**			
			SFI	13	**13**										
	Urine (sterile)	74	IVI	43	**26**	**2**	**1**	**8**	**3**	**1**			**2**		
			SFI	31	**13**	**1**	**3**	**5**	**2**	**1**	**1**	**3**	**1**	**1**	

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Table 3. Species distribution from all patients with reported antifungal agent (AFA) treatment (n=324), from patients with AFA-pre-treatment and FLC therapy (n=132), and per risk factors, gender, and age, associated to the initial patient infection-type (IVI ≙ invasive/systemic infections; SFI ≙ superficial infections).

Parameter		Pre-Treatment AFA	Frequency (N = 324)	Infection type:	N	Incidence per Species										
						C. glabrata	*C. tropicalis*	*C. albicans*	*C. parapsilosis*	*I. orientalis*	*D. hansenii*	*S. cerevisiae*	*K. marxianus*	*W. guilliermondii*	*Cl. lusitaniae*	*C. magnoliae*
				IVI	192	100	19	18	13	27	5	4	2	2	0	2
				SFI	132	66	5	26	11	6	5	5	4	2	2	0
AFA	**(Pre) Treatment reported**	FLC	191	IVI	60	**19**		**14**	**10**	**6**	**5**	**1**	**2**	**2**		**1**
				SFI	131	**65**	**5**	**26**	**11**	**6**	**5**	**5**	**4**	**2**	**2**	
		NYS	10	IVI	9	**6**						**2**				**1**
				SFI	1	**1**										
		VRC	123	IVI	123	**75**	**19**	**4**	**3**	**21**		**1**				
				SFI	0											
AFA	**Treatment, 400**mg FLC/ d/3w	**non** AMB	3	IVI	3	**1**	**2**									
		FLC	129	IVI	19	**9**		**6**	**2**						**2**	
				SFI	110	**55**	**3**	**20**	**9**	**7**	**5**	**5**	**4**	**2**		
Risk-factor	AM (pre) treatment		118	IVI	84	**34**		**12**	**6**	**13**	**4**	**3**	**1**			**2**
				SFI	34	**23**			**2**	**2**	**2**	**1**		**1**	**1**	
	Burn + AM		1	IVI	0											
				SFI	1						**1**					
	Catheter +AM		26	IVI	0											
				SFI	26	**10**			**1**	**2**	**2**	**2**	**1**	**1**		
	Catheter + ICU + AM		22	IVI	0											
				SFI	22	**9**			**3**	**1**		**1**	**1**			
	ICU + AM		156	IVI	108	**67**		**7**	**7**	**13**	**1**	**1**	**1**	**2**		
				SFI	48	**22**		**5**	**5**	**2**		**1**	**2**		**1**	
	Transplantation + AM		1	IVI	0											
				SFI	1	**1**										
Gender	Female		161	IVI	93	**59**		**8**	**3**	**16**	**3**		**1**			
				SFI	68	**39**		**4**	**7**	**2**	**3**	**2**	**3**		**1**	
	Male		160	IVI	96	**38**		**11**	**10**	**11**	**2**	**4**	**1**	**2**		**2**
				SFI	64	**27**		**1**	**4**	**4**	**2**	**3**	**1**	**2**	**1**	
	Not available		3	IVI	3	**3**										
				SFI	n.a.											
Age-range (years)	≤ 2		5	IVI	n.a											
				SFI	5	**2**			**1**		**2**					
	30-40		13	IVI	n.a.											
				SFI	12	**9**			**1**			**1**	**1**		**1**	
	41-50		14	IVI	n.a.											
				SFI	14	**6**		**2**	**1**	**1**		**1**	**1**			
	51-60		14	IVI	n.a.											
				SFI	16	**6**		**3**	**1**	**1**	**1**	**1**		**1**		
	61-70		39	IVI	n.a.											
				SFI	38	**22**		**9**		**1**	**1**	**2**	**1**	**1**		
	71-80		41	IVI	n.a.											
				SFI	41	**19**		**9**	**7**	**2**					**1**	
	≥ 81		6	IVI	n.a.											
				SFI	6	**2**		**3**					**1**			
	Not available		3	IVI	3	**3**										
				SFI	0											

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Table 4. Characteristic MIC-values, i.e. MIC range (MIC_{range}), MIC geometric mean (MIC_{gmean}), MIC mode (MIC_{mode}), the 50th, 75th and 90th percentile of the MIC (MIC_{50}, MIC_{75}, MIC_{90}) of the antifungal agents (AFA) bifonazole (BFZ), voriconazole (VRC), itraconazole (ITC), fluconazole (FLC), ciclopiroxolamine (CIC), griseofulvin (GRF), and terbinfine (TRF) for all dermatophytes (Total strains), derived from animals Animal) or patients (Human), and of the species: *Microsporum canis*, *Trichophyton interdigitale*, *T. mentagrophytes*, and *T. rubrum*.

AFA	MIC para-meter	Total strains	Origin		Dermatophyte species (mg/l):			
			Animal	Human	*M. canis*	*T. interdigitale*	*T. mentagrophytes*	*T. rubrum*
		n=170	n=70	n=100	n=2	n=5	n=159	n=4
BFZ	MIC_{range}	0.125-2	0.5-2	0.125-2	0.5-1	0.25-1	0.125-2	0.25-0.5
	MIC_{gmean}	0.9	0.9	0.8	0.8	0.6	0.9	0.4
	MIC_{mode}	1	1	1	-	0.5	1	025
	MIC_{50}	1	1	1	1	0.5	1	0.25
	MIC_{75}	1	1	1	1	0.5	1	0.5
	MC_{90}	2	2	2	1	1	2	0.5
VRC	MIC_{range}	0.008-0.5	0.008-0.5	0.008-0.5	0.063-0.063	0.016-0.063	0.008-0.5	0.016-0.125
	MIC_{gmean}	0.2	0.2	0.2	0.1	0.08	0.2	0.1
	MIC_{mode}	0.125	0.125	0.125	0.063	0.031	0.125	0.031
	MIC_{50}	0.063	0.125	0.063	0.063	0.031	0.063	0.031
	MIC_{75}	0.125	0.125	0.125	0.063	0.031	0.125	0.063
	MC_{90}	0.25	0.25	0.25	0.063	0.031	0.25	0.125
ITC	MIC_{range}	0.5-4	0.5-4	0.5-4	2-4	1-2	0.5-4	2-4
	MIC_{gmean}	1.3	1.2	1.3	2.1	1.1	1.2	2.1
	MIC_{mode}	1	1	1	-	1	1	2
	MIC_{50}	1	1	2	2	1	1	4
	MIC_{75}	2	2	2	4	1	2	4
	MC_{90}	2	2	2	4	2	2	4
FLC	MIC_{range}	2-128	2-128	2-128	32-32	8-32	2-128	2-32
	MIC_{gmean}	5.7	5.6	5.8	11.1	6.2	5.8	2.6
	MIC_{mode}	8	8	32	32	8	8	2
	MIC_{50}	16	8	16	32	16	16	2
	MIC_{75}	32	32	32	32	16	32	8
	MC_{90}	32	32	32	32	32	32	32
CIC	MIC_{range}	0.063-4	0.063-4	0.063-4	1-1	0.125-1	0.063-4	0.25-0.5
	MIC_{gmean}	1.0	1.2	0.9	1	0.6	1.1	0.4
	MIC_{mode}	2	2	2	1	1	2	0.25
	MIC_{50}	1	2	1	1	0.25	1	0.25
	MIC_{75}	2	2	2	1	0.25	2	0.5
	MC_{90}	4	4	4	1	0.5	4	0.5
GRF	MIC_{range}	0.125-1	0.125-1	0.125-1	0.25-0.25	0.25-0.5	0.125-1	0.5-1
	MIC_{gmean}	0.4	0.4	0.4	0.4	0.4	0.4	0.7
	MIC_{mode}	0.25	0.25	0.25	0.25	0.25	0.25	0.5
	MIC_{50}	0.25	0.031	0.25	0.25	0.25	0.25	0.5
	MIC_{75}	0.5	0.063	0.5	0.25	0.25	0.5	1
	MC_{90}	0.5	0.063	0.5	0.25	0.5	0.5	1
TER	MIC_{range}	0.008-0.125	0.008-0.125	0.008-0.125	0.008-0.016	0.031-0.125	0.008-0.125	0.016-0.063
	MIC_{gmean}	0.09	0.08	0.09	0.04	0.1	0.1	0.063
	MIC_{mode}	0.063	0.063	0.063	-	0.063	0.063	0.016
	MIC_{50}	0.031	0.031	0.031	0.016	0.063	0.031	0.016
	MIC_{75}	0.063	0.063	0.063	0.016	0.063	0.063	0.031
	MC_{90}	0.063	0.063	0.063	0.016	0.063	0.063	0.031

Note for all MIC-distribution graphs: Standard-error bars and the percentage of isolates at the appropriate log_2-dilutions are indicated on top of the graphs. The conversions of log_2-MIC-values to MICs (mg/l) are as follows:

-8=0.004; -7=0.008; -6=0.016; -5=0.031; -4=0.063; -3=0.125; -2=0.25; -1=0.5; 0=1; 1=2; 2=4; 3=5; 4=8; 5=16; 6=32; 7=64; 8=128

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Fig. 1. MIC (log_2-value) distribution of bifonazole (BFZ) of all isolates with normal distribution (red line: (-0.7377, 3.04187), and nonparametric density distribution (smooth blue curve: Kernel-Std 0.861536).

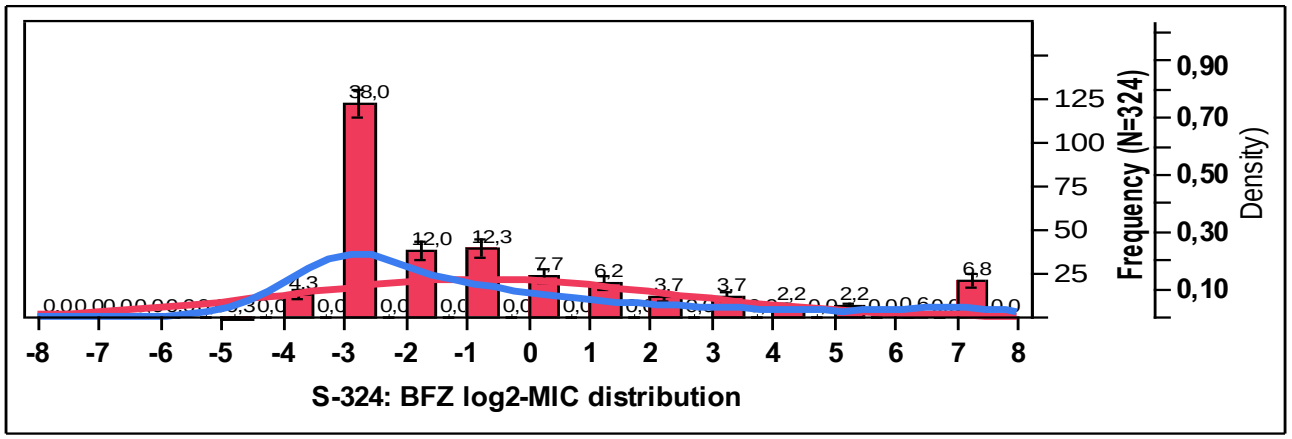

Fig. 2. MIC (log_2-value) distribution of fluconazole (FLC) in all isolates with normal distribution (red line: 2.80864, 2.07755), and nonparametric density distribution (smooth blue curve: Kernel-Std. 0.588416).

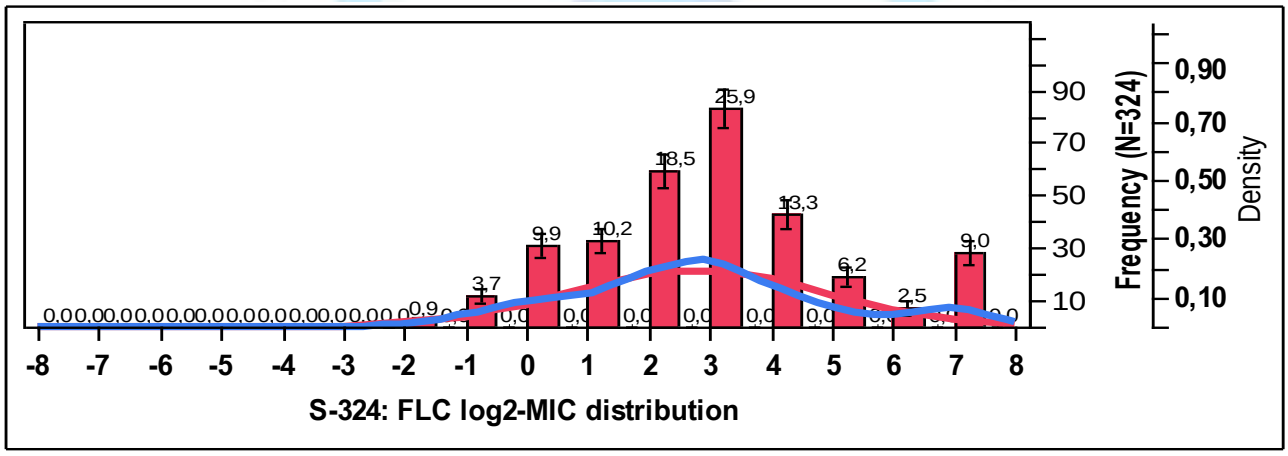

Fig. 3. MIC (log_2-value) distribution of itraconazole (ITC) in all isolates with normal distribution (red line: 1.10494, 2.49484), and nonparametric density distribution (smooth blue curve: Kernel-Std. 0,706605).

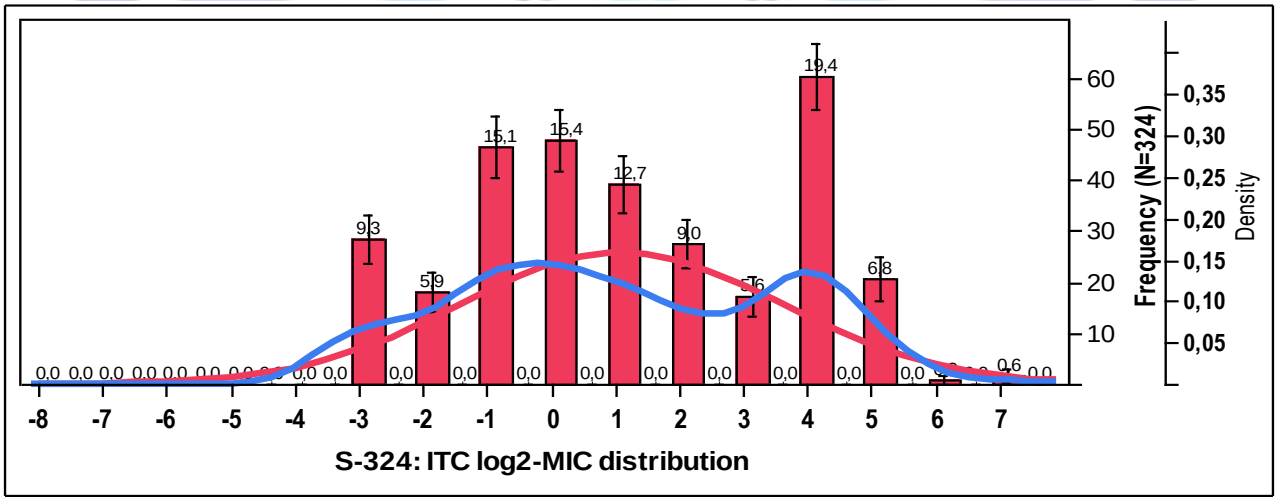

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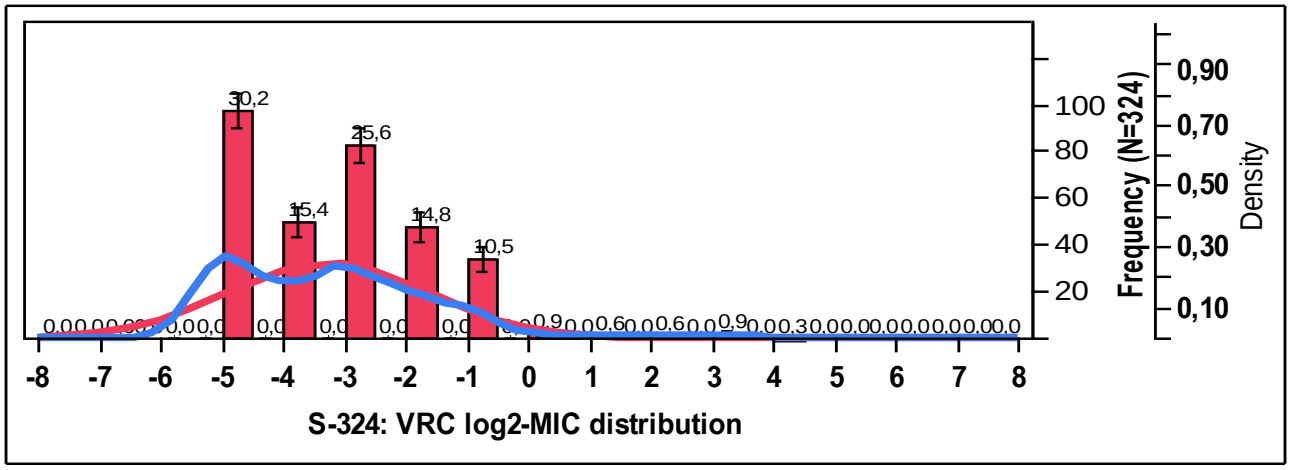
Fig. 4. MIC (log_2-value) distribution of voriconazole (VRC) in all isolates with normal distribution (red line: -3.2407, 1.64422), and nonparametric density distribution (smooth blue curve: Kernel-Std. 0.465688).



Although *C. albicans* is underrepresented in this collective, this species is still the most infectious pathogen in patients with SFIs and IVIs, and is widely documented in the literature [112]. However, more exact statements to the BFZ spectrum of activity, the resistance to NAC and NCS, and its clinical relevance would be possible, when on a broader scale these isolates could have been tested. Despite the higher MIC-levels, most likely due to the high amount of isolates of azole pre-treated patients in this study, low MIC-levels (high susceptibility) of BFZ in *C. glabrata* could be determined, and are demonstrated in Tables 5 and 6. 86% of the *C. glabrata* isolates were susceptible to BFZ, and 97% to VRC (Table 6). The similar in vitro performance of BFZ in comparison to VRC in *C. glabrata* is visualized in Figures 5 and 6, demonstrating peak performance in almost the same MIC range, and both drugs showed bi-modular MIC-distribution. This could be verified when the characteristic MIC-values of different *C. glabrata* collectives were compared (Table 8) showing similar azole MIC-profiles, even for the differing *C. glabrata* collectives. However, when other *Candida* species and most of the non-*Candida* isolates are taken into account, they are associated with elevated azole MICs, especially BFZ MICs. This had been also shown for NAC isolates by Carrillo-Muñoz and Torres-Rodriguez [113]. In a study with 88 vaginal isolates Dota et al. [114] found no fluconazole, miconazole and voriconazole, however, 48% ketoconazole and 29% itraconazole resistant yeast isolates. This is in contrast to reports in the literature [48, 115-117] and to the presented data, for which the possibility of higher azole MIC-levels exists than described in the literature because of preceding antifungal therapy. Thus, the overall resistance of all isolates (N=324) was 70%, 57%, 25%, and 3% for ITC, FLC, BFZ, and VRC, respectively.The results of this small-sized study confirm and update the earlier findings on the yeast antifungal activities of bifonazole [2, 5, 6, 93, 96, 118-119], demonstrating. that the treatment of superficial infections with topical antifungal agents and fluconazole is limited, especially in vulvovaginal and recurrent candidiasis [114, 120-122] where *C. glabrata* is the second most common cause after *C. albicans*, and often the primary species in elderly (> 65y) patients [123]. It also has been reported that resistant *C. glabrata* appear after fluconazole therapy, respectively, an increased number of infections with these species are encountered when fluconazole was used routinely for prophylaxis [124-127]. Therefore, resistance to azoles may develop if prior antimicrobial therapy is used [128] or are continuously applied in clinically unresponsive infections. Although bifonazole was the most effective drug after voriconazole to superficial *Candida* isolates, it should, however, be mentioned that newer antifungals such as voriconazole or echinocandins have not been properly evaluated in this indication field [129], and that VRC and BFZ, at least in Germany, are not licensed for such applications.

2.3 MIC assessment

As there are no breakpoints for the assessment of bifonazole *in vitro* data, the following facts for the chosen breakpoints had been considered: available MIC distributions in the literature and those shown in Fig. 2, to Fig. 7, the pharmacokinetic and lipophilic properties of BFZ, together with the achievable BFZ-concentration in different compartments. As reported, and when applied properly, 0.6±0.3% of the BFZ-dose applied is absorbed after six hours. The absorption rate for topical applications is approximately 0.008mg/100cm^2 per hour. In inflamed skin these values are higher by a factor of four. Similar results were obtained after the application of bifonazole as a 1% solution. Higher levels in the different compartments can be expected from the formulations on the market with up to 2.5% BFZ. Plasma-levels of up to 16 ng/ml were obtained in babies with nappy rash after a single 5g cream application [3]. For systemic applications no BFZ pharmacokinetic data are available. Except for BFZ, the listed FLC, ITC, and VRC-breakpoints were at test time similar to the now available EUCAST breakpoints [71]. For this collective, and with the MIC assessment in the three categories, susceptible (S), intermediate (I) and resistant (R), several inappropriate S-I-R ratings (MIC-categorizations without “S” or “R”, i.e. with only “IR” “IS” or “I”) or extremely high rates of intermediate tested azole isolates occurred (e.g. for itraconazole, Tables 5, 6). As this happens also in other *in vitro* evaluations of bacteria and fungi, biased reporting of susceptibility testing results and complications in objective (MIC) data comparison may be the outcome.

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Fig. 5. MAR-index-weighted log_2-MIC distribution of bifonazole (BFZ) of total *C. glabrata* isolates from study S-324 (n=166), with normal- (red curve: -1.9123, 1.05753), and nonparametric density distribution (smooth blue curve: Kernel-Std. 0.342383). The whisker plot with the diamond symbol above the bars is showing the mean MIC of the 95% confidence interval and the standard error bar. The error bars given additionally on top of the individual twofold dilutions indicate the standard deviation of the mean, and the number the percentage of strains at this log_2-dilution.

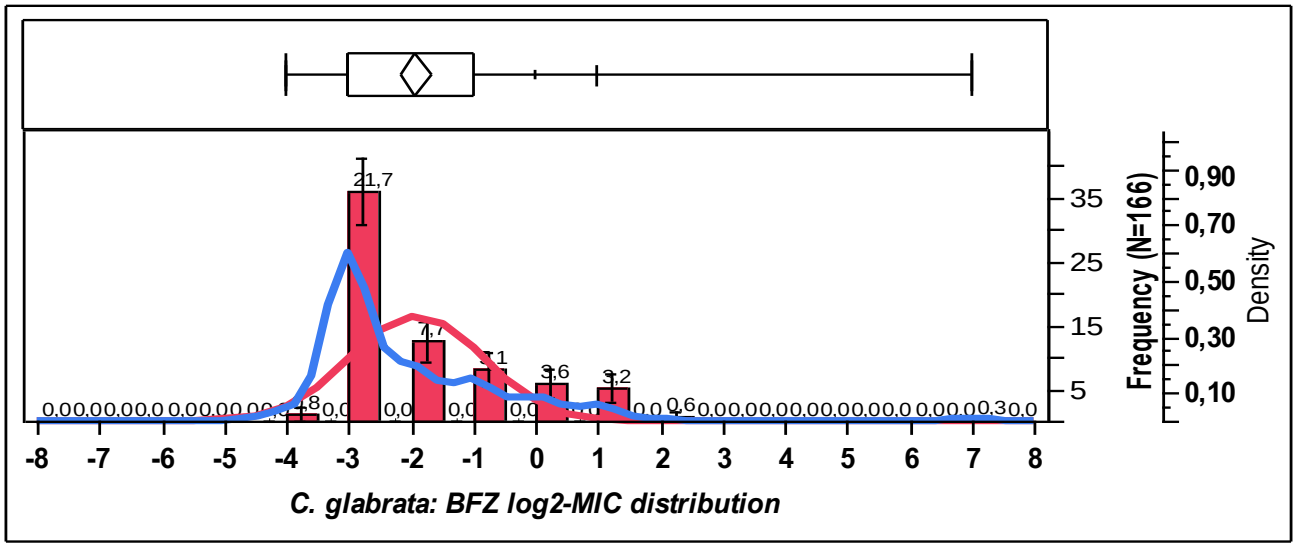
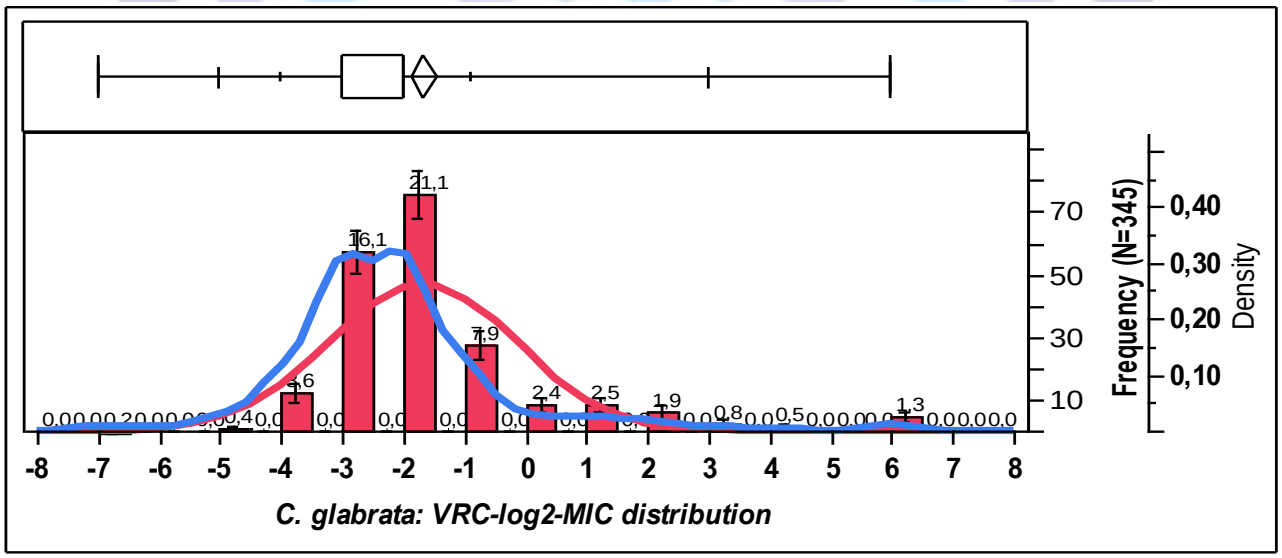


Fig. 6. MAR-index-weighted log_2-MIC distribution of voriconazole (VRC) of total *C. glabrata* isolates from study S-2029 (n=345), with normal- (red curve: -1.6714, 1.5279), and nonparametric density distribution (smooth blue curve: Kernel-Std. 0.424191). The whisker plot with the diamond symbol above the bars is showing the mean MIC of the 95% confidence interval and the standard error bar. The error bars given additionally on top of the individual twofold dilutions indicate the standard deviation of the mean, and the numbers the percentage of strains at this log_2-dilution.



The intermediate category was introduced to cover different factors influencing MIC-assessment such as:

- being a buffer zone to enshrine methodological aspects
- provide the possibility, if appropriate, to recommend drug doses for organisms which may be inhibited at drug concentrations above the therapeutically recommended but below the effective toxic dose;
- additional DD (dose-dependent inhibition) category for FLC in the CLSI recommendations [130].

Furthermore, due to the changes from general breakpoints to species specific breakpoints and according to the pharmacokinetic-pharmacodynamic properties of the antimicrobial substances, an intermediate category may not be appropriate and therefore has been dropped in recent CLSI and EUCAST recommendations. This again may result in situations of mixed MIC categorisations for several different drugs under comparative testings.

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Table 5. Percentage (% in round figures) of total and infection type dependent yeast isolates, susceptible (S) and resistant (R) to bifonazole (BFZ), fluconazole (FLC), itraconazole (ITC), and voriconazole (VRC), respectively, percentage of species-specific susceptibility and resistance of the azoles to either fluconazole (FLC^r) or bifonazole (BFZ^r) resistant isolates. The red marked figures correspondto S-R percentages which do not match 100% due to missing I-percentage values. S and R with 0% indicate that all MICs were assessed as intermediate.

Antifungal agent Status	Frequency Status	Infection Type	Antifungal agent	Total isolates		*C. glabrata*		*C. tropicalis*		*C. albicans*		*C. parapsilosis*		*i. orientalis*		*D. hansenii*		*S. cerevisiae*		*K. marxianus*		*M. guilliermondii*		*Cl. lusitaniae*		*C. magnoliae*	
			N/%	324/100	→	166/51		44/14		24/7		24/7		33/10		10/3		9/3		6/2		4/1		2/1		2/1	
			S-I-R*→	S N/%	R N/%	S %	R %	S %	R %	S %	R %	S %	R %	S %	R %	S %	R %	S %	R %	S %	R %	S %	R %	S %	R %	S %	R %
ALL	324	All	BFZ	217/67	82/25	86	6	45	55	79	17	46	42	21	73	30	60	67	0	83	17	50	0	50	50	0	100
			FLC	80/25	184/57	10	70	43	36	58	33	25	33	9	85	50	40	67	22	83	17	75	25	100	0	0	0
			ITR	30/9	225/70	4	78	9	55	21	50	4	71	18	73	0	60	22	56	50	33	50	50	0	50	0	100
			VOR	314/97	10/3	98	2	93	7	100	0	92	8	97	3	100	0	100	0	100	0	100	0	100	0	100	0
			N/%	192/100	→	102/53		18/9		19/10		12/6		26/14		5/3		4/2		2/1		2/1		0/0		2/1	
ALL	324	IVI	BFZ	129/67	49/26	88	6	39	61	79	16	33	50	23	69	20	60	75	0	100	0	50	0			0	100
			FLC	39/20	115/59	10	66	28	56	58	37	25	33	8	89	40	20	75	25	50	50	100	0			0	0
			ITR	25/13	117/61	7	65	17	56	26	42	8	58	19	69	0	60	50	25	50	50	50	50			0	100
			VOR	190/99	2/1	99	1	94	6	100	0	100	0	96	4	100	0	100	0	100	0	100	0			100	0
			N/%	132/100	→	64/48n		26/20		5/4		12/9		7/5		5/4		5/4		4/4		2/1		2/1		0/0	
ALL	324	SFI	BFZ	88/67	33/25	83	6	50	50	80	20	58	33	14	86	40	60	60	0	75	25	50	0	50	50		
			FLC	41/31	69/52	11	77	54	23	50	20	25	33	14	71	60	40	60	20	100	0	50	50	100	0		
			ITR	5/4	122/94	0	100	4	54	0	80	0	83	14	86	0	60	0	80	50	25	50	50	0	50		
			VOR	126/96	6/4	95	5	92	8	100	0	83	17	100	0	100	0	100	0	100	0	100	0	100	0		
			N/%	115/100	→	67/58		10/9		7/6		4/3		23/20		2/2		1/1		1/1		0/0		0/0		0/0	
FLC^r	184	IVI	BFZ	78/68	30/26	94	6	20	80	100	0	100	0	30	70	0	100	100	0	100	0						
			ITR	16/14	81/70	6	75	20	70	57	29	0	50	22	74	0	100	100	0	0	100						
			VOR	113/98	2/2	94	6	100	0	100	0	100	0	96	4	100	0	100	0	100	0						
			N/%	67/100	→	49/73		6/9		1/2		4/8		5/10		2/4		1/2		0/0		1/2		0/0		0/0	
	184	SFI	BFZ	48/70	15/22	94	6	67	33	100	0	25	75	0	100	0	100	100	0			100	0				
			ITR	1/1	66/96	0	100	0	67	0	100	0	100	20	80	0	100	0	100			0	100				
			VOR	65/94	4/6	94	6	100	0	100	0	75	25	100	0	100	0	100	0			100	0				
			N/%	49/100	→	6/12		11/23		3/6		6/12		18/37		3/6		0/0		0/0		0/0		0/0		2/5	
	82	IVI	FLC	9/18	30/61	0	67	18	73	0	33	33	0	11	89	33	67									0	0
			ITR	1/2	47/96	0	83	0	91	0	100	17	83	0	94	0	100									0	100
BFZ^r			VOR	47/96	2/4	83	17	100	0	100	0	100	0	94	6	100	0									100	0
			N/%	33/100	→	4/12		13/39		1/3		4/12		6/18		3/4		0/0		1/3		0/0		1/3		0/0	
	82	SFI	FLC	12/36	15/46	25	75	46	15	0	0	25	75	17	83	33	67			100	0			100	0		
			ITR	2/6	23/70	0	100	8	38	0	100	0	100	17	83	0	67			0	100			0	100		
			VOR	30/91	3/9	75	25	92	8	100	0	75	25	100	0	100	0			100	0			100	0		

* Intermediate assessed MIC-values are not displayed (table space reasons)

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Table 6. Comparison of the susceptibility profiles in percentage (% in round figures) of species-specific MICs categorized into "S", "I", and "R" (three-leg (**3-LS)** system), or "S*" and "R*" (two-leg (**2-LS**) system), respectively. Changes in percentages are indicated as bold numbers in red cells (2-LS) in contrast to grey shadowed figures (3-LS). The susceptibility/resistance profiles of IVI (rows in light blue) and SFI (rows in darker blue) patients were compared by 2-LS and displayed species-specific for bifonazole (BFZ), fluconazole (FLC) , itraconazole (ITC), and voriconazole (VRC).

Antifungal agent	Assessment method	N	S-I-R Category	Infection type	Frequency (N)	Percentage of N	*C. glabrata*	*C. tropicalis*	*C. albicans*	*C. parapsilosis*	*I. orientalis*	*D. hansenii*	*S. cerevisiae*	*K. marxianus*	*M. guilliermondii*	*Cl. lusitaniae*	*C. magnoliae*
BFZ	3-LS	324	S	All	217	**67**	**86**	45	**79**	**46**	**21**	**30**	**67**	83	**50**	50	0
			I		25	8	8	0	4	13	6	10	33	0	50	0	0
			R		82	**25**	**6**	55	**17**	**42**	**73**	**60**	**0**	17	**0**	50	100
	2-LS	324	S*	All	226	**70**	**89**	45	**81**	**51**	**24**	**34**	**80**	83	**70**	50	0
			R*		38	**30**	**11**	55	**19**	**49**	**76**	**66**	**20**	17	**30**	50	100
	2-LS	192	S*	IVI	135	70	91	39	81	40	26	28	85	100	70	-	0
			R*	IVI	57	30	9	61	19	60	74	72	15	0	30	-	100
	2-LS	132	S*	SFI	135	70	87	50	80	62	14	40	76	75	70	50	-
			R*	SFI	57	30	13	50	20	380	86	60	24	25	30	50	-
FLC	3-LS	324	S	All	80	**25**	**9**	**43**	**58**	**25**	**9**	**50**	**67**	83	75	100	0
			I		60	18	36	21	8	42	6	10	11	0	0	0	100
			R		184	**57**	**55**	**36**	**33**	**33**	**85**	**40**	**22**	17	25	0	0
	2-LS	324	S*	All	91	**28**	**24**	**68**	**62**	**42**	**12**	**54**	71	83	75	100	100
			R*		233	**72**	**76**	**32**	**38**	**58**	**88**	**46**	29	17	25	0	0
	2-LS	192	S*	IVI	61	32	20	34	60	42	9	48	75	50	100	-	40
			R*	IVI	131	68	80	66	40	58	91	52	25	50	0	-	60
	2-LS	132	S*	SFI	50	38	16	63	68	35	20	60	68	100	50	100	-
			R*	SFI	82	62	84	37	32	65	80	40	32	0	50	0	-
ITC	3-LS	324	S	All	30	**9**	**4**	**5**	**21**	**4**	**27**	**0**	**22**	**50**	50	**0**	0
			I		69	21	18	25	29	25	9	40	22	17	0	50	0
			R		225	**70**	**78**	**70**	**50**	**71**	**73**	**60**	**56**	**33**	50	**50**	100
	2-LS	324	S*	All	44	**23**	**11**	**15**	**33**	**14**	**22**	**16**	**31**	50	50	**20**	0
			R*		148	**77**	**89**	**85**	**67**	**86**	**78**	**84**	**69**	50	50	**80**	100
	2-LS	192	S*	IVI	19	10	18	28	13	22	31	16	60	50	50	-	0
			R*	IVI	173	90	82	72	87	78	69	84	40	50	50	-	100
	2-LS	132	S*	SFI	13	10	0	21	8	7	14	16	8	60	50	20	-
			R*	SFI	119	90	100	79	92	93	86	84	92	40	50	80	-
VRC	3-LS	324	S	All	314	97	93	91	100	92	97	100	100	100	100	100	100
			I		0	0	0	0	0	0	0	0	0	0	0	0	0
			R		10	3	7	9	0	8	3	0	0	0	0	0	0
	2-LS	324	S*	All	314	97	93	91	100	92	97	100	100	100	100	100	100
			R*		10	3	7	9	0	8	3	0	0	0	0	0	0
	2-LS	192	S*	IVI	189	98	99	94	100	100	96	100	100	100	100	-	100
			R*	IVI	3	2	1	6	0	0	4	0	0	0	0	-	0
	2-LS	132	S*	SFI	125	95	95	92	100	83	100	100	100	100	100	100	-
			R*	SFI	7	5	5	8	0	17	0	0	0	0	0	0	-

As with conventional MIC assessment inaptly results may be achieved, respectively, new substances tended to be shown in a "very poor light", Odds and Abbott [131] tried a novel approach to the assessment of antifungals by introducing the relative inhibition factors (RIFs). RIFs were defined there as "the area under a fixed portion of the antifungal dose-response curve, expressed as a percentage of the area under the dose-response curve for a theoretical non-inhibitory substance" [132], which may be impracticably for routine assessments. For bacterial MIC evaluations and for the generation of a susceptibility index, Grimm [73], described an easier way to achieve more balanced MIC-assessments, enabling also a better comparability of epidemiological and microbiological evaluations. To avoid assessment bias, and as the intermediate category may already contain strains with repeated exposure to the drug(s) [133], isolates with first step mutations [134], with activated regulative mechanisms or other mutations [135-137], i.e., strains which are on the way to resistance, the intermediate (I) category was split into two parts. The higher allotment of the intermediate category is

transferred to the resistant, and the lower proportion to the susceptible category. The results by adding 40% of the I-category to the susceptible assessed populations, and 60% to the resistant group are given in Table 6, when possible for both species from IVI and SFI patients. If minor or major changes in the MIC "S" and "R" assessment percentages occurred they are marked in bold face in grey-shadowed fields. The corresponding S*- and R*-values for the 2-leg system are given below in reddish marked cells. As it can be seen, 34% (Table 5, red marked S-R pairings) of the S-I-R assessments would be difficult to report, however, according to the mentioned transitions, more realistic, better comparable and more patient oriented results can be obtained. The fact that the readability and comparison of MIC results could be improved actually is shown in Table 7, e.g. by the differences in species-specific susceptibility/resistance to isolates from IVI and SFI patients S*/R* values of species from IVI patients in the light blue shadowed rows, and those of SFI patients shadowed as darker blue rows. The differences emerging by dividing the I-category may also be exemplarily seen by SPA, where about 50% of the SPs containing intermediate assessed AFAs disappear (Fig. 8; Table 10). That according to these transitions, more realistic, better comparable, and more patient oriented results may be obtained is exemplarily shown in Table 8, where azole susceptibility / resistance data associated to relevant epidemiological and patient related factors should be compared more clearly, space saving, and reliably.

Table 7. Characteristic MIC-values of *C. glabrata* strains derived of IVI and SFI patients ((INFT) from this study (S-324), and for comparative purposes from several other in parallel performed collaborative studies which included the antifungal agents (AFA) bifonazole (BFZ), fluconazole (FLC), itraconazole (ITC), ketoconazole (KTC), voriconazole (VRC), anidulafungin (ANF), caspofungin (CSF), micafungin (MCF), flucytosine (FCY), and amphotericin B (AMB).

Study			***C. glabrata***		**Characteristic MIC-values**						
Number	**INFT**	**AFA**	**frequency**		**MIC_{range}**	**MIC_{gmean}**	**MIC_{mode}**	**MIC_{50}**	**MIC_{75}**	**MIC_{90}**	**ECV**
[Reference]			**n**	**% of N**	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l	mg/l
		FLC			0.5-128	9.5	8	8	16	64	32
S-324	SFI	ITC	166	51.2	0.125-32	5.4	1	2	16	16	8
(N=324)		VRC			0.031-8	1.1	0.125	0.125	0.25	0.5	0.5
		BIF			0.063-128	1.2	0.125	0.125	0.5	1	0.5
		FLC			0.5-128	11.1	8	8	16	128	32
S-324	SFI*	ITC	62	48.1	1-32	9.6	16	16	16	32	64
(N=129)		VRC			0.031-8	1.1	0.125	0.125	0.25	0.5	0.5
		BIF			0.125-128	1.1	0.125	0.125	0.25	0.5	0.5
		FLC			0.031-128	4.9	4	4	16	32	16
S-4860		ITC			0.016-16	1.3	0.063	0.25	1	4	1
(N=4860)	IVI	VRC	889	18.3	0.008-16	1.1	0.125	0.125	0.5	1	0.5
[109]		KTC			0.016-16	1.1	0.031	0.25	1	2	1
S-60		FLC			0.25-32	3.7	4	4	8	8	16
(N=60)	IVI	ITC	60	100.0	0.063-4.0	1.5	1	1	2	2	4
[65]		VRC			0.008-4	1.0	0.125	0.125	0.125	0.25	0.5
		FLC			0.031-128	1.4	0.031	2	4	16	8
S-2029	IVI	ITC	258	12.7	0.008-8	1	0.008	0.063	0.25	1	0.25
(N=2029)		VRC			0.008-16	1	0.008	0.031	0.125	0.5	0.125
		FLC			0.063-128	10.5	8	8	16.0	128	32
		PSC			0.004-16	3.3	1	2	4.0	16	8
		VRC			0.004-16	1.5	0.5	0.5	1.0	16	2
S-1098		ANF			0.004-2	1.0	0.031	0.031	0.063	0.063	0.125
(N=1062)	IVI	CSF	236	22.2	0.008-1	1.1	0.063	0.063	0.125	0.25	0.25
[66]		MCF			0.004-2	1.0	0.016	0.016	0.016	0.016	0.125
		FCY			0.016-64	1.1	0.063	0.063	0.125	0.125	0.25
		AMB			0.125-2	1.3	0.5	0.5	1	1	2

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Table 8. Susceptibility / resistance (% in round figures) to bifonazole (BFZ), fluconazole (FLC), itraconazole (ITC), and voriconazole (VRC), assessed according to the two-leg (2-LS) system, associated with clinical speciality, specimen type, and demographic factors.

Parameter	Factor	Factor frequency		% Azole susceptibility (S) / resistance (R) of:							
				BFZ		FLC		ITC		VRC	
		N	%	S	R	S	R	S	R	S	R
Clinic speciality	Ear-Nose-Throat	2	1.5	0	100	50	50	20	80	100	0
	Gynaecology	3	2.3	100	0	47	53	33	67	100	0
	ICU	67	51.9	76	24	48	52	12	88	95	5
	Internal Medicine	30	23.3	61	39	47	53	13	87	97	3
	Paediatrics	5	3.9	40	60	36	64	0	100	100	0
	Surgery	1	0.8	100	0	0	100	0	100	100	0
	Transplantation	1	0.8	100	0	0	100	0	100	100	0
	Urology	1	0.8	100	0	0	100	0	100	100	0
	External	19	14.7	100	0	17	83	0	100	100	0
Specimen type	Aspirate	34	26.4	78	22	41	59	18	82	96	6
	Blood culture	6	4.7	50	50	50	50	7	93	100	0
	Catheter	4	3.1	79	21	15	85	0	100	75	25
	Fungal culture	19	14.7	100	0	18	82	9	91	100	0
	Fluid (n.st)	14	10.8	43	57	59	41	0	100	100	0
	Swab	25	19.4	74	26	39	61	10	90	96	4
	Urine	27	20.9	73	27	56	44	12	88	100	0
Patient risk	Burn	1	0.8	0	100	40	60	0	100	100	0
	Catheter	28	21.7	79	21	35	65	16	84	96	4
	Catheter + ICU stay >2d	24	18.6	70	30	51	49	12	88	92	8
	ICU stay >2d alone	34	26.4	75	25	55	45	15	85	97	3
	Transplantation+Catheter	1	0.8	100	0	0	100	0	100	100	0
	Low to negligible	22	17.0	56	44	41	59	5	95	100	0
	N.a.*	19	14.7	100	0	18	82	0	100	100	0
Gender	Female	47	36.4	75	25	51	49	13	87	98	2
	Male	63	48.8	67	33	41	59	11	89	95	5
	N.a.*	19	13.8	100	0	17	83	0	100	100	0
Age range (years)	≤ 2	5	3.9	40	60	36	64	0	100	100	0
	30-40	8	6.2	75	25	53	47	13	87	100	0
	41-50	10	7.8	90	10	48	52	18	82	100	0
	51-60	11	8.5	54	46	44	56	9	91	100	0
	61-70	34	26.4	60	40	45	55	15	85	94	6
	71-80	36	27.9	69	31	46	44	12	88	94	6
	≥ 81	6	4.6	83	17	50	50	93	7	100	0
	N.a.*	19	14.7	100	0	18	82	0	199	100	0

*N.a. = not applicable – *C. glabrata* direct cultures

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2.4 Azole parallel-resistance

In the literature, parallel resistance (two or more antimicrobial agents of the same substance class are resistant) is generally reported as cross-resistance (for more transparency the term should be allocated to the simultaneous resistance of two or more antimicrobial agents of different substance classes). As only AFAs from the same substance class were tested, and if not otherwise indicated, throughout the text “parallel-resistance" is used when pattern-profiles with two or more “R” are encountered.

SPA revealed that populations with zero to fourfold resistance occurred (Figure 9). Multiple-resistance was species-specific differently distributed, included bifonazole, and was most prominent in *C. tropicalis and C. glabrata*. Complete parallel resistance (2%) to all four azoles (Table 9).was seen in *C. glabrata* (n=2, 2%) and in *C. parapsilosis* (n=1, 1% of total isolates). That there is also a significant heterogeneity in respect to the azole susceptibility of the isolates is shown by cluster analysis in Fig. 7, and for different *C. glabrata* collectives in Table 10. It clearly could be demonstrated that complete susceptibility, exemplarily shown for to FLC, ITC, and VRC (SP: SSS), of treated, respectively pre-treated or fluconazole-treated patients are lower for *C. glabrata* (Table 10) than those of isolates from non-treated patients (NTP), when this study (S-324) is compared to others.

For the three leg MIC-assessment system and the 4 azoles, theoretically 3^4=81 SPs are possible, thereof 30 (39%) could be determined. That by applying the two-leg MIC assessment system the number of SPs is reduced by 45% is demonstrated in Figure 7, and Table 9. In Figure 7 the percentages of SPs obtained by 3-LS and 2-SPA are displayed as the example the number of SPs obtained for SFI isolates.

Fig. 7. Susceptibility patterns of the SP-profiles (FLC-ITC-VRC-BFZ) of populations of isolates from patients with superficial infections (n=129), when the MICs of isolates were assessed by the 3_LS (Frequency %-3L; 22 SPs) or the 2-LS (Frequency %-2LS; 10 SPs) method and evaluated by SPA.

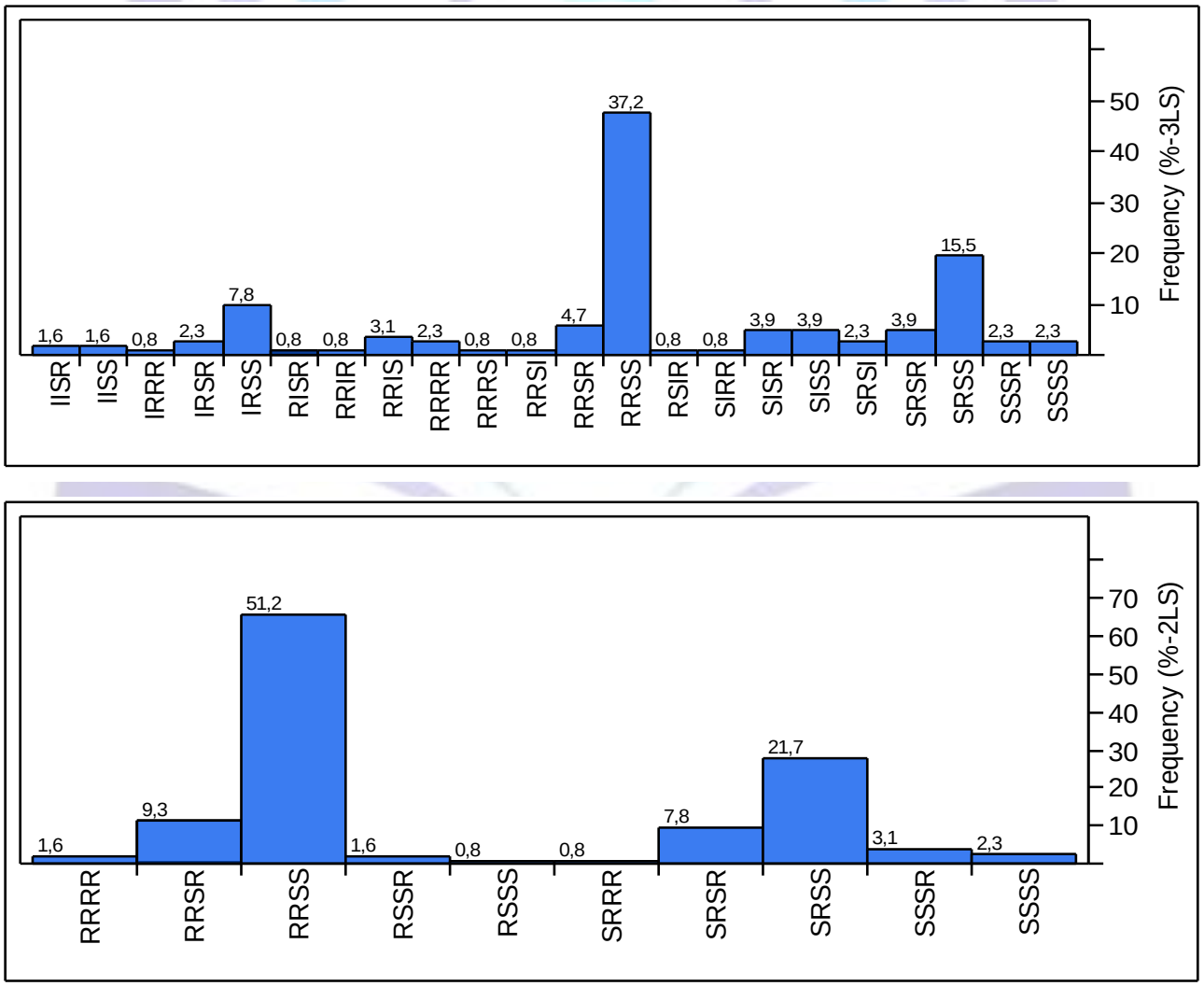

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Table 9. Qualitative and quantitative differences by SPA of the SP-profiles with MICs assessed by the 3-LS (dark grey shadowed cells) or 2-LS (reddish shadowed cells) method and evaluated by SPA. SPs are listed according to the frequency of multi-resistance (MR: 0xR to-4xR) together with the value of the appropriate MAR-index (MAR). The number of the different species-specific populations with resistance (**R**) to fluconazole (FLC), itraconazole (ITC), voriconazole (VRC), and bifonazole (BFZ) in the pattern (SP-profile) are given in the appropriate species column.

Number of multi-resistant (MR) AFA	MAR index	SPA: Susceptibility basis: FLC-ITC-VRC-BFZ				2-LS SP frequency 10 SP clsses)		3-LLS SP frequency 22 SP classes)		*C. glabrata*	*C. tropicalis*	*C. parapsilosis*	*I. orientalis*	*D. hansenii*	*S. cerevisiae*	*K. marxianus*	*M. guilliermondii*	*Cl. lusitaniae*	*C. albicans*	*C. magnoliae*
						N	%	N	%	62	26	11	8	5	5	4	3	2	2	1
n-fold MR	**MAR**	**SP-profile:**				129	100	129	100	48	20	8	6	4	4	3	2	2	2	1
						n	%	n	%	**No. of species-specific populations based on 3-LS (dark gray) and 2-LS (reddish figures)**										
		S	**S**	**S**	**S**	3	**2**	3	**2**							**2**	**1**			
0xR	**0.0**	S	I	S	S	0	**0**	5	**4**		**1**			**1**	**1**	**1**		**1**		
		I	I	S	S	0	**0**	2	**2**			**2**								
		S	S	S	**R**	0	**0**	3	**2**		**1**	**1**				**1**				
		S	S	S	**R**	4	**3**	0	**0**		2	1				1				
		S	I	S	**R**	0	**0**	5	**4**		**5**									
		I	I	S	**R**	0	**0**	2	**2**		**1**		**1**							
1xR	**0.25**	**R**	S	S	S	1	**1**	0	**0**			1								
		S	**R**	S	S	0	**0**	20	**15**	**9**	**5**	**1**		**1**	**1**	**1**	**1**		**1**	
		S	**R**	S	S	28	**22**	0	**0**	9	7	2	1	2	3	1	2		1	
		S	**R**	S	I	0	**0**	3	**2**		**1**		**1**		**1**					
		I	**R**	S	S	0	**0**	10	**7**	**4**	**2**	**3**			**1**					
		S	**R**	S	**R**	0	**0**	5	**4**	**1**	**1**		**1**	**1**				**1**		
		S	**R**	S	**R**	10	**8**	0	**0**	1	5		1	2				1		
		I	**R**	S	**R**	0	**0**	3	**2**		**2**									**1**
		S	I	**R**	**R**	0	**0**	1	**1**		**1**									
		R	S	S	**R**	2	**2**	0	**0**				2							
2xR	**0.5**	**R**	S	I	**R**	0	**0**	1	**1**				**1**							
		R	I	S	**R**	0	**0**	1	**1**		**1**									
		R	**R**	S	S	0	**0**	48	**37**	**41**	**4**	**1**			**1**				**1**	
		R	**R**	S	S	66	**51**	0	**0**	50	6	6			2			1	1	
		R	**R**	S	I	0	**0**	1	**1**								**1**			
		R	**R**	I	S	0	**0**	4	**3**	**4**										
		R	**R**	S	**R**	0	**0**	6	**5**		**1**	**1**	**4**							
		R	**R**	S	**R**	12	**8**	0	**0**		5	1	4	1						1
3xR	**0.75**	**R**	**R**	I	**R**	0	**0**	1	**1**					**1**						
		R	**R**	**R**	S	0	**0**	1	**1**	**1**										
		S	**R**	**R**	**R**	1	**1**	0	**0**		1									
		I	**R**	**R**	**R**	0	**0**	1	**1**					**1**						
4xR	**1.0**	**R**	**R**	**R**	**R**	2	**2**	3	**2**	**2**		**1**								

To further investigate and discriminate the susceptibility patterns, “multiple antibiotic resistance” (MAR) indexing was introduced for the yeasts, analogously as described by Krumperman [76] for bacteria. From the five MAR-groups obtained (MAR=0 to MAR=1.0), three were significantly above the factor 0.2 (0.5, 0.75, and 1.0), an artificial limit, which should indicate bacteria (here yeasts) from environments with the presence of several antimicrobial agents [76, 135], respectively, with a high risk to become multi-resistant. That the MAR-clusters are directly linked to the different susceptibility profiles was demonstrated in Tables 9 and 10. Whereas MAR=0 corresponds to the SPs of the populations with solely susceptibility to the individual azoles (SP: SSSS) or susceptible and/or intermediate assessed MICs, the MAR=0.25 group shows only populations which are resistant to only one antifungal agent in the pattern, the MAR=0.5 to MAR=1.0 groups harbour the multi-resistant populations with resistance to two to four azoles (Tables 9, 10). The manifold species-populations with their different SP-profiles have been visualized by cluster analysis in Fig. 8, considering the MAR indexes, the correlations of $\log_2$-MICs and SP-profiles. In this context, Cauwenberg [115], and Cross et al. [138] have shown that azole-based over-the-counter (OTC) antifungal agents used to treat vaginitis have the potential to contribute to the selection of highly resistant *Candida* strains in otherwise healthy women. In addition fluconazole-resistant *C. albicans* and C. glabrata of bloodstream isolates from cancer patients were “cross-resistant” to miconazole, clotrimazole, and tioconazole, but remained susceptible to butoconazole. These authors also provided evidence that spontaneous mutants of *C. glabrata* selected for resistance to clotrimazole were parallel-resistant to other azole-based drugs, including fluconazole. They also showed that OTC-azole antifungals, to which, aside of BFZ, belong the topically applicable agents butoconazole, clotrimazole, econazole, fenticonazole, fluconazole, itraconazole, ketoconazole, miconazole, omoconazole, oxiconazole, sertaconazole, sulconazole, and terconazole, can promote azole-resistance in *Candida*. This may be confirmed by the SPS results, showing exemplarily for BFZ its multi-fold parallel-resistance to FLC, ITC, and VRC (Table 10).

3. CONCLUSIONS

Cutaneous and superficial fungal infections are usually treated topically, but nail and hair infections, dermatophytosis and chronic non-responsive yeast infections are usually treated with oral antifungal drugs, which include griseofulvin, ketoconazole, fluconazole, itraconazole, bifonazole and terbinafine. In 2013, the European Medicines Agency’s Committee on Medicinal Products for Human Use (CHMP) recommended that the marketing authorisations of oral ketoconazole-containing medicines should be suspended throughout the European Union (EU), whereas topical formulations of ketoconazole (such as creams, ointments and shampoos) can continue to be used. Although bifonazole, which is available in about 100 trade products, demonstrates partly an *in vitro* antifungal activity beyond its granted marketing authorisation, it is licensed in Germany only for topical applications in topical formulations. By showing a high in vitro activity to relevant etiological agents for superficial and invasive yeast infections, especially *Candida glabrata*, these strains show at the same time azole parallel resistance, in which BFZ is included. Only for *C. glabrata* the antifungal activity came close to that of voriconazole, and BFZ was *in vitro* clearly less efficient to other NCA and NCS species. Susceptibility pattern analysis demonstrated low level, however, significant parallel-resistance of BFZ to other azoles. As by conventional S-I-R MIC-categorization alone cross-resistance patterns are qualitatively and quantitatively not detectable, suitable evaluation methods such as susceptibility pattern and cluster analysis, as demonstrated here, should be introduced to assure more reliable MIC-assessments and confident guidance to antimicrobial chemotherapy. As the identification of less frequently encountered species is problematic, and due to the diversification of old species, the detection of new species, and the emergence of cryptic strains (species complexes) by molecular-genetic methodologies, the accurate and rapid identification of the fungal pathogens is an equal important prerequisite for optimal antimicrobial therapy.

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Fig. 8. Dendrogram (spectral distances) by two-way hierarchical clustering (Ward's method) of MAR-index weighted log_2-MIC-values of bifonazole (L_BIF), fluconazole (L_FLC), itraconazole (L_ITR), and voriconazole (L_VOR) MICs, and SPs of all isolates of SFI patients (N=132).

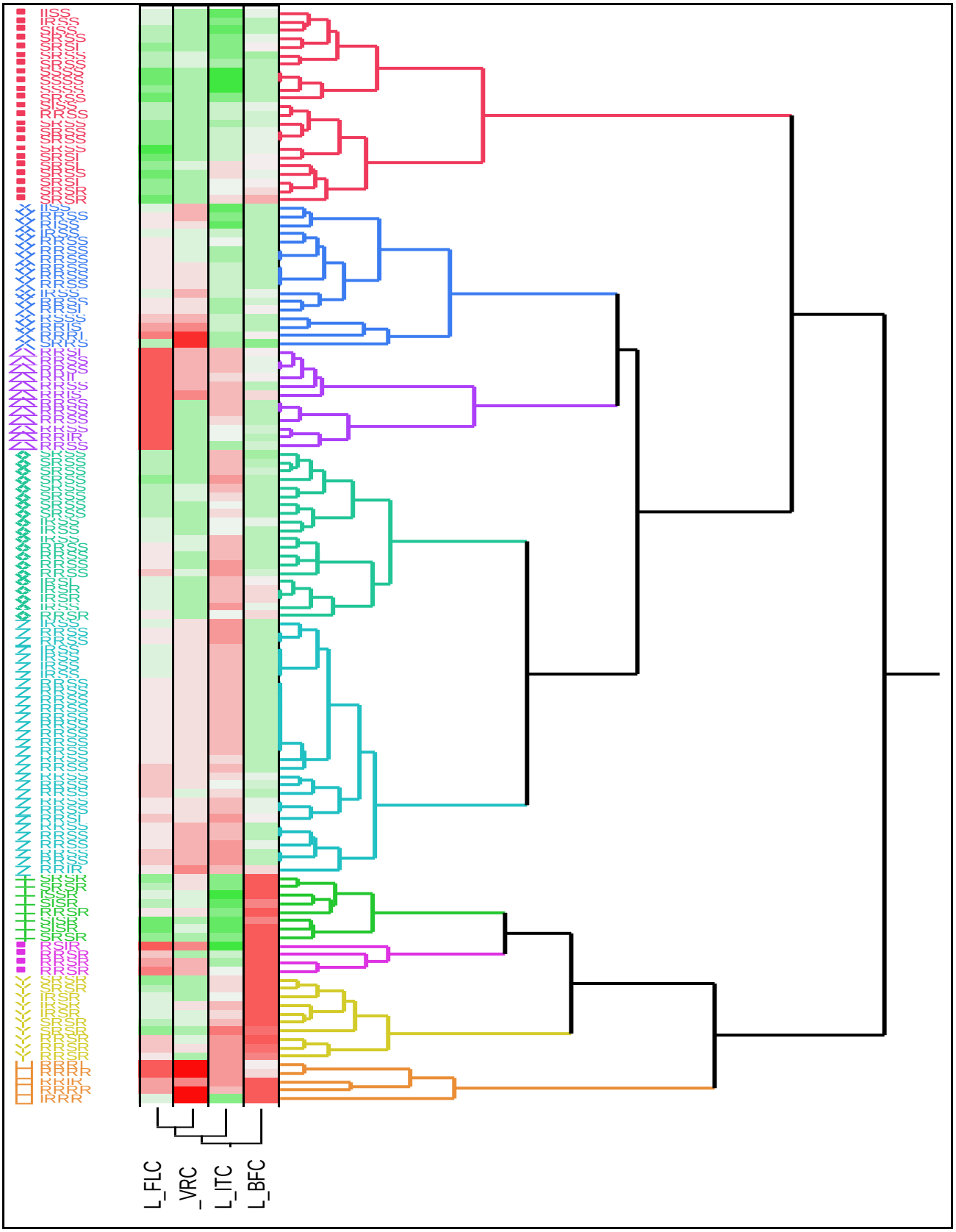

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Table 10. Comparison of SP-profiles for the same SP-basis (FLC-ITC-VRC) obtained by SPS of *C. glabrata* populations from different in parallel performed multicentre studies (S-xxx). The resistant antifungal agent (AFA) fluconazole (FLC), itraconazole (ITC), and voriconazole (VRC) in the SP is displayed as "R" and shadowed dark grey. The SPs without resistant AFAs and the corresponding percentages of occurrence in the different collectives are shadowed in light grey. The number of multi-resistant (MR) AFAs in the SP is given together with the calculated MAR-index (MAR) is given in the first two columns.

Parameter		SPA			*Candida glabrata* isolates from Study No.:																	
n-fold	Index	SP-basis:			S-324 (N=166)						S-2029 (N=358)			S-60 (N=60)			S-4860 (N=889)					
		FLC-ITC-VRC			SFI			IVI			IVI			IVI			ST			NST		
MR	MAR	SP-profile:			N	n	%	N	n	%	N	n	%	N (%)	n	%	N (%)	n	%	N (%)	n	%
		S	S	S		0	0		1	14		10	66		9	53		138	57		156	64
		S	S	I		0	0		0	0		1	7		1	6					1	0.01
		S	I	S		0	0		1	14		3	20		4	23		67	28		52	21
0xR	0.0	I	S	S	0	0	0	7	2	29	15	0	0	17	3	18	240	17	8	256	14	6
		S	I	I	(0)	0	0	(4)	0	0	(4)	0	0	(28)			(27)	0	0	(28)	1	0.01
		I	S	I		0	0		0	0		0	0					0	0		1	0.01
		I	I	S		0	0		2	29		1	7		3	18		18	8		20	8
		I	I	I		0	0		1	14												
		R	S	S		1	7		3	10								6	5		12	9
		R	S	I														3	2		6	5
		R	I	S								2	2		2	2		16	12		35	27
		R	I	I								1	1					5	4		3	2
		S	R	S	15	6	40	31	8	25	97			25	31	32	130	45	35	132	32	24
1xR	0.33	S	R	I	(9)			(19)			(27)			(42)			(14)	1	1	(15)	4	3
		I	R	S		8	53		20	65		61	63		23	92		32	25		35	27
		I	R	I								2	2					4	3		2	2
		S	I	R														2	1		0	0
		I	I	R														16	12		3	2
		R	I	R														3	5		2	4
		R	R	I	48	7	15	61	20	33	216	38	18	17	3	18	57	22	38	43	8	19
2xR	0.67	R	R	S	(29)	41	85	(36)	41	67	(60)	169	78	(28)	14	82	(6)	29	50	(5)	27	63
		R	S	R														3	5		5	12
		S	R	R								4	2					1	2		1	2
3xR	1.0	R	R	R	32	3	100	1 (1)	1	100	30 (8)	30	100	1 (2)	1	100	22 (3)	22	100	19 (2)	19	100

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