Emergence of resistant *Candida glabrata* in Germany

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**Background:** *Candida glabrata* is the second leading fungal pathogen causing candidaemia and invasive candidiasis in Europe. This yeast is recognized for its rapid ability to acquire antifungal drug resistance.

**Objectives:** We systematically evaluated 176 *C. glabrata* isolates submitted to the German National Reference Center for Invasive Fungal Infections (NRZMyk) between 2015 and 2019 with regard to echinocandin and fluconazole susceptibility.

**Methods:** Susceptibility testing was performed using a reference protocol (EUCAST) and a range of commercial assays. Hot spot regions of the echinocandin target *FKS* genes were sequenced using Sanger sequencing.

**Results:** In total, 84 of 176 isolates were initially classified as anidulafungin-resistant based on EUCAST testing. Of those, 71 harboured mutations in the glucan synthase encoding *FKS* genes (13% in *FKS1*, 87% in *FKS2*). Significant differences in anidulafungin MICs were found between distinct mutation sites. 11 *FKS* wild-type (WT) isolates initially classified as resistant exhibited anidulafungin MICs fluctuating around the interpretation breakpoint upon re-testing with multiple assays. Two *FKS* WT isolates consistently showed high anidulafungin MICs and thus must be considered resistant despite the absence of target gene mutations. Over one-third of echinocandin-resistant strains displayed concomitant fluconazole resistance. Of those, isolates linked to bloodstream infection carrying a change at Ser-663 were associated with adverse clinical outcome.

**Conclusions:** Resistant *C. glabrata* strains are emerging in Germany. Phenotypic echinocandin testing can result in misclassification of susceptible strains. *FKS* genotyping aids in detecting these strains, however, echinocandin resistance may occur despite a wild-type *FKS* genotype.

**Introduction**

*Candida* species are a leading cause of hospital-acquired infections.¹⁻³ A diverse spectrum of immunocompromised patients ranging from neonates to patients receiving organ transplants or suffering from malignancies are considered at risk.⁴⁻⁷

Within the phylogenetically heterogenous *Candida* genus, *Candida albicans* is the most frequently isolated species, followed by *Candida glabrata* as the second leading pathogen causing bloodstream infection in Europe.⁸⁻¹¹

Currently, the incidence of invasive *Candida* infections in ICU is estimated to range between 2.1 and 6.7 per 1000 admissions and candidaemia is recognized as the fourth most common cause of bloodstream infection worldwide.⁹,¹⁰ In Germany, the overall *Candida* species mortality of ICU mono-microbial bloodstream infection is 25.2% (*P < 0.001*).¹² Notably, non- *albicans Candida* species (27.1%; *P = 0.001*) seem to confer a slightly higher mortality than *C. albicans* (24.6%, *P < 0.001*), which may be linked to a higher degree of antifungal drug resistance.¹² In addition, a significant proportion of candidaemia cases occur outside the ICU. Thus, there is an estimated annual burden of 2000–12 000 cases of invasive candidiasis in Germany per year.⁷

Unlike *C. albicans*, which rarely develops antifungal drug resistance and is primarily susceptible to azole and echinocandin antifungals, *C. glabrata* shows an inherently reduced susceptibility to fluconazole. In addition, a significant proportion of clinical isolates show bona fide resistance to fluconazole.¹³⁻¹⁵

Since the introduction of echinocandins as first-line antifungal treatment for most cases of invasive candidiasis, the emergence of echinocandin resistance has also been observed in *C. glabrata*. Echinocandins target an essential element of the fungal cell wall...
by inhibition of the β-1,3-α-glucan synthase, which results in a fungicidal effect on Candida spp. Unlike C. albicans, C. glabrata harbours two FKS genes, FKS1 and FKS2, which are considered functionally redundant. Echinocandin resistance is mainly conferred by point mutations leading to amino acid substitutions within the two hot spots (HS) of the enzyme's catalytic subunits encoded by FKS1 and FKS2, which result in a substantial reduction of echinocandin affinity for its target enzyme. The haploid genome of C. glabrata as well as alterations within the DNA mismatch repair system contribute to rapid emergence of resistance during therapy. Studies have assessed the incidence of FKS-mutated C. glabrata strains ranging between 2%–18%. Due to these developments, antifungal susceptibility testing has become increasingly important. Phenotypic resistance testing is conducted by reference broth microdilution according to EUCAST or CLSI protocols. In routine daily use, commercial systems such as microdilution trays, semi-automated testing systems or agar gradient diffusion (AGD) tests facilitate antifungal susceptibility testing. However, the prolonged duration of all assays (24–48 h) and a recently observed overlap of wild-type and FKS-mutated populations limit the value of in vitro susceptibility testing. Due to these problems, some authors have suggested that molecular resistance testing should be performed to detect echinocandin resistance based on the presence of FKS mutations. Molecular analysis seems to predict therapeutic failure more precisely than phenotypic testing.

In this study, we assessed the current situation of phenotypic and genotypic echinocandin resistance in C. glabrata strains from Germany, focusing on differences in specific mutations and associated reduced susceptibility levels, the value of different testing methods and the emergence of MDR strains. Our data confirm the methodological problems of phenotypic testing. We showed that testing for micafungin is more discriminative than testing for anidulafungin with regard to detection of non-wild-type FKS isolates. However, we also identified two echinocandin-resistant isolates that do not harbour FKS mutations.

**Material and methods**

**Strain collection**

The National Reference Center for Invasive Fungal Infections (NRZMyk) serves as a national reference laboratory for Germany. In this study, 176 C. glabrata strains sent to the NRZMyk between 2015 and 2019 were analysed (see Table S1, available as Supplementary data at JAC-AMR Online). Strains were submitted to NRZMyk by German healthcare facilities and laboratories for species confirmation and susceptibility testing. Apart from isolates 2016–275 and 2016–293, which are two distinct strains from a single patient harbouring different FKS mutations, only initial isolates for a patient were included in this study.

**DNA extraction, species identification and FKS gene sequencing**

DNA extraction and PCR was conducted as described previously. Species identification was accomplished by sequencing the internal transcribed spacer of the ribosomal DNA (ITS-rDNA) using the primer pair V9G and LR3 for PCR and ITS4 as sequencing primer. Fungal DNA was amplified on a TProfessional Trio PCR thermocycler (Biometra GmbH, Göttingen, Germany). PCR products were visualized utilizing 1% agarose gels. SeqMan program version 11.0.0 (DNASTar; Lasergene) was used for the processing of sequences. Species identification was confirmed by GenBank basic local alignment search tool (BLAST) searches. HS of the FKS gene were amplified and sequenced as described previously. Relevant HS mutations were detected by ApE- A plasmid Editor v1.17 software.

**Susceptibility testing**

Initial in vitro susceptibility testing was performed by broth microdilution in accordance with EUCAST. Antifungal agents for susceptibility testing of the NRZMyk were provided by Pfizer Inc., Peapack, NJ, USA (anidulafungin and fluconazole) and MSD, Rahway, NJ, USA (caspofungin). The storage of plates did not exceed 6 months (at −80°C). RPMI supplemented with 2% glucose was used as a medium. The final inoculum ranged between 0.5 × 10⁵ and 2.5 × 10⁵ cfu/mL. MICs were assessed with a nephelometer (Labsystems Nepheloskan Ascent Microplate Reader Type 750) after 24 h of incubation at 35°C, defining the endpoint of growth as a ≥50% inhibition in comparison with the drug-free control. Current breakpoints (BP) were applied in accordance with CLSI or EUCAST as described in Table S2. Commercial testing devices used are listed in Table S3.

Reference strain ATCC 22019 Candida parapsilosis was used as a quality control.

**Data assessment and statistics**

Clinical data were extracted in an anonymized form from the NRZMyk database. Figures were designed in GraphPad Prism Version 7.05. The comparison of anidulafungin MICs and FKS HS mutations was performed using a Kruskal–Wallis test and Dunn’s multiple comparisons test (P values adjusted for multiple testing) in GraphPad Prism Version 7.05.

**Results**

**Characteristics of C. glabrata strains submitted to NRZMyk**

Over 5 years, a total of 178 C. glabrata clinical isolates were sent to the NRZMyk (20 strains in 2015; 47 strains in 2016; 35 strains in 2017; 41 strains in 2018; and 35 strains in 2019). Two isolates represented identical duplicates of initial isolates from the same patient and were thus excluded from further analysis. The remaining 176 C. glabrata isolates derived from 175 patients (Figure 1). Two non-identical isolates (2016–275 and 2016–293) were isolated from a single patient. The majority [14% (24/175)] of corresponding patients were between 61–65 years of age, 56% were male (Figure 2a). Most of the C. glabrata isolates (40%) had been grown from blood culture, 25% had been isolated from an intra-abdominal specimen and 7% from urine samples (Figure 2b). The most common reason for submission to the NRZMyk was a request for reference susceptibility testing. Thus, these strains represent a highly biased subset of C. glabrata isolates from Germany, where the primary laboratory had decided that submission to the NRZMyk was warranted. Importantly, this precludes any
estimations about the overall frequency of resistance in C. glabrata in Germany. Species identification as C. glabrata was confirmed for all isolates using ITS sequencing.

**Anidulafungin susceptibility testing**

Upon receipt of the strains at the NRZMyk, anidulafungin MICs were determined for all 176 isolates by broth microdilution according to EUCAST standards. Overall, anidulafungin MICs ranged from $0.016 \text{ mg/L}$ to $>8 \text{ mg/L}$. Ninety-two isolates (52%) were found to be susceptible to anidulafungin (AND$^S$) in accordance with EUCAST interpretation (MIC $<0.06 \text{ mg/L}$, Figure 1). Eighty-four isolates (48%) were classified as anidulafungin-resistant (AND$^R$) according to EUCAST reference testing without carrying any matching susceptibility tests for these isolates and included testing with micafungin and caspofungin to determine whether phenotypic resistance of these strains to echinocandins could be confirmed.

**FKS sequence analysis of C. glabrata isolates**

FKS sequencing was performed for all 176 isolates. None of the 92 AND$^S$ isolates harboured a mutation in the known HS regions of the FKS1 or FKS2 gene. In contrast, 71 out of 84 AND$^R$ isolates did contain a single mutation in one of the HS regions of either FKS1 or FKS2 (FKS$^{\text{mut}}$).

Most of these AND$^R$/FKS$^{\text{mut}}$ isolates (62/71; 87.2%) showed a mutation in FKS2, compared with only 12.7% (9/71) having an FKS1 mutation. All FKS1 mutations affected FKS1 HS1, whereas 60 FKS2 mutations affected FKS2 HS1 and 2 affected FKS2 HS2 (Figure 1).

The majority of mutations found (those located in FKS2 HS1) affected five different positions: Leu-662 ($n = 2$), Asp-666 ($n = 5$), Pro-667 ($n = 2$), Phe-659 ($n = 29$) and Ser-663 ($n = 22$), all of which have previously been identified to correspond to echinocandin resistance.\textsuperscript{33,34} Together, mutations affecting Phe-659 or Ser-663 accounted for nearly three-quarters of all mutations found in this study (Figure 2c). Other FKS mutations in AND$^R$ strains affected positions in FKS1 HS1 [Phe-625 ($n = 5$), Ser-629 ($n = 2$) Leu-630 ($n = 1$) and Asp-632 ($n = 1$)] or FKS2 HS2 [Arg-1378 ($n = 2$)]

Isolates harbouring relevant mutations in FKS2 HS1 position 663 (1× Ser-663→Phe; 1× Ser-663→Trp; 20× Ser-663→Pro; Figure 2e) were found to be associated with significantly higher anidulafungin MICs compared with mutations in position Phe-659 and position Asp-666 (P value $<0.001$; Figure 2c). This difference was confirmed by caspofungin MIC data for Ser-663 and Phe-659 using the same subset of mutated strains ($n = 71$) (Figure S1). The most frequent mutation Ser-663→Pro was associated with high anidulafungin MICs and the overwhelming majority of isolates harbouring this mutation showed an MIC of 2 mg/L (range: 1–4 mg/L).

In contrast, FKS2 HS1 Phe-659 mutations resulted in rather divergent phenotypes. Isolates with a deletion in position 659 showed higher anidulafungin MICs compared with other mutations within this position ($n = 9$; median: 2 mg/L; range: 0.5–4 mg/L), whereas Phe-659→Tyr ($n = 6$) and Phe-659→Ser ($n = 8$) mutations both showed a median anidulafungin MIC of 0.5 mg/L and thus seemed to be associated with a less-pronounced MIC increase (Figure 2c and e). Additional mutations affecting Phe-659 were observed only in a limited number of strains (3× Phe-659→Val, 2× Phe-659→Leu, 1× Phe-659→Cys), preventing further analysis of any correlation with MICs (Figure 2e).

**Echinocandin susceptibility in AND$^R$/FKS$^{\text{wt}}$ C. glabrata**

Initially, 13 C. glabrata isolates (7%) were identified as AND$^R$ by EUCAST reference testing without carrying any matching FKS HS mutations (AND$^R$/FKS$^{\text{wt}}$). Anidulafungin MICs for these strains ranged from 0.125 mg/L ($n = 7$), 0.25 mg/L ($n = 3$) to $\geq 0.5$ mg/L ($n = 3$) (Figure 3). We therefore performed a range of additional susceptibility tests for these isolates and included testing with micafungin and caspofungin to determine whether phenotypic resistance of these strains to echinocandins could be confirmed.
Figure 2. (a) Categorized age distribution of patients suffering from C. glabrata infection (n = 175). (b) Isolation site of obtained specimens (n = 175). (c) MIC values for anidulafungin (AND) at indicated amino acid positions in FKS2 hot spot 1. A significant difference between position 663 and 659 (P < 0.001) as well as 663 and 666 (P < 0.001) was observed. The box plots show the median, lower/upper quartile and range of each position. (d) Comparison of anidulafungin (AND) and micafungin (MIF) susceptibility results using different susceptibility testing devices and breakpoints on 13 selected borderline isolates: AND broth microdilution (AND BM) (according to EUCAST); AND and MIF Merlin Micronaut microdilution (AND MM; MIF MM) (according to EUCAST); AND and MIF Yeast One Sensititre microdilution (AND YO; MIF YO) (according to CLSI). MIC value of echinocandins (MIC of ECN). Scatter plots show the range and median. Coloured lines refer to the current MIC breakpoint applicable for the antifungal substance according to its testing method and affiliated reference society [green for EUCAST (AND ≥0.06 mg/L; MIF ≥0.03 mg/L); blue for CLSI (AND ≥0.5 mg/L; MIF ≥0.25 mg/L)]. The asterisk is used as specific marker for three strains with AND MIC values ≤0.016 mg/L in YO. (e) Distribution of MIC values concerning mutations in FKS2 hot spot 1. Scatter plots show the range and median of each amino acid exchange.
(Figure 3). For this, we used the Micronaut microdilution assay (MICRONAUT-AM, Merlin Diagnostika- A Bruker Company, Bornheim, Germany) (MM) interpreted according to EUCAST as well as the Sensititre Yeast One Y010 (Thermo Fisher Scientific, Massachusetts, USA) (YO) and the VITEK2 (AST-Y508 cartridge, bioMérieux, Paris, France) (VIT), both interpreted according to CLSI. Additionally, anidulafungin and micafungin agar gradient diffusion (AGD) tests (Etest, bioMérieux, Marcy-l’Etoile, France) were performed (Figure 3 and Figure 2d). As a result, 11 of the 13 ANDR/FKS\textsuperscript{wt} were tested and found to be susceptible to micafungin in two out of three (n = 1, 2016-172) or all three (n = 10) assays performed. Furthermore, test results for anidulafungin in the additional assays varied between susceptible and resistant (Figure 3), indicating that anidulafungin MICs for these strains are fluctuating around the breakpoint. In addition, all of these strains tested as intermediate (I) or resistant (R) in at least one test for caspofungin, interpreted according to CLSI. However, none of these 11 isolates consistently tested resistant to any of the echinocandins in all assays applied. Thus, we refer to these isolates as ‘borderline’ with regard to anidulafungin testing (ANDR, Figure 3).

Two of the 13 ANDR/FKS\textsuperscript{wt} strains (2016-252 and 2017-099) were found to be resistant to anidulafungin by all testing methodologies.

\textit{C. glabrata} 2016-252 was resistant to anidulafungin in all test systems, confirming the result obtained using the EUCAST reference method. Interestingly, 2016-252 was found to be susceptible to micafungin in all assays used, contradicting the paradigm of complete cross-resistance within the echinocandin class of antifungals (Figure 3). Thus, 2016-252 was categorized as ANDR/MIFR/FKS\textsuperscript{wt}.

\textit{C. glabrata} 2017-099 was consistently resistant to both anidulafungin and micafungin in all test systems and was thus categorized ANDR/MIFR/FKS\textsuperscript{wt} (Figure 3).

**Inter-laboratory susceptibility testing of ANDR/FKS\textsuperscript{wt}**

To address the potential consequences of MICs fluctuating around clinical breakpoints and echinocandin resistance in the absence of FKS mutations for phenotypic susceptibility testing, we conducted a blinded trial. \textit{C. glabrata} strains, 2016-252 (ANDR/MIFR/FKS\textsuperscript{wt}), 2018-172 (ANDR/FKS\textsuperscript{wt}) and 2017-099 (ANDR/MIFR/FKS\textsuperscript{wt}) were sent to 10 different microbiology laboratories in our InfectControl sentinel laboratory network for susceptibility testing. 2015-136 (ANDR/MIFR/FKS\textsuperscript{wt}) and 2016-205 (ANDR/MIFR/FKS\textsuperscript{mut}; FKS mutation: Ser-663→Pro in FKS2 HS1) were added as controls. In total, the 10 laboratories used four different assays generating 29 MIC values for each strain (Figure 4). With one exception, the control strains were correctly tested as susceptible or resistant by all labs, although one laboratory generated a false susceptible result for 2016-205 by anidulafungin AGD test, which, however, would have been ignored during result interpretation (Figure 4). Strain 2018-172 was tested and found to be susceptible to anidulafungin and micafungin by all labs, indicating that the borderline phenotype did not occur in a significant proportion of blinded routine testing (Figure 4). \textit{C. glabrata} 2016-252 was tested as anidulafungin resistant by six laboratories, anidulafungin susceptible by three laboratories and generated contradictory results with two test systems used in one laboratory. In line with the results from the NRZMyk, nine labs evaluated this strain as micafungin susceptible, while one laboratory found this strain to be micafungin resistant. Except for a single susceptible anidulafungin AGD test, \textit{C. glabrata} 2017-099 was scored as resistant to all echinocandins tested, confirming phenotypic resistance despite the absence of an FKS mutation (Figure 4).

**Fluconazole and multidrug resistance**

Fluconazole MICs were determined using EUCAST reference methodology. MICs were distributed from 0.25 mg/mL to >64 mg/mL (median 4 mg/L). Resistance to fluconazole was found in 38% (n = 67) of the strains (those with MICs >16 mg/L; Table S1). Of those, 26 isolates showed combined fluconazole and echinocandin resistance, all of them with a concordant FKS mutation (14% of all isolates).

The type of FKS mutations for these strains was overall similar to that of all ANDR isolates. In 23 strains an FKS2 HS1 mutation (11→Phe-659; 7→Ser-663; 4→Asp-666; 1→Pro-667) was detected, whereas in three cases an FKS1 HS1 mutation (2→Phe-625; 1→Ser-629) was confirmed (Table S1).

Within the group of multidrug-resistant \textit{C. glabrata}, nine isolates (three each from 2016, 2017 and 2018) were from bloodstream infections (Table 1). Three of those (2016-058, 2016-064 and 2017-252) showed elevated echinocandin and fluconazole MIC values, at the upper end of the tested ranges (anidulafungin 2–4 mg/L; caspofungin >8 mg/L; micafungin >64 mg/L) and all carried Ser-663→Pro mutations (Table 1). 2016-058 was obtained from a patient suffering from acute myeloid leukaemia, with graft versus host disease of the skin and gastrointestinal tract, under triple immunosuppressive therapy after transplantation. Antifungal therapy was initially switched from voriconazole to caspofungin. Upon further clinical deterioration (multiple organ failure) a combination of anidulafungin and amphotericin B was administered (Table 1). Isolate 2016-064 had been isolated from a patient with cirrhosis due to alcoholic liver disease, lactic acidosis and pneumonia. Treatment was escalated from anidulafungin to amphotericin B (Table 1). The strain 2017-252 was isolated from a patient with acute liver failure and transjugular intrahepatic portosystemic shunt (TIPS) infection. Candidaemia was treated with fluconazole and caspofungin (Table 1). All three patients succumbed to the infection. In contrast five of the remaining patients with MDR \textit{C. glabrata} bloodstream infection survived. In one case (1/9) no outcome data was available (Table 1).

**Discussion**

Antifungal drug resistance has become a major concern and limits therapeutic options in life-threatening invasive fungal infections. New resistant fungal pathogens, such as \textit{Candida auris}, emerge and spread globally.\textsuperscript{5} In addition, well-known fungal pathogens, including \textit{Candida} spp., \textit{Aspergillus} spp. and \textit{Cryptococcus} spp. have acquired resistant phenotypes in recent decades.\textsuperscript{45–47}

The widespread use of echinocandins as first-line therapy for candidaemia promotes the emergence of resistant strains. The haploid genome of \textit{C. glabrata} enables rapid mutation that can occur during therapy and result in treatment failure.\textsuperscript{48} This may be further enhanced by alterations within the DNA mismatch repair gene resulting in mutator phenotypes.\textsuperscript{22,23} Our study confirms that echinocandin resistance in \textit{C. glabrata} is emerging in
Germany. We can confirm previous findings showing that mutational H51 of both FKS genes plays a major role in mediating echinocandin resistance, as 97% (69/71) of all relevant mutations are detected in this region (Figure 1). Zhao et al. designed a rapid FKS1/FSK2 H51 genotyping tool based on melting curve analysis which includes the most relevant mutations of H51 (8xFKS1 H51; 7xFKS2 H51) and showed 100% specificity and 100% sensitivity in WT/non-WT discrimination validated by 186 clinical C.

| Assay | Minimal inhibitory concentrations (MICs) in mg/L | Ref. |
|-------|---------------------------------------------|------|
|       | AND9/FKS^wt |                                |      |
|       | 2018-257 | 2015-140 | 2015-190 |
|       | AND | MIF | CAS | AND | MIF | CAS | AND | MIF | CAS |
| BM    | 0.125 | – | – | 0.125 | – | – | 0.125 | – | – |
| MM    | 0.031 | 0.016 | 0.125 | 0.031 | 0.016 | 0.125 | 0.031 | 0.016 | 0.125 |
| VIT   | – | <0.06 | 0.25 | – | <0.06 | 0.5 | – | <0.06 | 0.5 |
| YO    | <0.015 | 0.015 | 0.06 | 0.015 | 0.015 | 0.06 | ≤0.015 | 0.015 | 0.03 |
| AGD   | 0.032 | 0.023 | – | 0.023 | 0.023 | – | 0.016 | 0.012 | – |
|       | 2018-258 | 2017-098 | 2015-007 |
|       | AND | MIF | CAS | AND | MIF | CAS | AND | MIF | CAS |
| BM    | 0.125 | – | – | 0.125 | – | – | 0.125 | – | – |
| MM    | 0.031 | 0.016 | 0.125 | 0.063 | ≤0.002 | 0.25 | 0.25 | 0.016 | 0.25 |
| VIT   | – | <0.06 | 0.5 | – | <0.06 | 0.5 | – | <0.06 | 0.5 |
| YO    | 0.06 | 0.015 | 0.012 | 0.25 | 0.015 | 0.5 | 0.06 | 0.03 | 0.5 |
| AGD   | 0.032 | 0.023 | – | 0.125 | 0.016 | – | 0.064 | 0.047 | – |
|       | 2018-150 | 2017-555 | 2018-129 |
|       | AND | MIF | CAS | AND | MIF | CAS | AND | MIF | CAS |
| BM    | 0.125 | – | – | 0.25 | – | – | 0.25 | – | – |
| MM    | 0.125 | 0.016 | 0.125 | 0.125 | 0.016 | 0.125 | 0.125 | 0.016 | 0.125 |
| VIT   | – | <0.06 | 0.5 | – | <0.06 | 0.25 | – | <0.06 | 0.25 |
| YO    | 0.25 | 0.03 | 0.25 | 0.03 | 0.015 | 0.12 | 0.12 | 0.015 | 0.25 |
| AGD   | 0.19 | 0.032 | – | 0.032 | 0.008 | – | 0.032 | 0.012 | – |
|       | 2018-291 | 2018-172 |
|       | AND | MIF | CAS | AND | MIF | CAS |
| BM    | 0.25 | – | – | 0.5 | – | – |
| MM    | 0.125 | 0.016 | 0.25 | 0.125 | 0.016 | 0.125 |
| VIT   | – | ≤0.06 | 0.5 | – | ≤0.06 | 0.5 |
| YO    | 0.03 | 0.03 | 0.12 | 0.12 | 0.25 | 0.5 |
| AGD   | 0.047 | 0.016 | – | 0.125 | 0.016 | – |
|       | 2016-252 | 2017-099 |
|       | AND | MIF | CAS | AND | MIF | CAS |
| BM    | 0.5 | – | – | 2 | – | – |
| MM    | 0.25 | ≤0.002 | 0.125 | 0.125 | 0.125 | 0.25 |
| VIT   | – | ≤0.06 | 0.5 | – | 0.5 | 1 |
| YO    | 0.5 | 0.03 | 0.12 | 0.5 | 0.5 | 1 |
| AGD   | 0.38 | 0.064 | – | 0.38 | 0.25 | – |

Figure 3. Susceptibility testing of 11 AND^9/FKS^wt isolates and 2 AND^9/FKS^wt isolates using multiple assays. Initial broth microdilution (BM) was extended with the commercial testing devices Yeast one (YO), VITEK 2 (VIT), Merlin Micronaut (MM) and agar gradient diffusion tests (AGD, Etest). Antifungal agents: AND, anidulafungin; MIF, micafungin; CAS, caspofungin. Colours according to breakpoints of either EUCAST or CLSI: red (resistant); orange (intermediate); green (susceptible).
glabrata isolates. Applied to our results, 69% (49/71) of all mutations found would have been detected within a timeframe of only 3 h.

The majority (72%, 51/71) of all echinocandin-resistant strains with a mutated FKS revealed an amino acid change at positions Phe-659 and Ser-663 (both FKS1 H52) (Figure 2c). In particular, Ser-663 exchange the polar amino acid serine (Ser) with a hydrophobic Phe-659 and Ser-663 (both with a mutated FKS1 H52). As a result, complete deletion at Phe-659 appears to have the highest impact on resistance to echinocandins (Figure 2e).

**Figure 4.** Susceptibility testing results for one AND/FKS wt strain, two AND/FKS mut and two control strains by 10 German microbiology laboratories. 2015-136 (FKS wild-type) and 2016-205 (mutation in FKS2 H51 S663P) were added as controls. Assays used: MM, Merlin Micronaut; YO, Yeast one; VIT, VITEK 2; AGD, agar gradient diffusion test. Antifungal agents: AND, anidulafungin; MIF, micafungin; CAS, caspofungin. Colours are applied according to breakpoints of either EUCAST or CLSI: red (resistant); orange (intermediate); green (susceptible). Susceptibility interpretation of CAS MIC data marked with an asterisk are derived from AND and/or MIF, as CAS lacks official EUCAST BP.

![Figure 4](image)

The majority (72%, 51/71) of all echinocandin-resistant strains concerning different echinocandins have been observed, questioning the paradigm of complete cross-resistance in this class of antifungals. We identified one isolate (2016-252) which did not carry an FKS mutation and was consistently tested ANDR by 8 of 11 labs, including the NRZMyk, but was susceptible to micafungin (10/11). It remains unclear whether this reflects an extreme borderline phenotype in a susceptible isolate or points to an as yet unknown mechanism conferring selective resistance to anidulafungin.

However, in 7% (13/176) of the isolates a decreased susceptibility to anidulafungin was observed phenotypically, while genotypic resistance could not be confirmed (Figure 1). The majority (11/13) showed MICs fluctuating around the clinical breakpoints upon re-testing indicating a ‘borderline’ resistance (ANDB). Notably, these 11 strains were found to be micafungin susceptible in all assays conducted, suggesting a higher discriminative power in detecting FKSmut strains compared with anidulafungin. An adaption of the current anidulafungin EUCAST BP by implementing an area of technical uncertainty (ATU) at anidulafungin MICs of 0.125 mg/L or recommending additional testing of micafungin in these rare cases might address this issue.

Differences in the in vitro activity of mutated C. glabrata strains concerning different echinocandins have been observed, questioning the paradigm of complete cross-resistance in this class of antifungals. We identified one isolate (2016-252) which did not carry an FKS mutation and was consistently tested ANDR by 8 of 11 labs, including the NRZMyk, but was susceptible to micafungin (10/11). It remains unclear whether this reflects an extreme borderline phenotype in a susceptible isolate or points to an as yet unknown mechanism conferring selective resistance to anidulafungin.
Table 1. Outcome and patient data for MDR C. glabrata strains associated with bloodstream infection between 2016 and 2019

| MIC (µg/L) | Pre-existing conditions | Reason for admission | Age category (years) | Sex | Specimen | Antifungal therapy | Outcome of candidaemia | Outcome of candidemia AND CAS FLC |
|-----------|-------------------------|----------------------|---------------------|-----|----------|--------------------|------------------------|---------------------------------|
| 8         | FKS 64 S663P (2 HS1)    | ALRD, cirrhosis      | 2016-064 f 61–65    | female | blood culture | caspofungin + AMB | deceased | deceased, Survived     |
| 0.5       | FKS 64 F659S (2 HS1)    | pneumonia             | 2016-144 m 76–80    | male   | blood culture | voriconazole        | Survived               |                                 |
| 2         | FKS 64 F625C (1 HS1)    | lung cancer           | 2017-022 m 51–55    | male   | blood culture | micafungin + FLC   | Survived               |                                 |
| 2         | FKS 64 P667T (2 HS1)    | liver failure         | 2017-252 m 51–55    | male   | blood culture | anidulafungin       | deceased               | Survived, Survived              |
| 2         | FKS 64 D666N (2 HS1)    | chronic heart failure | 2018-462 m 56–60    | male   | blood culture | amphotericin B      | Survived               |                                 |
| 1         | FKS 8 32 F659del (2 HS1) | aspiration            | 2019-615 m 76–80    | male   | blood culture | caspofungin         | Survived               |                                 |

Abbreviations: AML, acute myeloid leukaemia; ALRD, alcohol-related liver disease; GvHD, graft versus host disease; LVAD, left ventricular assist device; TIPS, transjugular intrahepatic portosystemic shunt; AND, anidulafungin; AMB, amphotericin B; CAS, caspofungin; 5FC, flucytosine; VRC, voriconazole.

Notably, Healy et al. described a related phenotype showing reduced susceptibility to caspofungin while micafungin susceptibility was paradoxically increased. More importantly, we identified one FKS<sup>mut</sup> isolate (2017-099) that exhibits reduced susceptibility to all echinocandins, contradicting the paradigm that echinocandin resistance is only mediated by such mutations. This in vitro resistance (all MICs were found to be above the current BP) may not serve as a sufficient proof of a refractory therapeutic response in vivo, but it strongly hints that there may be relevant FKS-independent resistance mechanisms. Functional, biochemical and genomic analyses are underway to further elucidate this phenomenon.

In conclusion, our data emphasize the clinical importance of susceptibility testing for C. glabrata strains but also highlight technical difficulties. Our finding, that infection with MDR C. glabrata strains with Ser-663 mutations result in adverse outcomes is in line with studies indicating that certain genetic phenotypes may be connected to increased mortality. These evolving multiresistant strains represent a major threat and need to be monitored closely in epidemiological surveillance studies. Micafungin testing is clearly more robust than anidulafungin testing and better suited to identify isolates with a mutated FKS gene. If available, genotypic resistance testing is a rapid and reliable tool to identify resistant strains, although our findings suggest that FKS-independent resistance mechanisms may occur in rare cases. Diagnostic laboratories urgently need to optimize their susceptibility testing portfolio for Candida.

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Transparency declarations
None to declare.

Author contributions
O.K. designed the study. G.W., R.M. and A.M.A. supplied the NRZMyk data. O.K., G.W., R.M., M.H. and A.M.A. interpreted the findings. M.H. contributed to the statistical analysis. Members of the InfectControl Fungal Infections Study Group conducted a round robin test and contributed test results. A.M.A. wrote the first manuscript version. All authors contributed to subsequent and critical revisions and approved the final version of the manuscript.

Supplementary data
Figure S1 and Tables S1 to S3 are available as Supplementary data at JAC-AMR Online.

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