Echinocandin resistance and population structure of invasive *Candida glabrata* isolates from two university hospitals in Germany and Austria

Ulrike Klotz[1], Dirk Schmidt[1], Birgit Willinger[2], Eike Steinmann[3], Jan Buer[1], Peter-Michael Rath[1], and Joerg Steinmann[1,#]

[1]Institute of Medical Microbiology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany

[2]Division of Clinical Microbiology, Department of Laboratory Medicine, Medical University of Vienna, Vienna, Austria

[3]Institute of Experimental Virology, Twincore Centre for Experimental and Clinical Infection Research, Hannover, Germany

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[#]To whom correspondence should be addressed:

Joerg Steinmann, MD

Institute of Medical Microbiology

University Hospital Essen, University of Duisburg-Essen

Hufelandstr. 55

D-45122 Essen

Phone: + 49-201-72385771; Fax: + 49-201-7235602

E-Mail: Joerg.Steinmann@uk-essen.de

Abstract

Backround: Echinocandin resistance in *Candida glabrata* is emerging and is associated with the presence of *FKS* mutations.

Objective: In this study we analyzed the antifungal susceptibility, presence of *FKS* mutations and clonality of *C. glabrata* blood culture isolates from two hospitals in Germany and Austria.

Methods: Susceptibility testing of 64 *C. glabrata* bloodstream isolates from 2 university hospitals was performed with broth microdilution method according to EUCAST. In addition, all isolates were screened for *FKS* mutations. Molecular fingerprinting was performed by microsatellite PCR with 3 separate primer pairs and semi-automated repetitive sequenced-based PCR (rep-PCR).

Results: One *C. glabrata* isolate from Germany (1.5%) was echinocandin resistant, with a corresponding mutation in *FKS*2 gene hot spot 1. The discriminatory power of microsatellite PCR was higher than that of rep-PCR (Simpson Index of 0.94 vs. 0.88); microsatellite PCR created 31 separate genotypes, whereas rep-PCR created 17. Predominant genotypes or clusters of isolates from Germany and Austria were present, with no epidemiological evidence of nosocomial transmissions.

Conclusion: Although we found a low incidence of echinocandin resistance in *C. glabrata* in our settings, further surveillance projects in central Europe are warranted for monitoring future epidemiological trends. The genetic population structure of *C. glabrata* demonstrates overrepresented geographical clusters.

Introduction

Candida species are the most common cause of fungal bloodstream infections world-wide.[1-3] The incidence of candidemia and invasive candidiasis has increased in the last decade.[4-6] After *C. albicans, C. glabrata* is the second most common cause of bloodstream infections,[6-8] with a mortality rate ranging from 58% to 61%.[1,7,9]

In recent years, several studies have reported increasing rates of resistance of *C. glabrata* to various antifungal agents, especially azoles and echinocandins.[8-11] The reduced susceptibility to fluconazole (FLC) has increased the value of the echinocandins as the most appropriate therapy for *C. glabrata* candidemia.[2,7] However, resistance to echinocandins has also emerged, especially to caspofungin (CAS) as one of the representatives of this class of antifungal agents.[8,11]

Phenotypic resistance to the echinocandins is associated with mutations in the *FKS*1 or *FKS*2 genes,[11-13] which encode the 1,3-beta-D-glucan synthase enzyme complex that synthesizes an important component of the fungal cell wall.[14] Most published studies reporting the increasing problem of echinocandin resistance in *C. glabrata* are from North America[15] but for Europe limited data are available.

For epidemiological reasons it is also important to evaluate whether antifungal resistance is associated with a certain genetic population structure. Several studies have shown that clonal clusters of *C. glabrata* blood culture isolates are overrepresented within a geographical association.[16-18] The use of microsatellites, defined as short tandem repeats of 2 to 6 nucleotides known to be highly polymorphic, was shown to be useful for DNA fingerprinting of *C. glabrata*.[18] In this study we investigated the prevalence of caspofungin resistance and the molecular epidemiology (microsatellite PCR and repetitive sequenced-based PCR, DiversiLab) of a set of *C. glabrata* blood culture isolates from 2 university hospitals in Germany and Austria.

Material and Methods

Isolates. This study used 64 *C. glabrata* blood culture isolates from 64 patients (45 isolates from the University Hospital Essen, Essen, Germany, collected between October 2008 and November 2012; and 19 isolates from the Medical University of Vienna, Vienna, Austria, collected between January 2008 and June 2010). Both institutions are tertiary care teaching hospitals with large transplant programs (e.g. bone marrow, liver, kidney) and a neonatal intensive care unit. We included only the first isolate of an episode and only one isolate per patient. All isolates were cultured on Sabouraud glucose agar (Oxoid, Wesel, Germany) at 37°C for 48 h and were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF, Vitek MS; bioMérieux, Marcy l'Etoile, France). DNA was extracted from each isolate with the Maxwell 16 Tissue Low Elution Volume Total RNA Purification Kit (Promega Corporation, Fitchburg, WI, USA) according to the manufacturer's recommendations and was stored at -20°C until amplification was conducted.

Susceptibility testing. The European Committee on Antimicrobial Susceptibility (EUCAST) broth microdilution (BMD) reference method was used to determine the *in vitro* susceptibility of all isolates. EUCAST BMD testing was applied according to the EUCAST definitive document EDef 7.2 Revision.[19] The stock solutions of the antifungal agents were prepared with concentrated antifungal agents dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich, Steinheim, Germany) as recommended. RPMI-1640 medium (Sigma-Aldrich) with 2% glucose and 3-(N-Morpholino) propanesulfonic acid buffer (MOPS; Sigma-Aldrich) were used as recommended to generate the dilutions of the antifungal agents in flat-bottomed microdilution plates

(Brand, Wertheim, Germany). Results were read spectrophotometrically at 405 nm after 24-h incubation.

The MIC of amphotericin B (AMB) was determined as the lowest concentration of AMB that achieved an inhibition of growth of at least 90% with regard to the drug-free control. MIC values of the other antifungal agents were clarified as the lowest concentration of drug that achieved an inhibition of growth of at least 50% with regard to the drug-free control.

The assays included the following antifungal agents (Sigma-Aldrich) with test ranges in BMD: AMB (0.03-16.0 mg/l), FLC (0.12-64.0 mg/l), voriconazole (VRC; 0.016-8.0 mg/l), anidulafungin (AND; 0.016-8.0 mg/l), micafungin (MCF; 0.016-8.0 mg/l) and CAS (0.016-8.0 mg/l). *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 reference strains were used in each attempt as internal quality controls, as recommended by the EUCAST definitive document EDef 7.2 Revision.[19]

Interpretation of MICs. To interpret the MICs of each sample in the categories *susceptible*, *intermediate*, and *resistant*, we used the breakpoint tables of EUCAST version 7.0, valid from 2014-08-12.[20] Breakpoints were available for AMB (susceptible isolates, ≤1 mg/l; resistant isolates, >1 mg/l), FLC (susceptible isolates, ≤0.002 mg/l; intermediate isolates, >0.002-32.0 mg/l; resistant isolates, >32 mg/l), AND (susceptible isolates, ≤0.06 mg/l; resistant isolates, >0.06 mg/l) and MCF (susceptible isolates, ≤0.03 mg/l; resistant isolates, >0.03 mg/l). Epidemiological cut-off values for VRC (>0.5 mg/l) were obtained from Posteraro *et al.*,[21] and the CAS MIC breakpoint (>0.25 mg/l) from the Clinical and Laboratory Standards Institute (CLSI 2012) was used.[22] For the analysis, the low off-scale MIC results were left unchanged, and the high off-scale MIC results were converted to the next highest concentration, as previously reported.[23,24]

FKS-*PCR*. Four primers were used for sequencing the amplified DNA and

identifying mutations in the *FKS*1 gene, hot spot (HS) 1 and 2 region, and in the *FKS*2 gene, HS 1 and 2 region, as previously described.[25] *FKS*-PCR was performed in a volume of 5 µl (1ng/µl) of *C. glabrata* DNA and 45 µl PCR mixture with the Taq-PCR core kit (Qiagen, Hilden, Germany). The PCR mixture was composed in a total volume of 90 µl of 10 µl 10× PCR buffer (15 mM $MgCl_2$), 2 µl $MgCl_2$ (2 mM), 20 µl Q-solution , 2 µl deoxynucleoside triphosphate (dNTP; 10nM each), 1 µl of each primer (100 µM), 0.5 µl Taq polymerase (5 U/µl), and 53.5 µl of nuclease-free water. Thermal cycling parameters were as follows: initial duration, 5 min at 94°C, followed by 40 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by an additional step of 5 min at 72°C. The purification of the PCR products was performed using a PCR purification kit (QIAquick; Qiagen). The purified PCR products were diluted to a final concentration of 20 ng DNA/µl. Sequencing was performed by LGC Standards GmbH (Wesel, Germany). The obtained sequences were compared with the reference sequence for FKS1 GenBank accession number XM_446406 and for FKS2 GenBank accession number XM_448401.

Molecular typing by microsatellite PCR. Microsatellite PCR was performed with three primer pairs (RPM2, MTI, and ERG3) containing repeated sequences of potential microsatellites.[18] Amplification was performed in a 20 µl volume containing 2 µl of *C. glabrata* DNA (1 ng/µl) and 18 µl PCR mixture. The PCR mixture was composed in a total volume of 90 µl of 10 µl 10× PCR buffer (15 mM $MgCl_2$), 2 µl dNTP (10 nM each), 1 µl RPM and ERG primer pair (20 mM each), 2 µl of the MTI primer pair (40 mM each), 0.5 µl of Taq polymerase (5 U/µl), and 75.5 µl (alternatively, 73.5 µl for the MTI primer pair) of nuclease-free water. Thermal cycling parameters were as follows: initial denaturation for 10 min at 95°C, followed by 30 cycles for 30 sec at 95°C, for 30 sec at 55°C, for 30 sec at 72°C, followed by an additional step for 5 min at 72°C. The PCR product was added to an ALFexpress

DNA sequencer (size, 50-500 bp; Cy5-labelled; Amersham Biosciences, Piscataway, NJ, USA). Denaturation followed for 3 min at 90°C and kept on ice. The products were analyzed in an automated fluorescence-based DNA sequencing system (Long Read Tower System; Visible Genetics, Toronto, Ontario, Canada), and the data were analyzed with GeneScan software (Applied Biosystems, Foster City, CA, USA) for detecting the length of the amplification products. The fingerprint of each DNA sample was clustered into genotypes, depending on the fragment size of the three amplification products. Similarities between genotypes were visualised graphically with a minimum spanning tree created by BioNumerics, version 7.50 (Applied Maths, Sint-Martens-Latem, Belgium); the data were treated as categorical information, and the unweighted pair group method with arithmetic mean (UPGMA) was used.

Molecular typing by rep-PCR (DiversiLab). Rep-PCR was performed with the DiversiLab Candida kit for DNA fingerprinting (bioMérieux), according to the manufacturer's instructions. Rep-PCR products were separated by a microfluidic LabChip (PerkinElmer, Waltham, MA, USA) and detected with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The rep-PCR amplicons were analyzed with DiversiLab system software version B.02.05.SI360. Pearson's correlation coefficient was used to create genotypes for each isolate. Isolates with a similarity of 98% or higher were considered to be a single genotype.

Statistical analysis. The Simpson Index (SI) was applied to discriminate the two methods, as previously described.[26]

Results

The results of the *in vitro* susceptibility testing are summarized in Table 1. Of the isolates tested, 48 (75%) of the isolates were intermediate and 16 (25%) were resistant to FLC. Cross-resistance to VRC was seen in 14 (88%) of 16 FLC-resistant isolates. Most of the patients with FLC-resistant isolates had a solid tumor (31%),

leukemia (31%), or hematologic malignancy (6%) or were older men who had undergone a long stay in an intensive care unit (19%). Of the isolates tested, 63 (98%) were susceptible to the three echinocandins.

All 64 *C. glabrata* isolates were analyzed for the presence of *FKS* mutations. DNA sequencing revealed 4 mutations in 3 separate strains. In 3 cases the mutations were located outside the HS regions. Only one isolate from Germany showed an alteration of serine to proline at position 663 in the *FKS*2 gene (S663P). This isolate was resistant to the echinocandins. In our case, the patient with a echinocandin-resistant *C. glabrata* bloodstream infection had also undergone pretreatment (>6 weeks) with CAS and a long stay in the intensive care unit. None of the other isolates exhibited mutations in the *FKS*1 or *FKS*2 gene.

Microsatellite PCR found a total of 31 separate genotypes, whereas rep-PCR discriminated 17 separate genotypes (Fig. 1). Microsatellite PCR found that 25 (56%) of the 45 isolates from Essen clustered in 17 separate genotypes with no Vienna isolate and that 10 (53%) of the 19 isolates from Vienna clustered in 9 separate genotypes. Three genotypes were overrepresented in the Essen center, whereas rep-PCR demonstrated four overrepresented genotypes. The genetic distance of all isolates, as determined by categorical analysis, is illustrated as a minimum spanning tree in Fig. 2. The CAS-resistant isolate created its own genotype by both methods. The discriminatory power, calculated by the SI, was 0.94 for microsatellite PCR and 0.88 for rep-PCR (Fig. 1). No epidemiological link was found between the patients, as determined by temporal-spatial relationships.

Discussion

This is the first study to systematically investigate the prevalence of echinocandin resistance and *FKS* mutations in invasive *C. glabrata* in samples from

2 university hospitals in Germany and Austria. One (2.2%) of the 45 isolates collected from 2008 to 2012 at the University Hospital Essen, Germany, exhibited echinocandin resistance and a mutation in the *FKS*2 HS1 region (S663P). The other 63 isolates, which were also screened for *FKS* mutations, were echinocandin susceptible.

An alarming increase in echinocandin resistance (from 2-3% to >13%) has been described over the past ten years in the United States.[11] The resistance rate depends on study design, patient´s population and geographical location.[27] For example, a recent study found that 10% of *C. glabrata* isolates from cancer patients at the M.D. Anderson Cancer Center, Houston, Texas, were resistant to CAS.[28] In this study it was demonstrated that CAS resistance is also associated with a higher rate of 28-day all-cause mortality.[28] Furthermore, it was shown that the presence of *FKS* mutants is associated with therapeutic failure.[11,29]

The prevalence of echinocandin-resistant *C. glabrata* in Europe also varies by countries and studies: the resistance rate was 0% in a study including 79 *C. glabrata* bloodstream isolates from Italy and Spain.[30] On the other hand, 2 (2.1%) of 94 invasive *C. glabrata* isolates obtained mainly from Lombardy, Italy, and 2 (1%) of 193 isolates (both with a *FKS*2 mutation) obtained mainly from urine samples from France were resistant to an echinocandin.[31,32] Researchers from Turkey reported that 1 (2%) of the 50 isolates they analyzed were resistant to caspofungin,[33] whereas a multicenter study from Switzerland found that 18 (9.4%) of 191 isolates were resistant.[34] Data from Germany are limited and from Austria do not exist; two global surveillance studies performed between 2001 and 2011 found 5 *C. glabrata* isolates in a set of 119 non-wild-type strains with mutations in the *FKS*2 HS1 region in samples collected in Germany.[35]

The geographical variations of echinocandin resistance rates may also be

influenced by the site- or country-specific use of antifungal prophylaxis and therapy. A main risk factor for the development of *FKS* mutations in *C. glabrata* is pretreatment with echinocandins[36,37,29] as it was also the case in our patient. It has to be noted that CLSI and EUCAST do not recommended susceptibility testing of CAS due lack of reproducibility and standardization.[38] It was shown that testing of AND and MCF are good markers to predict CAS susceptibility.[39,40]

Only a few studies have investigated the molecular epidemiology of *C. glabrata*. Genotyping was initiated to exclude an oligo- or monoclonal epidemiology on the one hand, and to investigate a possible association between particular genotypes and resistance on the other hand. In the case of *C. glabrata*, it is not clear whether the semi-automated rep-PCR assay is sufficient for epidemiological studies. To the best of our knowledge, the DiversiLab system has not yet been compared with another method for the tracing of *C. glabrata*.

We could demonstrate that the population structure of *C. glabrata* is mainly polyclonal, with site-specific clusters. The discriminatory power of microsatellite PCR is higher than that of rep-PCR. Both methods arrange the same isolates in similar clusters, but microsatellite PCR builds more unique genotypes. These facts are confirmed by the finding that the SI of microsatellite PCR is 0.94, whereas that of rep-PCR is 0.88. In the Essen center, three microsatellite genotypes were predominant, but there was no evidence of spatial-temporal clusters.

Diab-Elschahawi et al. [41] compared the two methods in genotyping 99 *Candida parapsilosis* isolates*.* They also found that the discriminatory power of microsatellite PCR was higher than that of rep-PCR. Foulet et al. achieved a SI of 0.84 by using the same three primer pairs in microsatellite PCR, whereas Abbes et al. (2012) achieved a discriminatory power of 0.94 when comparing 6 primer pairs

and 0.95 when comparing four primer pairs.[17,18] Another study achieved a SI of 0.97 by using 8 markers.[42]

Several studies have reported that there is no relationship between a prominent genotype and the pathogenicity or the location of the collected sample.[17,18,43] Moreover, it has been reported that predominant genotypes are related to the continent or country from which the strain originates.[16,44,45] We found no concordance between any of our genotypes from Germany and Austria and the fragment lengths of microsatellites from France and Tunisia, a finding that indicates geographical selection.[17,18,44] Some researchers have speculated that predominant genotypes may have a selective ecological advantage.[17,18,43,44] Therefore, it would be interesting to determine whether the predominant genotypes found in the blood are more virulent than unique clones.

It was not possible to predict a susceptibility pattern (e.g., to FLC) by using molecular methods to determine a genotype from *C. glabrata*.[46] We also found no correlation of any genotype with a phenotype (data not shown). The echinocandin-resistant *C. glabrata* isolate was classified as a single genotype. In addition, multilocus sequence typing (MLST) of 10 *C. glabrata* isolates with S663P mutation indicated polyclonality.[47]

Our study has some limitations. Since we performed broth microdilution susceptibility testing on only the first blood culture isolate from each patient, the rate of echinocandin resistance may have underestimated. Although, we screened all isolates for the presence of *FKS* mutations from one study site in each country, our set of isolates is not representative of the whole of Germany and Austria. Therefore, future multi-centre studies analyzing *C. glabrata* echinocandin susceptibility in central Europe are warranted.

In summary, echinocandin-resistant *C. glabrata* was rare in our study. The genetic population structure of *C. glabrata* demonstrates overrepresented geographical clusters with site-specific features. Our findings are also relevant for therapeutic considerations: the low prevalence of echinocandin resistance justifies the recommended empirical use of echinocandins for the treatment of candidemia in our settings.

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Table 1: Antifungal susceptibility testing of 64 *C. glabrata* isolates by EUCAST broth microdilution method

Antifungal agent	MIC (mg/l)			Susceptible	Intermediate	Resistant
	Range	MIC_{50}	MIC_{90}	n (%)	n (%)	n (%)
AMB	0.125-0.5	0.25	0.5	64 (100)	0	0
FLC	4-128	16	128	0	48 (75)	16 (25)
VRC	0.03-16	0.5	4	45 (70)[a]	NA[b]	19 (30)[a]
AND	0.03-2	0.06	0.06	63 (98)	0	1 (1.6)
CAS	0.016-16	0.03	0,06	63 (98)[c]	NA[b]	1 (1.6)[c]
MCF	0.016-1	0.016	0.016	63 (98)	0	1 (1.6)

AMB, amphotericin B; FLC, fluconazole; VRC, voriconazole; AND, anidulafungin; CAS, caspofungin; MCF, micafungin; [a] according to Posteraro et al. (21); [b] Not available; [c] according to CLSI 2012 (22)

Figure 1. Overview of results for genotyping of 64 *Candida glabrata* isolates with microsatellite PCR and repetitive sequenced-based

PCR (rep-PCR, Dive