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Candida albicans bloodstream isolates in a German university hospital are genetically heterogenous and susceptible to commonly used antifungals

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ABSTRACT

From an eight-year-span, 99 *Candida* bloodstream isolates were collected at the University Hospital Wuerzburg, Germany. In this study, all strains were analyzed using molecular and phenotypic typing methods. Confirmatory species identification revealed three isolates that were initially diagnosed as *C. albicans* to be actually *C. dubliniensis*. Two isolates contained a mixed culture of *C. albicans* and *C. glabrata*, in one of the specimens both species could be separated while it was not possible to recover *C. albicans* in the other sample. The remaining 95 *C. albicans* isolates were profiled by multilocus sequence typing (MLST). Phylogenetic analyses showed a highly heterogenous collection of strains, associated with many different clades and constituting a set of new diploid sequence types (DST). For all strains with identical DST, patient data were reviewed for potential nosocomial transmission. In addition, all isolates were tested for their susceptibility to amphotericin B, caspofungin, fluconazole, itraconazole, posaconazole and voriconazole. No clinically relevant resistance could be detected. Furthermore, these data underline that correlation between minimal inhibitory concentrations for caspofungin and anidulafungin is low.
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1. Introduction

Candida species are yeasts of the phylum *Ascomycota* and can be isolated from various environmental sources as well as from mammals and humans (McManus and Coleman, 2014). Within the genus, *C. albicans* is the most prevalent and pathogenic of the *Candida* species, causing the majority of systemic and oral candidiasis (Sullivan et al., 2004; Thompson et al., 2010; Zomorodian et al., 2011). In the last decades, *C. albicans* has evolved as a leading cause of hospital acquired infections (Pfaller and Diekema, 2010; Pfaller et al., 2011). In the EPIC-II study, a 1-day point prevalence study involving more then 13,000 patients in 1265 intensive care units, fungi accounted for 19% of all infections and more than 85% of these infections were caused by *Candida* spp. (Vincent et al., 2009). A retrospective analysis of the EPIC-II cohort showed that 12.6% of all positive blood cultures were positive for *Candida* spp. (Kett et al., 2011). Other species in the genus *Candida* can also cause infections and non-*albicans* species generally account for ~50% of hospital acquired *Candida* infections (Perlroth et al., 2007). Although closely related to *C. albicans*, *C. dubliniensis* is a much less virulent pathogen and has initially been associated mainly with oropharyngeal candidiasis in HIV-positive patients (Kurzai et al., 2000; Asmundsdottir et al., 2009; Stokes et al., 2007). *Candida glabrata* on the other hand emerged as a major fungal pathogen during the last decades, mainly due to its resistance against azole antifungals (Rodrigues et al., 2014). In contrast, antifungal drug resistance is generally not a problem in *C. albicans*. Although fluconazole resistant strains have been described, antifungal drug resistance in *C. albicans* remains at very low levels (Alcazar-Fuoli and Mellado, 2014). In a large study, analyzing 256,882 isolates of *Candida* spp. from 41 countries, 98% of *C. albicans* isolates were susceptible to fluconazole (Pfaller et al., 2010).

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To learn more about the epidemiology and transmission of nosocomial *C. albicans* infections, it is important to characterize single isolates in a distinctive and reproducible way, yielding results that can be shared and compared between different labs. Multilocus sequence typing (MLST) has been shown to fulfill all these requirements (Bougnoux et al., 2002). Single nucleotide polymorphisms in DNA fragments of seven conserved housekeeping genes provide enough discriminatory power to distinguish even closely related isolates. The occurrence of heterozygous positions in the sequenced loci contributes to the individual MLST profile of a *C. albicans* isolate (Bougnoux et al., 2002, 2003; Tavanti et al., 2003). Nowadays, this method is commonly used for molecular typing and epidemiological studies of *C. albicans* (McManus and Coleman, 2014). So far, 18 clades of *C. albicans* were described with one supposed to be more specific for Asia (Odds et al., 2007a; Shin et al., 2011). To date, more than 2000 isolate profiles were collected in the *C. albicans* MLST database (http://calbicans.mlst.net). MLST has proven especially useful for analyzing potential nosocomial transmission of *C. albicans* in a hospital setting (Song et al., 2014). Although most *C. albicans* infections are considered to arise from endogenous infection, horizontal transmission in hospitals has been described (Pfaller, 1996).

Here, we report a MLST- based genotyping of 95 *C. albicans* bloodstream isolates which were collected in a German university hospital. All isolates were tested for antifungal drug resistance. Two *C. albicans* strains were found to be identical and isolated from premature twins, indicating perinatal rather than nosocomial transmission.

2. Material and methods

2.1. Strains and media

Candida bloodstream isolates from the Wuerzburg university hospital were collected over the years 2005–2012 by methods routinely used in diagnostic laboratories. For this study, strains were grown in either liquid or solid yeast extract peptone dextrose (YPD) medium at 37 °C/180 rpm or 35 °C, respectively.

2.2. MLST analysis

According to previous works, we have used seven different loci for the MLST analysis. Therefore, the recommended oligonucleotide primers for the amplification of *AAT1a*, *ACC1*, *ADP1*, *MPI1*, *SYA1*, *VPS13* and *ZWF1b* were used (Bougnoux et al., 2002, 2003; Tavanti et al., 2003). Sequences of these primers are publicly available at the *C. albicans* MLST database homepage (http://calbicans.mlst.net/misc/info.asp).

The PCR was performed on isolated genomic DNA. PCR products were purified using the Invitrogen PureLink Quick PCR Purification Kit (Life Technologies). Purified PCR products were then sequenced by an external company (GATC Biotech, Konstanz, Germany). Both DNA-strands were sequenced and all sequences were reviewed and compared to the MLST database manually. Each sequence chromatogram was scanned in search for heterozygous nucleotide positions, which are characterized by two overlayed, equally strong fluorescence peaks. The MLST profile for each allele was double checked in both forward and reverse sequence. Strain information and diploid sequence types (DSTs) were consequently added to the MLST database (http://calbicans.mlst.net/misc/info.asp).

2.3. Phylogenetic analysis

Phylogenetic analysis was performed based on the MLST information of all strains available in the database. The BURST algorithm was used to cluster the bloodstream isolates into groups and to compare them with the other isolate profiles available in the MLST database (Feil and Enright, 2004; Feil et al., 2004).

2.4. Discrimination of *Candida* species

Additional tests to secure the species affiliation were conducted on those isolates that posed problems during the MLST analysis. Identification of *C. dubliniensis* included sequencing of the internal transcribed spacer sequence (ITS) of the large ribosomal subunit and failure to form hyphae in liquid RPMI (5 h at 37 °C, 180 rpm). *C. glabrata* and *C. albicans* were identified on CHROMagar Candida after growth for 48 h at 35 °C, in case of *C. glabrata* the ITS region was amplified via PCR and compared to reference strains.

2.5. Antifungal susceptibility testing

To test the susceptibility of the *Candida* bloodstream isolates, we used commercially available E-tests for amphotericin B, caspofungin, fluconazole, itraconazole, posaconazole and voriconazole (Biomerieux). E-tests were applied following the EUCast guidelines, using RPMI agar and 35 °C for incubation. MIC values were read 24 h and 48 h after application. MIC values for fluconazole and anidulafungin were additionally determined using the EUCAST reference methodology. For analysis of the correlation of outcomes for anidulafungin and caspofungin, the MICs for each antifungal drug were plotted using the plotrix package in the statistical environment R. Point size indicates the number of multiple points at the specific position. The dashed regression line was calculated using the lm function and the denoted R-squared coefficient estimates the quality of this linear fit.

3. Results

3.1. MLST-based genotyping of C. albicans bloodstream isolates

The aim of this study was to address population heterogeneity and potential antifungal drug resistance in *C. albicans* bloodstream isolates. For our study we selected 99 *C. albicans* bloodstream isolates that had been stored in the strain collection of the Institute of Hygiene and Microbiology, University of Wuerzburg. All isolates had been retrieved from patients of the University Hospital in Wuerzburg during the years 2005–2012 and identified as *C. albicans*. As an internal control we included three sequential isolates from the same patient (CABI93, -94, and -97). Genotyping of all isolates was performed using multilocus sequence typing (MLST) as described previously (Bougnoux et al., 2003; Tavanti et al., 2003), by determining the diploid sequence types (DST) of defined internal regions of seven housekeeping genes (*AAT1a*, *ACC1*, *ADP1*, *MPIb*, *SYA1*, *VPS13*, and *ZWF1b*). All data were entered into the MLST online database which contains more than 2500 DSTs (http://calbicans.mlst.net). Typing PCRs repeatedly failed to yield amplicons for three isolates (CABI6, CABI11 and CABI49). To confirm their species identity, internal transcribed spacer regions were amplified and sequenced. Based on this, all three isolates could be unequivocally identified as *C. dubliniensis* and were excluded from further analysis. CABI22 was excluded from further analysis as we were unable to retrieve the *C. albicans* isolate from a case of mixed *C. albicans*/*C. glabrata* infection with subcultures repeatedly only growing *C. glabrata*. CABI8, initially identified as *C. albicans*, turned out to be mixed culture of *C. albicans* and *C. glabrata* on CHROMagar. This was confirmed by the sequencing of the ITS region. Consequently, 95 bloodstream *C. albicans* isolates were used in further analyses.

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