Analysis of antifungal resistance genes in *Candida albicans* and *Candida glabrata* using next generation sequencing

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**Abstract**

**Introduction/Objectives**

An increase in antifungal resistant *Candida* strains has been reported in recent years. The aim of this study was to detect mutations in resistance genes of azole-resistant, echinocandin-resistant or multi-resistant strains using next generation sequencing technology, which allows the analysis of multiple resistance mechanisms in a high throughput setting.

**Methods**

Forty clinical *Candida* isolates (16 *C. albicans* and 24 *C. glabrata* strains) with MICs for azoles and echinocandins above the clinical EUCAST breakpoint were examined. The genes *ERG11*, *ERG3*, *TAC1* and *GSC1 (FKS1)* in *C. albicans*, as well as *ERG11*, *CgPDR1*, *FKS1* and *FKS2* in *C. glabrata* were sequenced.

**Results**

Fifty-four different missense mutations were identified, 13 of which have not been reported before. All nine echinocandin-resistant *Candida* isolates showed mutations in the hot spot (HS) regions of *FKS1*, *FKS2* or *GSC1*. In *ERG3* two homozygous premature stop codons were identified in two highly azole-resistant and moderately echinocandin-resistant *C. albicans* strains. Seven point mutations in *ERG11* were determined in azole-resistant *C. albicans* whereas in azole-resistant *C. glabrata*, no *ERG11* mutations were detected. In 10 out of 13 azole-resistant *C. glabrata*, 12 different potential gain-of-function mutations in the transcription factor *CgPDR1* were verified, which are associated with an overexpression of the efflux pumps CDR1/2.
**Conclusion**

This study showed that next generation sequencing allows the thorough investigation of a large number of isolates more cost efficient and faster than conventional Sanger sequencing. Targeting different resistance genes and a large sample size of highly resistant strains allows a better determination of the relevance of the different mutations, and to differentiate between causal mutations and polymorphisms.

**Introduction**

*Candida* spp. has emerged as an important pathogen causing bloodstream infections associated with a high mortality. *C. albicans* and the less susceptible *C. glabrata* are the most common species causing candidemia and candidiasis [1, 2]. Echinocandins and azoles play an important role in the therapeutic management of invasive candidiasis. In recent years, *Candida* isolates with acquired resistance to azoles and echinocandins have been reported more frequently [3, 4]. Therefore, antifungal susceptibility testing and the detection of mutations in resistance genes are becoming increasingly important to detect antifungal resistance and determine the underlying resistance mechanisms.

Echinocandins inhibit the glycosyltransferase 1,3-β-D-glucan synthase (*FKS*) non-competitively. This enzyme is responsible for the biosynthesis of the oligosaccharide 1,3-β-D-glucan, an important structural component of the fungal cell wall [5]. Decreased susceptibility to echinocandins is associated with target mutations in the hot spot (HS) regions of Fks proteins, which represent the putative binding domain of the echinocandins. Point mutations in these regions can reduce the affinity of the echinocandins to 1,3-β-D-glucan synthase [3, 6, 7].

The pharmacological target of azoles is the enzyme 14-α-demethylase (encoded by *ERG11*), an important enzyme in ergosterol biosynthesis. Acquired resistance to azoles may be caused by several mechanisms. Mutations of the pharmacological target are able to change the enzyme’s structure and may result in reduced binding affinity of the azoles to Erg11p [8, 9]. Frequently, efflux pumps reduce the intracellular accumulation of azoles. The increased efflux is based on overexpression of *CDR1/CDR2* (Candida Drug Resistance) and *MDR1* (Multi Drug Resistance). Gain-of-function mutations in the transcription factors *TAC1* and *CgPDR1* can lead to higher gene expression of drug efflux pumps [10–12].

Loss-of-function mutations in the enzyme Erg3p are another mechanism of azole resistance. In addition to the inhibition of Erg11p, azoles cause a metabolic bypass resulting in the accumulation of toxic concentrations of 14α-methyl-3,6-diol. This metabolite blocks fungal growth. Loss-of-function mutations in *ERG3* inhibit the conversion of 14α-methylfecosterol to toxic 14α-methyl-3,6-diol thereby decreasing azole efficacy. Additionally, the precursor 14α-methylfecosterol can be used to substitute ergosterol [13, 14].

The purpose of this study was to investigate clinical isolates of *C. albicans* and *C. glabrata* showing either echinocandin or azole resistance or both in vitro, and to correlate the resistant phenotypes with described mutations in resistance genes.

Despite generating much data, whole genome sequencing has crucial disadvantages like low coverage levels and a high data analysis burden. Sanger Sequencing is not suitable either, because of the time-consuming process and high costs per sequenced base. Targeted resequencing design offers many advantages over conventional sequencing approaches for the parallel sequencing of a high number of isolates, like higher reliability, the fast sequencing process and more manageable data analysis. Next generation sequencing (NGS) is a very efficient tool to
study a set of genes that are known to be involved in antifungal-resistance in a comprehensive strain set. Therefore, NGS based on targeted resequencing design was used to investigate the underlying resistance mechanisms in our clinical isolates.

We sequenced the genes involved in echinocandin resistance (GSC1 in C. albicans and FKS1 and FKS2 in C. glabrata) and the genes involved in azole resistance (ERG11, TAC1 and ERG3 in C. albicans and ERG11 and CgPDR1 in C. glabrata). The aim of this study was not only to detect established but also to identify novel point mutations associated with echinocandin or azole-resistance in the described resistance genes.

Methods

Sampling and antifungal susceptibility testing

Forty isolates obtained from specimens such as swabs (4), sterile fluids (8), blood cultures (12), a central venous catheter (1), as well as urine (3), feces (4), sputum (1) as well as not specified (7) from various centres in Austria and Germany were investigated. In addition, the susceptible strains ATCC 90030 and ATCC Y33.90 for C. glabrata, and ATCC 90028, ATCC 10231, as well as the azole-resistant ATCC 64124 for C. albicans were included as controls for the validation of the sequencing process. Antifungal susceptibility testing using the broth microdilution method was performed for all strains and C. parapsilosis ATCC 22019 and C. krusei ATCC 6258 as control strains as described by the European Committee of Antimicrobial Susceptibility Testing (EUCAST E.DEF 7.3 December 2015) [15]. Minimal inhibitory concentrations (MIC) were determined for anidulafungin, micafungin and caspofungin, as well as for fluconazole, posaconazole, voriconazole, itraconazole and isavuconazole. Strains with a MIC one to twofold dilutions above the clinical breakpoint for echinocandins were classified as borderline echinocandin resistant. Multi-resistant isolates were defined as resistant to all tested echinocandins and azoles.

DNA extraction

Due to better quantitative and qualitative results in comparison to commercial DNA extraction kits, a modified SDS CTAB chlorophorm based method was used [16]. DNA extraction was performed from a 24h Candida culture on Sabouraud-dextrose agar (SAB). Mechanical lysis was carried out using 1mm silica spheres under addition of the detergents SDS (sodium dodecyl sulfate) and CTAB (cetyltrimethylammonium bromide) as well as proteinase K (Qiagen, Venlo, Netherlands). After adding chloroform-isoamy lalcohol 24:1, the water-soluble polar layer was transferred to a new tube followed by precipitation with ammonium acetate and isopropanol and was subsequently washed using ethanol. The air-dried DNA was then resuspended in 10 mM Tris-EDTA buffer. After extraction, the amount of DNA was determined with Qubit 2.0 via the dsDNA HS kit (Life technologies, Carlsbad, California) and NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, Massachusetts). Additionally, the ratios A260/280 and A260/230 were used to estimate the purity of the DNA.

Next generation sequencing and library preparation

Sequencing was carried out using a targeted resequencing design on the MiSeq platform (Illumina, San Diego, California). Sequence analysis was performed for the whole gene sequence of ERG11 and ERG3, the HS regions of FKS1, FKS2 and GSC1, as well as relevant regions of TAC1 and CgPDR1 (Table 1). Sequencing of ERG11 and ERG3 was achieved using overlapping primers and subsequent assembly. The amplicon sequencing was based on the 16s protocol as described by Illumina [17]. PCR 1 was performed with locus-specific primers with the
additional overhang sequence that is mandatory for sequencing with Illumina technology. Four of the 26 primer pairs published by Garnaud et al. were newly designed by Primer3 Tool because of the formation of hairpins and primer dimers (S1 and S2 Tables) [18, 19]. The library amplification was performed using the KAPA HiFi Hot Start Ready Mix Kit (Kapa Biosystems, Wilmington, Massachusetts), a high fidelity polymerase with proofreading activity which is well suited for the production of NGS-libraries [20]. 12.5 ng genomic DNA was added to the PCR-mix. Afterwards, the amplified PCR products of each isolate were pooled. The washing steps were based on Ampure Beads (Beckman Coulter, Brea, California). Subsequently, an index PCR was performed to tag the amplicons for identification of the different isolates after pooling. The DNA quantification of the PCR products was carried out using Qubit 2.0 via the dsDNA HS kit (Life technologies, Carlsbad, California). DNA was diluted to a concentration of 8pM for sequencing on the V2-Flowcell 2x250bp (Illumina, San Diego, California) and all isolates were pooled. The DNA library was denatured according to the protocol [17]. For quality control, the library was spiked with 5% PhiX DNA.

### Bioinformatic analysis

The quality of the NGS run was verified using the software FASTQC 0.11.4 [21]. The removal of low-quality bases was carried out with the Trimmomatic-0.35 software [22]. This tool also removed all reads under a minimum length of 90bp. In addition, the first 24bp were removed to exclude the primer sequences. The reads were assembled with Bowtie2-2.2.7 [23]. Subsequently, alignment to the reference sequence was carried out. The strains SC5314 for *C. albicans* and CBS138 for *C. glabrata* were used as reference sequences. The gene sequences were downloaded from www.candidagenome.org [24]. To determine variants from the reference sequence Samtools 0.1.19 and VarScan.v2.3.9 [25] were used. After this, SnpEff 4.270 was used to detect alterations causing amino acid substitutions. Finally, a visual validation of the mutations in the assembly files was performed to exclude bias variants. Table 2 shows the sources of the reference sequences. The sequences of the isolates have been deposited in the BioProject database under accession number PRJNA510782.

### Results

#### EUCAST microdilution

Among the 19 *C. albicans* isolates, two were susceptible control strains, seven were resistant to azoles, six were echinocandin-resistant, two borderline echinocandin-resistant, and two were

**Table 1. Overview of the sequenced regions from *C. albicans* and *C. glabrata.***

| Species       | Gene   | Gene length (bp) | Coordinates (bp) | Sequenced gene length (bp) |
|---------------|--------|------------------|------------------|---------------------------|
| *C. albicans* | GSC1   | 5694             | 1752–2130; 3885–4273 | 768                       |
|               | TAC1   | 2946             | 1879–2253; 2720- +166 | 768                       |
|               | ERG11  | 1587             | -71 - +22         | 1680                      |
|               | ERG3   | 1161             | -33 - +28         | 1222                      |
|               | Total  |                  |                  | 4438                      |
| *C. glabrata* | FKS1   | 5592             | 1693–2075; 3831–4225 | 778                       |
|               | FKS2   | 5694             | 1802–2198; 3935–4326 | 789                       |
|               | ERG11  | 1602             | -89 - +45         | 1736                      |
|               | CgPDR1 | 3324             | 804–1168; 1526–1922; 2355–2753; 2968- +31 | 1549                      |
|               | Total  |                  |                  | 4852                      |

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resistant against all tested echinocandins and azoles and were classified as multi-resistant isolates (Table 3). Among the 26 C. glabrata isolates, 13 were resistant to the tested azoles and three to the tested echinocandins. In addition, two isolates were borderline echinocandin-resistant, and six were multi-resistant. Each azole-resistant isolate exhibited a complete cross-resistance against all tested azoles with consistently high MIC values. In the case of echinocandins, with the exception of isolate Cg41, all strains showed a complete cross-resistance against echinocandins. Strain Cg41 revealed high MIC values for anidulafungin and caspofungin, but was susceptible to micafungin with a MIC value of 0.016 mg/l. Tables 3 and 4 show all MIC data.

Validation of the sequencing process

For the validation of the sequencing process, different control strains were sequenced and no discrepancies to the published sequences were found. The Q30 quality score was 87% and the total number of reads was 11.7 million. The number of reads of each isolate was between 250,000 and 400,000. The minimal and maximal mean coverages of the genes were 2,250x and 32,000x respectively. NGS data revealed the presence of heterozygous mutations in C. albicans with a variant (frequency rate) in approximately 50% of the reads (48.2%-51.7%). For homozygous mutations close to 100% (98.6%-100%) of the reads showed the presence of the variant. As C. glabrata is haploid, variants in the reads of this species had a frequency of approximately 100% (97.9%-100%). In isolate Cg28, we found two mutations with a frequency of 26% and 73%, which could be caused by a subpopulation. This observation was verified with Sanger sequencing. The frequencies of the mutations of all other isolates indicate that they are clonal and without subpopulations.

Non causal polymorphisms

Mutations present in both susceptible and resistant isolates were defined as polymorphisms non-causal for antifungal resistance development. Of the detected 54 missense mutations, seven were already defined as polymorphisms and four mutations were displayed by susceptible isolates and thus were classified as polymorphisms. The missense mutations S935L [18] and S941P [18] in TAC1 have already been described as polymorphisms. The heterozygous mutation S937L was also present in two azole-susceptible isolates. This mutation has not been described, and causality in homozygous cases cannot be ruled out. In heterozygous cases, however, this mutation does not cause azole resistance. In ERG11, the mutations D116E [18], K128T [26] and E266D [26] were observed homozygous as well as heterozygous in susceptible strains and have already been described as non-causal mutations. The mutation V488I in ERG11 was described as causal by Manastir et al., and as non-causal by Wang et al. [26, 27].
| ID   | Resistance          | Origin       | Gene       | Nucleotide Substitution | Aminoacid Substitution | Frequency (%) | Hot Spot | Literature            | AFG  | CAS  | MFG  | FLC  | POS | ISA | ITC | VRC |
|------|---------------------|--------------|------------|-------------------------|------------------------|---------------|----------|------------------------|------|------|------|------|-----|-----|-----|-----|
| Ca1  | susceptible         | ATCC 90028  |            |                         |                        |               |          |                        | 0.008| 0.016| 0.016| 0.25 | 0.032| <0.008| 0.032| <0.016|
| Ca2  | susceptible         | ATCC 10231  |            |                         |                        |               |          |                        | 0.008| 0.016| <0.008| <0.125| 0.016| <0.008| <0.008| <0.016|
| Ca3  | azole-resistant     | ATCC 64124  | TAC1       | 2929A>G                 | N977D                  | 49.26         |          |                        | 0.016| 0.032| 0.008| 128  | 4   | 4   | >16 | 8   |
|      |                     |              | ERG11      | 214T>C                  | F72L                   | 99.79         |          | Coste (2009)           |      |      |      |      |     |     |     |     |
|      |                     |              | ERG11      | 394T>C                  | Y132H                  | 99.9          |          | Favre (1999)           |      |      |      |      |     |     |     |     |
|      |                     |              | ERG11      | 1349G>A                 | G450E                  | 99.73         |          | Favre (1999)           |      |      |      |      |     |     |     |     |
|      |                     |              | ERG3       | 503C>T                  | A353T                  | 84.71         |          | Morio (2012)           |      |      |      |      |     |     |     |     |
|      |                     |              | ERG3       | 986C>G                  | T329S                  | 99.83         |          | Morio (2012)           |      |      |      |      |     |     |     |     |
| Ca5  | azole-resistant     | not specified| ERG11      | 1309G>A                 | V437I                  | 48.2          | HS 3     | Favre (1999)           | 0.016| 0.032| 0.016| >256 | >32 | >16 | >16 | >8  |
| Ca6  | azole-resistant     | drainage fluid| TAC1       | 2810C>T                 | S937L                  | 51.06         |          |            | 0.064| 0.032| 0.016| 128  | >32 | >16 | >16 | >8  |
|      |                     |              | ERG11      | 1309G>A                 | V437I                  | 99.87         | HS 3     | Favre (1999)           |      |      |      |      |     |     |     |     |
|      |                     |              | ERG3       | 571T>C                  | S191P                  | 99.0          |          |            |      |      |      |      |     |     |     |     |
| Ca8  | azole-resistant     | mouth swab   | TAC1       | 2218A>G                 | N746D                  | 100.0         |          |            |      |      |      |      |     |     |     |     |
|      |                     |              | ERG11      | 394T>C                  | Y132H                  | 99.84         | HS 1     |            |      |      |      |      |     |     |     |     |
|      |                     |              | ERG11      | 1349G>A                 | G450E                  | 99.46         | HS 3     | Favre (1999)           |      |      |      |      |     |     |     |     |
| Ca9  | azole-resistant     | vaginal swab | ERG11      | 622G>A                  | E208K                  | 50.0          |          |            | 0.064| 0.064| 0.032| >256 | >32 | >16 | >16 | >8  |
|      |                     |              | ERG11      | 1574C>T                 | T525I                  | 49.59         |          |            |      |      |      |      |     |     |     |     |
|      |                     |              | ERG3       | 782G>A                  | G261E                  | 99.83         |          |            |      |      |      |      |     |     |     |     |
| Ca10 | azole-resistant     | mouth swab   | TAC1       | 2939G>A                 | G980E                  | 99.86         |          |            | 0.016| 0.032| 0.016| 64   | 1   | 0.5 | 0.5 | 0.25|
|      |                     |              | ERG11      | 428A>G                  | K143R                  | 99.84         | HS 1     |            |      |      |      |      |     |     |     |     |
|      |                     |              |           |                         |                        |               |          | Manastir (2009) Flowers (2015) |      |      |      |      |     |     |     |     |
| Ca11 | azole-resistant     | not specified| TAC1       | 1946G>A                 | G649D                  | 99.85         |          |            | 0.016| 0.032| 0.016| 64   | 0.5 | 0.25| 1   | 0.5 |
|      |                     |              | TAC1       | 2920T>C                 | F974L                  | 99.7          |          |            |      |      |      |      |     |     |     |     |
|      |                     |              | ERG11      | 428A>G                  | K143R                  | 99.86         | HS 1     |            |      |      |      |      |     |     |     |     |
|      |                     |              |           |                         |                        |               |          | Manastir (2009) Flowers (2015) |      |      |      |      |     |     |     |     |
| Ca13 | echinocandin-resistant | bile drainage| GSC1      | 1922T>G                 | F641C                  | 50.56         | HS 1     |            | 0.125| 0.125| 0.064| 0.25 | 0.032| <0.008| 0.064| <0.016|
|      |                     |              | GSC1      | 1923C>T                 | F641C                  | 49.17         | HS 1     |            |      |      |      |      |     |     |     |     |
|      |                     |              | ERG3      | 1057G>A                 | A353T                  | 51.19         |          | Morio (2012)           |      |      |      |      |     |     |     |     |

(Continued)
Table 3. (Continued)

| ID     | Resistance            | Origin     | Gene   | Nucleotide Substitution | Aminoacid Substitution | Frequency (%) | Hot Spot | Literature                                      | AFG | CAS | MFG | FLC | POS | ISA | ITC | VRC |
|--------|-----------------------|------------|--------|-------------------------|------------------------|---------------|----------|-----------------------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| Ca14   | echinocandin-resistant | blood culture | GSC1   | 1922T>C                 | F641S                  | 99.75         | HS1      | Wiederhold (2011) Balashov (2006)*            | 0.5 | 1   | 1   | 0.25| 0.032| 0.008| 0.032| <0.016|
| Ca15   | echinocandin-resistant | not specified | GSC1   | 1933T>C                 | S645P                  | 99.66         | HS1      | Garnaud (2015)                                | 0.5 | 2   | 1   | 0.25| 0.032| 0.008| 0.032| <0.016|
| Ca16   | echinocandin-resistant | not specified | GSC1   | 1933T>C                 | S645P                  | 48.64         | HS1      | Garnaud (2015)                                | 0.5 | 1   | 0.5 | 0.25| 0.125| 0.008| 0.064| <0.016|
| Ca17   | echinocandin-resistant | jugularis catheter | GSC1   | 1933T>C                 | S645P                  | 99.49         | HS1      | Garnaud (2015)                                | 0.5 | 2   | 2   | 0.25| 0.032| 0.008| 0.032| <0.016|
| Ca18   | echinocandin-resistant | ascites     | GSC1   | 1946C>A                 | P649H                  | 99.85         | HS1      | Garcia-Effron (2008) Desnos-Ollivier (2008) Dudiuk (2015) | 0.25 | 0.5 | 0.5 | 0.125| 0.016| 0.008| 0.016| <0.016|
| Ca19   | borderline echinocandin-resistant | blood culture | GSC1   | 2086A>G                 | M696V                  | 98.61         | 90 bp after HS1 30 bp before HS3 |                       | 0.064| 0.064| 0.064| 0.25| 0.125| 0.008| 0.064| <0.016|
| Ca21   | borderline echinocandin-resistant | blood culture | GSC1   | 570T>G                  | Y190                   | 99.84         | 99.95             | Morio (2012) | 0.016| 0.064| 0.032| 0.5 | 0.064| 0.008| 0.125| <0.016|
| Ca12   | multi-resistant       | feces  | ERG3   | 975C>A                  | Y325                   | 99.00          |                    |                         | 0.125| 0.064| 0.064| 256 | >32   | >16   | 8    | 4    |
| Ca22   | multi-resistant       | feces  | ERG3   | 570T>G                  | Y190                   | 99.84         | 99.95             | Morio (2012) | 0.25 | 0.5  | 0.125| 256 | >32   | >16   | >16  | >8   |

* same position, different aminoacid substitution

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Analysis of antifungal resistance genes
Table 4. Potentially causal missense mutations and MIC values in *C. glabrata* isolates.

| ID   | Resistance         | Origin                | Gene   | Nucleotide Substitution | Aminoacid Substitution | FREQ | Hot Spot | Literature  | AFG | CAS | MFG | FLC | POS | ISA | ITC | VRC |
|------|--------------------|-----------------------|--------|-------------------------|------------------------|------|----------|-------------|-----|-----|-----|-----|-----|-----|-----|-----|
| Cg23 | susceptible        | ATCC 90030            |        |                         |                        |      |          |             | 0.064 | 0.032 | 0.016 | 16 | 1.0 | 0.5 | 0.5 | 0.25 |
| Cg24 | susceptible        | ATCC Y33.90           |        |                         |                        | 0.032 | 0.032 | 0.016 | 4.0 | 0.25 | 0.125 | 0.125 | 0.25 |
| Cg26 | azole-resistant    | blood culture         | PDR1   | 3235G>A                 | G1079R                 | 97.86 |          | Ferrari (2009) | 0.064 | 0.064 | 0.016 | 128 | >32 | 8   | >16 | 4   |
| Cg27 | azole-resistant    | blood culture         |        |                         |                        |      |          |             | 0.064 | 0.064 | 0.016 | 128 | >32 | 8   | >16 | 4   |
| Cg28 | azole-resistant    | blood culture         | PDR1   | 2626G>T                 | D876Y                  | 26.0  |          | Sanglard (2016) Ferrari (2009) | 0.064 | 0.064 | 0.032 | 128 | >32 | 8   | >16 | 4   |
|      |                    |                       | PDR1   | 3236G>T                 | G1079V                 | 73.04 |          |            | Ferrari (2009) | 0.064 | 0.064 | 0.016 | 128 | >32 | 8   | >16 | 4   |
| Cg30 | azole-resistant    | blood culture         | PDR1   | 1043G>A                 | G348D                  | 99.87 |          |             | Ferrari (2009) Tsai (2010) | 0.064 | 0.064 | 0.016 | 256 | >32 | 8   | >16 | 8   |
| Cg31 | azole-resistant    | blood culture         | PDR1   | 871T>C                  | L291P                  | 99.95 |          | Ferrari (2009) | 0.064 | 0.125 | 0.032 | 256 | >32 | 8   | >16 | 4   |
|      |                    |                       | PDR1   | 872T>C                  | L291P                  | 99.78 |          | Ferrari (2009) |            | 0.064 | 0.125 | 0.032 | 256 | >32 | 8   | >16 | 4   |
| Cg32 | azole-resistant    | sputum                |        |                         |                        |      |          |             | 0.064 | 0.125 | 0.064 | 128 | >32 | 8   | >16 | 4   |
| Cg33 | azole-resistant    | urine                 | PDR1   | 1042G>A                 | G348S                  | 99.8  |          |             | Ferrari (2009) Tsai (2010) | 0.064 | 0.064 | 0.032 | 256 | >32 | 16  | >16 | 8   |
| Cg34 | azole-resistant    | not specified         | PDR1   | 1114T>C                 | Y372H                  | 99.83 |          |             | Ferrari (2009) | 0.064 | 0.125 | 0.032 | 256 | >32 | 8   | >16 | 8   |
| Cg35 | azole-resistant    | vaginal swab          | PDR1   | 1037G>A                 | G346D                  | 99.74 |          | Ferrari (2009) | 0.064 | 0.125 | 0.032 | 256 | >32 | 8   | >16 | 8   |
| Cg36 | azole-resistant    | urine                 | PDR1   | 862C>G                  | H288D                  | 99.0  |          | Ferrari (2009) | 0.064 | 0.125 | 0.032 | 256 | >32 | 8   | >16 | 8   |
| Cg37 | azole-resistant    | ascites               | PDR1   | 1037G>C                 | G346A                  | 99.74 |          | Ferrari (2009) | 0.064 | 0.125 | 0.032 | 256 | >32 | 16  | >16 | >8  |
| Cg38 | azole-resistant    | ascites               | PDR1   | 1042G>A                 | G348S                  | 99.72 |          |             | Ferrari (2009) Tsai (2010) | 0.125 | 0.064 | 0.064 | 256 | >32 | 8   | >16 | 8   |
| Cg39 | azole-resistant    | feces                 |        |                         |                        | 0.125 | 0.064 | 0.064 | 256 | >32 | 16  | >16 | >8  |
| Cg41 | echinocandin-      | capillary drainage    | FKS2   | 1999C>A                 | P667T                  | 99.7  | 1 bp after HS1 | Spreghini (2012) Garcia-Effron (2009) | 0.25 | 2.0 | 0.016 | 4.0 | 0.125 | 0.032 | 0.125 | 0.064 |
|      | resistant          |                       |        |                         |                        |      |          |             |             | 0.25 | 2.0 | 0.016 | 4.0 | 0.125 | 0.032 | 0.125 | 0.064 |
| Cg45 | echinocandin-      | drainage fluid        | FKS2   | 1987T>C                 | S663P                  | 99.0  | HS 1     |             | Garnaud (2015) Beyda (2015) Zimbeck (2010) | 2.0 | >16 | 2    | 4   | 0.25 | 0.125 | 0.25  | 0.125 |
|      | resistant          |                       |        |                         |                        |      |          |             |             |     |     |     |     |     |     |     |     |
| Cg51 | echinocandin-      | blood culture         | FKS2   | 1977_1979 delCTT        | F659del                | 99.8  | HS 1     |             | Saraya (2014) | 2.0 | >16 | 4    | 32  | 2   | 2   | 2   | 0.5 |
|      | resistant          |                       |        |                         |                        |      |          |             |             | 0.25 | 0.125 | 0.032 | 8.0 | 1.0 | 0.25 | 0.5  | 0.125 |

(Continued)
We found this mutation homozygous in an azole susceptible strain, which supports the study published by Wang et al. In ERG3 we detected the hitherto unknown homozygous mutations A138T, P181A and the heterozygous P267S, which could also be found in susceptible isolates in our study.

**Potentially causal resistance mutations**

In all strains, 87 different silent mutations compared to the reference sequences of the strains CBS138 and SC5314 were found. Fifty different missense mutations as well as an in frame deletion and two premature stop codons were detected. All mutations including silent mutations are listed in the supplementary data (S3 Table). We identified 43 missense mutations as potentially causal, as they were only present in resistant isolates [28]. Of these, 30 have already been described as causative for resistance acquisition. In our study, 13 potentially causal mutations are reported for the first time. Tables 3, 4, 5 and 6 show the potentially causal mutations of the respective genes. Only missense mutations, which are classified as potentially causal, are listed.

In *C. albicans*, all seven azole-resistant strains showed a mutation in the target gene ERG11. In ERG3 and TAC1 four and five potentially causal mutations could be found respectively. In comparison, no mutations in ERG11 could be detected in *C. glabrata*, but 10 of 13 azole-resistant strains showed a mutation in CgPDR1. GSC1 or FKS mutations were found in all echinocandin-resistant isolates. No FKS mutations could be found in the four borderline echinocandin-resistant *C. albicans* and *C. glabrata* isolates. All eight multi-resistant isolates had at least one mutation possibly leading to azole or echinocandin resistance (Table 5).

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Table 4. (Continued)

| ID  | Resistance                | Origin      | Gene  | Nucleotide Substitution | Aminoacid Substitution | FREQ | Hot Spot | Literature                | AFG | CAS | MFG | FLC | POS | ISA | ITC | VRC |
|-----|---------------------------|-------------|-------|-------------------------|------------------------|------|----------|---------------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| Cg43| borderline echinocandin-resistant | blood culture | FKS1  | 3967A>G                  | K1323E                 | 99.79| 15 bp before HS2 | Garnaud (2015) | 0.25 | 0.125 | 0.032 | 8 | 1 | 0.25 | 0.5 | 0.25 |
| Cg29| multi-resistant           | blood culture | FKS1  | 1874T>C                  | F625S                  | 99.68| HS1      | Garnaud (2015) | 0.064 | 0.25 | 0.125 | 128 | 16 | 8 | 8 | 8 |
| Cg46| multi-resistant sterile fluid | FKS1  | 889T>C                  | W297R                  | 99.62                  |      |          | Ferrari (2009)       | 0.125 | 0.25 | 0.125 | 0.064 | 128 | 16 | 8 | 8 |

*same position, different aminoacid substitution

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Table 5. Potentially causal missense mutations in *C. albicans* isolates, homozygous mutations in bold.

| Gene | Mutation | Literature | susceptible | azole-resistant | echinocandin-resistant | Borderline echinocandin-resistant | multi-resistant |
|------|----------|------------|-------------|-----------------|-------------------------|----------------------------------|-----------------|
| TAC1 | G649D    | Siikala et al. 2010 |             | N740D           |                         |                                  |                 |
|      | N740D    | Siikala et al. 2010 | G649D       |                 |                         |                                  |                 |
|      | F974L    | Coste et al. 2006  | F974L       |                 |                         |                                  |                 |
|      | N977D    | Coste et al. 2006  | N977D       |                 |                         |                                  |                 |
|      | G980E    | Coste et al. 2009  | G980E       |                 |                         |                                  |                 |
| ERG11| F72L     | Faye et al. 1999   | F72L        |                 |                         |                                  |                 |
|      | N740D    | Faye et al. 1999   | N740D       |                 |                         |                                  |                 |
|      | F974L    | Faye et al. 1999   | F974L       |                 |                         |                                  |                 |
|      | N977D    | Faye et al. 1999   | N977D       |                 |                         |                                  |                 |
|      | G980E    | Faye et al. 1999   | G980E       |                 |                         |                                  |                 |
|      | E208K    | E208K          |             |                 |                         |                                  |                 |
|      | V437I    | Faye et al. 1999   | V437I       |                 |                         |                                  |                 |
|      | G450E    | Faye et al. 1999   | G450E       |                 |                         |                                  |                 |
|      | T525I    | T525I          |             |                 |                         |                                  |                 |
| ERG3 | A168V    | Morio et al. 2012  | A168V       |                 |                         |                                  |                 |
|      | Y190'    | Y190'         |             |                 |                         |                                  |                 |
|      | S191P    | S191P         |             |                 |                         |                                  |                 |
|      | G261E    | G261E         |             |                 |                         |                                  |                 |
|      | Y325'    | Y325'         |             |                 |                         |                                  |                 |
|      | T329S    | Morio et al. 2012  | T329S       |                 |                         |                                  |                 |
|      | A353T    | Morio et al. 2012  | A353T       |                 |                         |                                  |                 |
| GSC1 | F641C    | Wiederhold et al. 2011; Balbashov et al. 2006 | F641C |                 |                         |                                  |                 |
|      | F641S    | Wiederhold et al. 2011; Balbashov et al. 2006 | F641S |                 |                         |                                  |                 |
|      | S645P    | Garnaud et al. 2013 | S645P |                 |                         | S645P | S645P | S645P | P649H | M696V |
| Gene | Mutation | LITERATURE | susceptible | azole-resistant | echinocandin-resistant | Borderline echinocandin-resistant | multi-resistant |
|------|----------|------------|-------------|-----------------|------------------------|-------------------------------|-----------------|
|      |          |            | Cg 23       | Cg 24           | Cg 26                  | Cg 27                        | Cg 28           |
|      |          |            | Cg 29       | Cg 30           | Cg 31                  | Cg 32                        | Cg 33           |
|      |          |            | Cg 34       | Cg 35           | Cg 36                  | Cg 37                        | Cg 38           |
|      |          |            | Cg 39       | Cg 40           | Cg 41                  | Cg 42                        | Cg 43           |
|      |          |            | Cg 44       | Cg 45           | Cg 46                  | Cg 47                        | Cg 48           |
|      |          |            | Cg 49       | Cg 50           | Cg 51                  | Cg 52                        | Cg 53           |
|      |          |            | Cg 54       | Cg 55           | Cg 56                  | Cg 57                        |
|      |          |            | Cg 58       | Cg 59           | Cg 60                  | Cg 61                        |

| Fg11 | –        | –          | H291F       | L291P            | FERRARI ET AL. 2009         | FERRARI ET AL. 2009         |
| FDR1 | H291F    | –          | –           | –                | W297R                      | H291F                        |
|      | L291P    | FERRARI ET AL. 2009 | –       | –                | W297R                      | H291F                        |
|      | W297R    | FERRARI ET AL. 2009 | –       | –                | W297R                      | H291F                        |
|      | V329F    | HEALEY ET AL. 2016 | –       | –                | V329F                      | –                            |
| G44D | FERRARI ET AL. 2009 | –          | G346D       | G346A            | FERRARI ET AL. 2009         | FERRARI ET AL. 2009         |
|      | G346D    | FERRARI ET AL. 2009 | –       | –                | G346A                      | FERRARI ET AL. 2009         |
|      | G346S    | TSI et al. 2010  | G346S       | G346S            | FERRARI ET AL. 2009         | FERRARI ET AL. 2009         |
|      | G346D    | TSI et al. 2010  | G346D       | G346D            | FERRARI ET AL. 2009         | FERRARI ET AL. 2009         |
|      | V372H    | FERRARI ET AL. 2009 | –       | –                | V372H                      | V372H                        |
|      | DB76Y    | SOHNRLAND AND GREVE 2016 | –       | –                | DB76Y                      | DB76Y                        |
|      | G1079R   | FERRARI ET AL. 2009 | G1079R    | G1079R           | FERRARI ET AL. 2009         | FERRARI ET AL. 2009         |
|      | G1079V   | FERRARI ET AL. 2009 | G1079V    | G1079V           | FERRARI ET AL. 2009         | FERRARI ET AL. 2009         |
|      | F625S    | GERNAND ET AL. 2015 | –       | –                | F625S                      | F625S                        |
|      | F132S    | SANCHEZ ET AL. 2014 | –       | –                | K132S                      | K132S                        |
| FKS1 | F695I    | GARCIA-BILBON ET AL. 2009 | –       | –                | F695I                      | F695I                        |
|      | S663P    | GARCIA-BILBON ET AL. 2009 | –       | –                | S663P                      | S663P                        |
|      | F667T    | SPROFFERTI ET AL. 2012; GARCIA-BILBON ET AL. 2009 | –       | –                | F667T                      | F667T                        |

* same position, different amino acid substitution

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Mutations in azole-resistant *C. albicans* and *C. glabrata*

The seven potentially causal mutations F72L [29], Y132H [29], K143R [27, 30], E208K, V437I [29], G450E [29] and T525I were found in *ERG11* in *C. albicans*. Isolate Ca9 showed the two heterozygous mutations E208K and T525I, which have not been described so far. In *ERG3*, we detected the seven potentially causal mutations A168V [14], S191P, G261E, T329S [14] and A353T [14]. The mutation A353T is shown as a homozygous substitution in an azole-resistant strain, and as a heterozygous mutation in the azole-susceptible isolates Ca13 and Ca14. In *TAC1*, the five potentially causal mutations G649D, N740D [31], F974L, N977D [10, 32] and G980E [32] are shown. (Table 5).

In contrast to *C. albicans*, none of the azole-resistant *C. glabrata* isolates showed any mutations in *ERG11*. In *CgPDR1*, 10 different mutations were observed. H288D has not yet been described. The mutations L291P, G346D, G346A, G348S, G348D and Y372H have been described in these positions but with different amino acid substitutions [33, 34]. The mutations D876Y, G1079R and G1079V have been described as causal by Ferrari et al. [33]. No mutations in the sequenced regions of *CgPDR1* have been detected in the isolates Cg27, Cg32 and Cg39 (Table 6).

Mutations in echinocandin-resistant *C. albicans* and *C. glabrata*

In all six echinocandin-resistant *C. albicans* isolates, a mutation was found in the target gene *GSC1*. All mutations were detected in *GSC1* HS 1 or its immediate vicinity. The mutations observed were S645P [18], which was detected three times, F641C, F641S [7, 35], P649H [36–38] and M696V. For the isolates Ca13 and Ca16 the mutations F641C and S645P, respectively, are shown as heterozygous. Isolate Ca14 shows the mutation F641S, which was homozygous in this strain and was associated with higher MIC than for isolate Ca13. Isolate Ca18 showed the two homozygous mutations P649H and M696V, which are located in HS 1 and 90 nucleotides downstream of HS 1, respectively. The borderline echinocandin-resistant isolates Ca19 and Ca21, which exhibit a MIC of micafungin of 0.064 mg/l and 0.032 mg/l respectively, did not have any mutations in *GSC1*. Our strains showed no missense mutations in the HS regions of *GSC1* genes whenever the MIC was lower than 0.125 mg/l in anidulafungin and caspofungin, and lower than 0.064 mg/l in micafungin (Table 5).

In the echinocandin-resistant isolates of *C. glabrata* we identified the mutations S663P [18, 39, 40], F659del [41–43] in *FKS2*, but none in *FKS1*. The isolate Cg51 showed very high MIC values for anidulafungin (2 mg/l), caspofungin (> 16 mg/l) and micafungin (4 mg/l). In this isolate the deletion F659del in *FKS2* HS 1 was detected. The borderline echinocandin-resistant isolates Cg42 and Cg43 showed no mutations in *FKS1* and *FKS2*. In these isolates the MIC for anidulafungin were twofold dilutions above the breakpoint and for micafungin exactly at the breakpoint (Table 6).

Mutations in multi-resistant *C. albicans* and *C. glabrata*

The multi-resistant *C. albicans* isolates Ca12 and Ca22 showed the homozygous premature stop codons Y325* and Y190* in *ERG3*, respectively. In both *GSC1* HS regions, no mutations were detected despite elevated echinocandin MIC values (Table 5).

In the six multi-resistant *C. glabrata* isolates, five mutations were verified in *FKS* genes. In *FKS1*, the mutations K1323E and F625S [18] could be detected in isolates Cg29 and Cg46, respectively. In *FKS2*, the mutations F659S and S663P could be detected in isolates Cg50 and Cg47 respectively. For three of the six multi-resistant isolates, potentially causal mutations were found in *CgPDR1*: W297R [33, 34] for isolate Cg46, V329F [44] for isolate Cg49 and G1088E for isolate Cg50. Only isolates Cg46 and Cg50 showed a mutation in *FKS* genes and
CgPDR1. Isolate Cg49, which displayed moderately elevated MIC values for echinocandins showed no mutations in FKS1 and FKS2 (Table 6).

Discussion

NGS has previously been shown to successfully detect antifungal resistance mutations in clinically important Candida species [18]. With targeted resequencing resistance genes of a high number of isolates can be studied simultaneously. Compared to whole genome sequencing (WGS), this approach reduces sequencing costs, generates more manageable raw data and reduces the burden of data analysis. Investigating 50 isolates the costs would reach 16,000 € using WGS. In this study, the costs were only 3000 €. Thus, the sequencing costs could be reduced to at least 5x due to the targeted resequencing design. Additionally, targeted resequencing results in coverage levels much higher than those achieved with WGS. Thus, this method is very reliable and allows the detection of low frequent variants, e.g. resistant subpopulations. Furthermore, sequencing runs are much faster than with the conventional Sanger method. The sequencing process in our project took about 22 hours for 51 strains with 13 amplicons each. In comparison, using Sanger sequencing on a 4-capillary sequencer, the run time would have been about four weeks. Sanger sequencing is more expensive, and the analysis of this extensive sequencing data would be time-consuming.

In this study, a high number of phenotypically resistant isolates obtained from various centres in Austria and Germany were investigated in order to find mutations causing resistance, and to examine if strains categorized as resistant using the clinical breakpoints possess already described or hitherto unknown resistance mutations. Due to high throughput sequencing, we were able to detect established as well as novel resistance mutations in a large sample of antifungal-resistant strains.

Azole resistance

Target mutations in ERG11 were detected only in C. albicans. In every azole-resistant isolate, potentially causal mutations were detected in this gene. Five of these mutations have already been described as inducing resistance, based on the putative mechanism that the change in the protein sequence leads to a reduced binding affinity of azoles [27, 29, 30]. In contrast to C. albi- cans, there were no mutations in ERG11 in any of the 16 azole-resistant C. glabrata strains. The absence of ERG11 mutations in azole-resistant C. glabrata has already been described [45, 46]. This suggests that our results are in concordance with other investigations and ERG11 mutations have no impact onazole resistance in our C. glabrata isolates.

CgPDR1 appears to be a more important cause for azole resistance in C. glabrata. CgPDR1 is a transcription factor, which induces the gene expression of the efflux pumps CgCDR1/2p and CgSNQ2p. In CgPDR1 67 gain-of-function mutations have been described hitherto [33]. These mutations were associated with intrinsically high expression of the efflux pumps and specifically were related to azole resistance [12]. In our study, 13 potentially causal mutations were found. In 10 out of 13 azole-resistant strains, at least one mutation was found in CgPDR1. Eleven of these mutations are known, the remaining mutations H288D and G1088E have been detected for the first time. Tsai et al. described the domains of CgPDR1 based on the homology between S. cerevisiae PDR1 and C. glabrata CgPDR1 [34]. The DNA-binding domain is positioned at residues 26–59, the regulatory domain at 322–465 and the activation domain at 903–1107. They found four mutations at residues 280–391. Nine of 13 mutations in CgPDR1 found in our study were located at residues 288–372, near the putative regulatory domain. Accordingly, these mutations could be associated with altered expression of efflux pumps. Three mutations (G1079R, G1079V and G1088E) were identified in the putative activation domain.
Thus, mutations in these regions could be associated with overexpression of drug efflux pumps. To confirm the impact of these mutations, the analysis of the expression levels of efflux pumps could be performed in future studies.

Gain-of-function mutations in the transcription factor TAC1 lead to inherently increased expression of efflux pumps CDR1p and CDR2p and were associated with azole resistance [47]. In our study, five potentially causal mutations in the sequenced areas of TAC1 could be identified. Of these, G649D and F974L have not been described so far. However, since none of the sequenced strains showed mutations in TAC1 only, the relevance of this mechanism could not be clarified in this study.

We could also detect loss-of-function mutations in ERG3 which presumably leads to azole resistance in combination with moderate echinocandin resistance and is described in the multi resistance section.

**Echinocandin resistance**

In our study, mutations in GSC1, FKS1 and FKS2 were detected in all isolates showing an increase of MIC values (>2 dilution folds above CB), which was associated with complete cross-resistance with the exception of one C. glabrata isolate. Several missense mutations were found in these isolates. For the isolates with moderately elevated MICs, i.e. one to twofold dilutions above the clinical breakpoint and classified as borderline resistant, neither cross-resistance within the echinocandins nor FKS mutations were found.

In our study, a single mutation in the HS regions of the target genes led to complete cross-resistance in the class of echinocandins and was seen for both heterozygous and homozygous mutations. In contrast to this observation, the C. glabrata isolate Cg41 displayed an isolated susceptibility to micafungin (MIC 0.016 mg/l) whereas anidulafungin showed an elevated MIC of 0.25 mg/l and caspofungin 2 mg/l. In this isolate the already known mutation P667T in FKS2 HS 1 was detected. The absence of cross-resistance has already been reported [3, 48]. This is of particular interest as anidulafungin has been discussed to serve as a surrogate marker for all echinocandins [49, 50]. In our case, however, micafungin could have been an important therapeutic alternative. These results indicate that changes in conformation of 1,3-β-D-glucan synthase may lead to an incomplete cross-resistance in the class of echinocandins depending on the specific structure of the respective agent. Therefore, susceptibility testing of every echinocandin seems to be preferable to using anidulafungin as an indicator for echinocandin resistance.

The external localization of some regions of the putative transmembrane protein 1,3-β-D-glucan synthase seems to reflect the HS regions [51]. All of our six echinocandin-resistant C. albicans isolates displayed a mutation in GSC1 HS 1. No mutations were detected in HS 2. Therefore, HS 1 appears to play a more important role in the development of echinocandin resistance in our C. albicans strains. The C. albicans isolate Ca18 showed two homozygous mutations in GSC1. P649H is located at the downstream end of HS 1 and M696V is located 90 nucleotides downstream HS 1 and 30 nucleotides upstream HS 3, which is described by Johnson et al. [51]. Thus, it could be assumed that in rare cases other HS regions may be involved in acquisition of echinocandin resistance.

Out of six echinocandin-resistant C. glabrata strains, four showed a mutation in FKS2 HS 1. The multi-resistant isolates Cg29 and Cg48 showed the mutation K1323E in FKS1, which is five amino acids upstream HS 2. These isolates displayed a minimal rise of MIC values being only onefold dilution above the clinical breakpoint. In these cases, the mutation outside the HS was associated with minimally elevated MIC values.

In isolate Cg51 the in frame deletion F659del in FKS2 HS 1 was detected. This strain showed MIC values in the resistant range for anidulafungin (2 mg/l), caspofungin (> 16 mg/l),
and micafungin (4 mg/l). This mutation has already been described associated with very high as well as low MIC values, which might be caused by different expression rates [41–43].

Multi-resistance

Out of 40 clinical strains, two C. albicans and six C. glabrata were multi-resistant. In C. glabrata strains, only two isolates showed mutations in both FKS1 and CgpDR1, which explainsazole and echinocandin resistance. In the other four isolates, only a mutation in either the FKS genes or in CgPDR1 could be detected.

The multi-resistant C. albicans strains Ca12 and Ca22 only showed a loss-of-function mutation in ERG3 due to the premature stop codons Y325* and Y190*, which presumably leads toazole resistance and moderate resistance to echinocandins without displaying FKS mutations. Although the molecular mechanism for the moderate echinocandin resistance is not clear, this observation is in concordance with Rybak et al. [52]. Thus, the importance of ERG3 loss-of-function mutations for resistance development should not be overlooked.

The fact that a potentially causal mutation forazole and echinocandin resistance is not present in every resistant isolate suggests that different—hitherto unknown—resistance mechanisms are involved.

Conclusion

NGS proved to be a suitable method to detect resistance mutations. This technique allows a thoroughly, more cost-efficient and much faster sequencing method than conventional Sanger sequencing. Furthermore, the targeted resequencing design enables the investigation of a larger sample size than WGS. In combination with the phenotypic analysis of resistance patterns, conclusions can be drawn about the underlying molecular mechanisms.

We investigated 40 resistant clinical C. albicans and C. glabrata isolates and found 30 described and 13 novel mutations in six resistance genes. In addition, a high rate of polymorphisms was found in the coding sequences. This observation underlines the importance of the differentiation between polymorphisms and causal mutations. This applies especially for azole resistance, where several mechanisms can lead to resistance and interact with each other. As a consequence, a SNP database, which includes each variant as well as the phenotype, would be helpful to distinguish between polymorphisms and relevant mutations.

In conclusion, an association between mutations in FKS genes and echinocandin resistance can be confirmed. However, the acquisition of azole resistance seems to have multifactorial causes. The mutations in ERG11 appear to play a role only in C. albicans. In C. glabrata, over-expression of efflux pumps is conceivable instead. In C. albicans, homozygous ERG3 nonsense mutations seem to be associated with azole resistance and moderately elevated echinocandin MICs. Four of the multi-resistant C. glabrata isolates showed no underlying mutations for both echinocandin and azole resistance. Hence, there are still other cellular mechanisms, which require further investigations.

Supporting information

S1 Table. C. albicans primers used in this study.
(DOCX)

S2 Table. C. glabrata primers used in this study.
(DOCX)

S3 Table. List of mutations detected in this study.
(DOCX)
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References
1. Pfaller MA, Diekema DJ, Gibbs DL, Newell VA, Ellis D, Tullio V et al. Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: a 10.5-year analysis of susceptibilities of Candida Species to fluconazole and voriconazole as determined by CLSI standardized disk diffusion. J Clin Microbiol 2010 [cited 2016 Sep 9]; 48(4):1366–77. Available from: URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2849609/pdf/2117-09.pdf. PMID: 20164282

2. Yapar N. Epidemiology and risk factors for invasive candidiasis. Ther Clin Risk Manag 2014; 10:95–105. https://doi.org/10.2147/TCRM.S40160 PMID: 24611015

3. Alexander BD, Johnson MD, Pfeiffer CD, Jimenez-Ortigosa C, Catania J, Booker R et al. Increasing echinocandin resistance in Candida glabrata: clinical failure correlates with presence of FKS mutations and elevated minimum inhibitory concentrations. Clin Infect Dis 2013 [cited 2016 Sep 4]; 56(12):1724–32. Available from: URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3658363/pdf/cit136.pdf. PMID: 23487382

4. Pfaller MA, Messer SA, Hollis RJ, Boyken L, Tendolkar S, Kroeger J et al. Variation in susceptibility of bloodstream isolates of Candida glabrata to fluconazole according to patient age and geographic location in the United States in 2001 to 2007. J Clin Microbiol 2009; 47(10):3185–90. https://doi.org/10.1128/JCM.00946-09 PMID: 19656983

5. Denning DW, Bromley MJ. Infectious Disease. How to bolster the antifungal pipeline. Science 2015; 347(6229):1414–6. https://doi.org/10.1126/science.aaa6097 PMID: 25814567

6. Katiyar S, Pfaller M, Edlind T. Candida albicans and Candida glabrata clinical isolates exhibiting reduced echinocandin susceptibility. Antimicrob Agents Chemother 2006; 50(8):2892–4. https://doi.org/10.1128/AAC.00349-06 PMID: 16870797

7. Balashov SV, Park S, Perlin DS. Assessing resistance to the echinocandin antifungal drug caspofungin in Candida albicans by profiling mutations in FKS1. Antimicrob Agents Chemother 2006; 50(6):2058–63. https://doi.org/10.1128/AAC.01653-05 PMID: 16723566

8. Lamb DC, Kelly DE, Schunck W-H, Shyadethi AZ, Akhtar M, Lowe DJ et al. The Mutation T315A in Candida albicans Sterol 14α-Demethylase Causes Reduced Enzyme Activity and Fluconazole Resistance through Reduced Affinity. J. Biol. Chem. 1997; 272(9):5682–8. PMID: 9038178

9. Xu Y, Sheng F, Zhao J, Chen L, Li C. ERG11 mutations and expression of resistance genes in fluconazole-resistant Candida albicans isolates. Arch Microbiol 2015; 197(9):1087–93. https://doi.org/10.1007/s00203-015-1146-8 PMID: 26349561

10. Coste A, Turner V, Ischer F, Morschhauser J, Forche A, Selmecki A et al. A mutation in Tactp, a transcription factor regulating CDR1 and CDR2, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in Candida albicans. Genetics 2006; 172(4):2139–56. https://doi.org/10.1534/genetics.105.054767 PMID: 16452151
11. Tsai H-F, Krol AA, Sarti KE, Bennett JE. Candida glabrata PDR1, a transcripational regulator of a pleiotropic drug resistance network, mediates azole resistance in clinical isolates and petite mutants. Antimicrob Agents Chemother 2006 [cited 2016 Sep 10]; 50(4):1384–92. Available from: URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1426987/pdf/1629-05.pdf. PMID: 16569856

12. Ferrari S, Sanguinetti M, Torrelli R, Posterraro B, Sanglard D. Contribution of CgPDR1-regulated genes in enhanced virulence of azole-resistant Candida glabrata. PLoS One 2011; 6(3):e17589. https://doi.org/10.1371/journal.pone.0017589 PMID: 21408004

13. Miyazaki Y, Geber A, Miyazaki H, Falconer D, Parkinson T, Hitchcock C et al. Cloning, sequencing, expression and allelic sequence diversity of ERG3 (C-5 sterol desaturase gene) in Candida albicans. Gene 1999; 236(1):43–51. PMID: 10433965

14. Morio F, Pagniez F, Lacroix C, Miegeville M, Le Pape P. Amino acid substitutions in the Candida albicans sterol Delta5,6-desaturase (Erg3p) confer azole resistance: characterization of two novel mutants with impaired virulence. J Antimicrob Chemother 2012; 67(9):2131–8. https://doi.org/10.1093/jac/dks186 PMID: 22678731

15. M. C. Arendrup, J Guinean, M. Cuenca-Estrella, J. Meletiadis, J. W. Mouton, K. Lagrou, S. J. Howard and the Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST). EUCAST DEFINITIVE DOCUMENT E.DEF 7.3: Method for the determination of broth dilution minimum Inhibitory concentrations of antifungal agents for yeasts [cited 2016 Aug 29]. http://www.eu cast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Files/EUCAST_E_Def_7_3_Yeast_testing_definitive.pdf.

16. Umesha S, Manukumar HM, Raghava S. A rapid method for isolation of genomic DNA from food-borne fungal pathogens. 3 Biotech 2016 [cited 2018 Dec 18]; 6(2):123; https://doi.org/10.1007/s13205-016-0436-4 PMID: 28330193

17. Illumina. 16S metagenomic sequencing library preparation guide.; 2013 [cited 2014 Aug 13]. support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-1504223-b.pdf.

18. Garnaud C, Botterel F, Sertour N, Bougnoux M-E, Dannaoui E, Larrat S et al. Next-generation sequencing offers new insights into the resistance of Candida spp. to echinocandins and azoles. J Antimicrob Chemother 2015; 70(9):2556–65. https://doi.org/10.1093/jac/dkv139 PMID: 26017039

19. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M et al. Primer3—new capabilities and interfaces. Nucleic Acids Res 2012 [cited 2017 Jan 7]; 40(15):e115. Available from: URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3424584/pdf/gks596.pdf. PMID: 22730293

20. Quail MA, Smith M, Coupland P, Otto TD, Harris SR, Connor TR et al. A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. BMC Genomics 2012; 13:341. https://doi.org/10.1186/1471-2164-13-341 PMID: 22827831

21. Simon Andrews. FastQC: A quality control tool for high throughput sequenct data.; 2010 [cited 2018 Sep 12]. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

22. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 2014; 30(15):2114–20. https://doi.org/10.1093/bioinformatics/btu170 PMID: 24695404

23. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods 2012; 9(4):357–9. https://doi.org/10.1038/nmeth.1923 PMID: 22386286

24. Skrzypek MS, Binkley J, Binkley G, Miyazaki SR, Simmon M, Sherlock G. The Candida Genome Database (CGD): incorporation of Assembly 22, systematic identifiers and visualization of high throughput sequencing data. Nucleic Acids Res 2017; 45(D1):D92–D96. https://doi.org/10.1093/nar/gkw924 PMID: 27738138

25. Koboldt DC, Chen K, Wylie T, Larson DE, McLellan MD, Mardis ER et al. VarScan: variant detection in massively parallel sequencing of individual and pooled samples URL: http://varscan.sourceforge.net. Bioinformatics 2009; 25(17):2283–5. PMID: 19542151

26. Wang H, Kong F, Sorrell TC, Wang B, McNicholas P, Pantarat N et al. Rapid detection of ERG11 gene mutations in clinical Candida albicans isolates with reduced susceptibility to fluconazole by rolling circle amplification and DNA sequencing. BMC Microbiol 2009; 9:167. https://doi.org/10.1186/1471-2180-9-167 PMID: 19682357

27. Manasrl F, Ergon MC, Yucosy M. Investigation of mutations in Erg11 gene of fluconazole resistant Candida albicans isolates from Turkish hospitals. Mycoses 2011; 54(2):99–104. https://doi.org/10.1111/j.1439-0507.2009.01766.x PMID: 19732347

28. MacArthur DG, Manolio TA, Dimmock DP, Rehm HL, Shendure J, Abecasis GR et al. Guidelines for investigating causality of sequence variants in human disease. Nature 2014; 508(7497):469–76. https://doi.org/10.1038/nature13127 PMID: 24759409

29. Favre B, Didmon M, Ryder NS. Multiple amino acid substitutions in lanosterol 14alpha-demethylase contribute to azole resistance in Candida albicans. Microbiology 1999; 145 (Pt 10):2715–25.
30. Flowers SA, Colon B, Whaley SG, Schuler MA, Rogers PD. Contribution of clinically derived mutations in ERG11 to azole resistance in Candida albicans. Antimicrob Agents Chemother 2015; 59(1):450–60. https://doi.org/10.1128/AAC.03974-14 PMID: 25380595

31. Siikala E, Rautemaa R, Richardson M, Saxen H, Bowyer P, Sangiardi D. Persistent Candida albicans colonization and molecular mechanisms of azole resistance in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) patients. J Antimicrob Chemother 2010; 65(12):2505–13. https://doi.org/10.1093/jac/dkq354 PMID: 20876623

32. Coste A, Crittin J, Bauser C, Rohde B, Sangiardi D. Functional analysis of cis- and trans-acting elements of the Candida albicans CDR2 promoter with a novel promoter reporter system. Eukaryot Cell 2009; 8(8):1250–67. https://doi.org/10.1128/EC.00069-09 PMID: 19561319

33. Ferrari S, Ischer F, Calabrese D, Postera B, Sanguinetti M, Fadda G et al. Gain of function mutations in the fks1 gene accounts for reduced echinocandin susceptibility. Antimicrob Agents Chemother 2008; 52(7):3254–60. https://doi.org/10.1128/AAC.00262-08 PMID: 18443110

34. Desnos-Ollivier M, Bretagne S, Raoux D, Hoinard D, Dromer F, Dannaoui E. Mutations in the fks1 gene in Candida glabrata not only mediate antifungal resistance but also enhance virulence. PLoS Pathog 2009, 5(1):e1000268. https://doi.org/10.1371/journal.ppat.1000268 PMID: 19148266

35. Wiederhold NP, Najvar LK, Bocanegra RA, Kirkpatrick WR, Patterson TF. Caspofungin dose escalation for invasive candidiasis due to resistant Candida albicans. Antimicrob Agents Chemother 2011; 55(7):3254–60. https://doi.org/10.1128/AAC.01750-10 PMID: 20547810

36. Garcia-Effron G, Katlyar SK, Park S, Edlind TD, Perlin DS. A naturally occurring proline-to-alanine amino acid change in Fks1p in Candida parapsilosis, Candida orthopsilosis, and Candida metapsilosis accounts for reduced echinocandin susceptibility. Antimicrob Agents Chemother 2008; 52(7):2305–12. https://doi.org/10.1128/AAC.00989-08 PMID: 18591282

37. Katiyar SK, Alastruey-Izquierdo A, Healey KR, Johnson ME, Perlin DS, Edlind TD. Fks1 and Fks2 are functionally redundant but differentially regulated in Candida glabrata: implications for echinocandin resistance. Antimicrob Agents Chemother 2012 [cited 2016 Sep 4]; 56(12):6304–9. Available from: URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3497156/pdf/zac6304.pdf. PMID: 23027185

38. Zimbeck AJ, Iqbal N, Ahquist AM, Farley MM, Harrison LH, Chiller T et al. FKS mutations and elevated echinocandin MIC values among Candida glabrata isolates from U.S. population-based surveillance. Antimicrob Agents Chemother 2010; 54(12):5042–7. https://doi.org/10.1128/AAC.00836-10 PMID: 20837754

39. Saraya T, Tanabe K, Araki K, Yonetani S, Makino H, Watanabe T et al. Breakthrough invasive Candida glabrata in patients on micafungin: a novel FKS1 gene conversion correlated with sequential elevation of MIC. J Clin Microbiol 2014; 52(7):2709–12. https://doi.org/10.1128/JCM.03593-13 PMID: 24789192

40. Flowers SA, Colon B, Whaley SG, Schuler MA, Rogers PD. Contribution of clinically derived mutations in ERG11 to azole resistance in Candida albicans. Antimicrob Agents Chemother 2015; 59(1):450–60. https://doi.org/10.1128/AAC.03974-14 PMID: 25380595

41. Zimbeck AJ, Iqbal N, Ahquist AM, Farley MM, Harrison LH, Chiller T et al. FKS mutations and elevated echinocandin MIC values among Candida glabrata isolates from U.S. population-based surveillance. Antimicrob Agents Chemother 2010; 54(12):5042–7. https://doi.org/10.1128/AAC.00836-10 PMID: 20837754

42. Klotz U, Schmidt D, Willinger B, Steinmann E, Buer J, Rath P-M et al. Echinocandin resistance and population structure of invasive Candida glabrata isolates from two university hospitals in Germany and Austria. Mycoses 2016; 59(5):312–8. https://doi.org/10.1111/myc.12472 PMID: 26806376

43. Desnos-Ollivier M, Bretagne S, Raoux D, Hoinard D, Dromer F, Dannaoui E. Mutations in the fks1 gene in Candida albicans, C. tropicalis, and C. krusei correlate with elevated caspofungin MICs uncovered in AM3 medium using the method of the European Committee on Antibiotic Susceptibility Testing. Antimicrob Agents Chemother 2008; 52(9):3092–8. https://doi.org/10.1128/AAC.00088-08 PMID: 18591282

44. Garcia-Effron G, Lee S, Park S, Cleary JD, Perlin DS. Effect of Candida glabrata FKS1 and FKS2 mutations on echinocandin sensitivity and kinetics of 1,3-beta-D-glucan synthase: implications for the existing susceptibility breakpoint. Antimicrob Agents Chemother 2009; 53(9):3690–9. https://doi.org/10.1128/AAC.00443-09 PMID: 19546367

45. Klotz U, Schmidt D, Willinger B, Steinmann E, Buer J, Rath P-M et al. Echinocandin resistance and population structure of invasive Candida glabrata isolates from two university hospitals in Germany and Austria. Mycoses 2016; 59(5):312–8. https://doi.org/10.1111/myc.12472 PMID: 26806376

46. dos Santos Silva Danielley Beraldo, Rodrigues LMC, Almeida AA, Almeida AA, Almeida AA, Almeida AA, Almeida AA. Novel point mutations in the ERG11 gene in clinical isolates of azole resistant Candida species. Mem Inst Oswaldo Cruz 2016; 111(3):192–9. https://doi.org/10.1590/0074-02760150400 PMID: 26982177
47. Coste AT, Karababa M, Ischer F, Bille J, Sanglard D. TAC1, transcriptional activator of CDR genes, is a new transcription factor involved in the regulation of Candida albicans ABC transporters CDR1 and CDR2. Eukaryot Cell 2004 [cited 2017 Jan 5]; 3(6):1639–52. Available from: URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC539021/pdf/0132-04.pdf. PMID: 15590837

48. Johnson ME, Katiyar SK, Edlind TD. New Fks hot spot for acquired echinocandin resistance in Saccharomyces cerevisiae and its contribution to intrinsic resistance of Scedosporium species. Antimicrob Agents Chemother 2011; 55(8):3774–81. https://doi.org/10.1128/AAC.01811-10 PMID: 21576441

49. Pfaller MA, Diekema DJ, Jones RN, Castanheira M. Use of anidulafungin as a surrogate marker to predict susceptibility and resistance to caspofungin among 4,290 clinical isolates of Candida by using CLSI methods and interpretive criteria. J Clin Microbiol 2014 [cited 2017 Aug 5]; 52(9):3223–9. https://doi.org/10.1128/JCM.00782-14 PMID: 24951808

50. European Committee on Antimicrobial Susceptibility Testing. Anidulafungin: Rationale for the clinical breakpoints, version 2.0, 2013.

51. Johnson ME, Edlind TD. Topological and mutational analysis of Saccharomyces cerevisiae Fks1. Eukaryot Cell 2012; 11(7):952–60. https://doi.org/10.1128/EC.00082-12 PMID: 22581527

52. Rybak JM, Dickens CM, Parker JE, Caudle KE, Manigaba K, Whaley SG et al. Loss of C-5 Sterol Desaturase Activity Results in Increased Resistance to Azole and Echinocandin Antifungals in a Clinical Isolate of Candida parapsilosis. Antimicrob Agents Chemother 2017; 61(9).