Mechanisms of echinocandin resistance in *Candida* species

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Abstract

Echinocandins are antifungals used against *Candida* infections and resistant strains have been emerging. Among these strains are *Candida albicans* and non-*albicans*. Given the widespread use of echinocandins, it is important to study how resistance mechanisms act and evolve. Antifungal susceptibility tests for echinocandins were performed for 29 isolates of *Candida* species. Two resistant isolates were obtained, *Candida krusei* (4 mg/L MIC for the three echinocandins) and *Candida glabrata* (0.5 mg/L MIC for anidulafungin and caspofungin and 1 mg/L for micafungin) and two isolates of *Candida inconspicua* and *Candida palmioleophila* with high MIC values of 4 mg/L for the three echinocandins, something that had not been previously reported. Resistance to echinocandins is based on the occurrence of point mutations of the *FKS* gene. PCR identification using a single primer for the *FKS* gene was performed to obtain multilocal patterns that would allow quick identification. A total of 70 isolates of *Candida* species were tested. It was possible to obtain identifiable patterns for *Candida albicans*, *Candida palmioleophila* and *Candida krusei*. The last two presented different patterns according to their susceptibility to echinocandins. The genes *CHS1*, *PST1*, *CWP1*, and *CPW2* encoding cell wall proteins, possibly upregulated when there are alterations in the *FKS1* gene, were investigated for 8 *Candida* isolates. Upregulation of all genes was verified for one susceptible isolate of *Candida albicans* and two resistant isolates of *Candida palmioleophila* and *Candida krusei*, pointing to a possible alteration in the *FKS1* gene of these isolates. In general results have shown that amongst *Candida* species, there are important connections between cell wall dynamics and resistance to antifungal drugs. It is important to continue to try to understand how the mechanisms of resistance and the cellular responses to maintain cell wall integrity work in order to develop new strategies to fight these infections.

Keywords: *Candida albicans*, non-*albicans Candida* species, antifungal drug resistance, echinocandins, PCR fingerprinting, real time PCR, *FKS* gene

1. Introduction

1.1. Emergence of *Candida* species

Candida is a genus of yeasts and one of the most common cause of fungal infections worldwide. *Candida* species exist as commensals of the skin, mouth and gastrointestinal tract (1). These yeasts possess the ability to act as pathogens that cause superficial and systemic infections. The incidence of fungal infections caused by *Candida* spp. has increased dramatically during the last decades. This is mainly due to the rise in number of immunocompromised patients (2). *Candida* infections are derived from the individual's own endogenous reservoir when the host presents certain risk factors (3). Mortality due to systemic candidiasis remains high (46% to 75% for *C. albicans* alone) (4). There are over 150 heterogeneous species included in the *Candida* genus (5). *C. albicans* is the most common cause of candidemia, but there have been increased numbers of isolations of non-*albicans* species in recent years, with the most prominent being *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei*. Together they account for 92–95% of all cases of *Candida* infection (6). Other rare species of *Candida* are also emerging. There's lack of susceptibility data for these species thanks to the limited clinical experience. Consequently, infections caused by these rare pathogens are linked to high mortality and therapeutic failure (7). Examples of these non-*albicans* species are *Candida palmioleophila* and *Candida inconspicua*. The rise in the non-*albicans Candida* spp. is possibly due to their high levels of intrinsic antifungal drug resistance, but it is also possible that improvements to laboratory detection and identification may provide more specific identification than in the past and account for emergence of less common species (8).

1.2. Antifungal drugs

Candida infections can be treated using four main drug classes: azoles, polyenes, pyrimidine analogues and echinocandins. Echinocandins are recommended as the first-line empirical treatment for invasive candidiasis (9). They specifically inhibit the biosynthesis of the fungal-specific enzyme (1,3)-β-D-glucan synthase, and (1,3)-β-D-glucan is a major structural component of fungal cell walls. This enzyme inhibition leads to the formation of fungal cell walls with impaired structural integrity, which results in cell vulnerability to osmotic lysis. All three agents, caspofungin (CSP), micafungin (MCF), and anidulafungin (AND), exhibit concentration-dependent fungicidal activity against most species of *Candida*. These antifungals have generally favourable safety and tolerability profiles with adequate pharmacokinetics and few drug interactions. The use of echinocandins for prophylaxis and treatment has been expanding, and more than 60% of candidemia patients are now reported to receive an echinocandin (10). In treatment with echinocandins, there are many changes in cellular and cell wall composition in *Candida* spp. treated with these antifungals, not only the increase in cell wall chitin content, but also the decrease in β-glucans and the upregulation of cell surface proteins (11).

1.2.1. Antifungal susceptibility

High rates of morbidity and mortality associated with fungal infections have to do with the current limited antifungal options and the rise of antifungal drug resistance. Resistant strains can be either primary resistant strains that are inherently less prone to a specified antifungal agent or secondary resistant strains that attain a resistance feature following drug exposure in an otherwise sensitive strain (12). Antifungal susceptibility testing (AFST) is important for resistance surveillance, epidemiological studies and for comparison of the in vitro activity of new and existing agents (13). MICs are defined as the lowest drug concentration resulting in a significant reduction of growth (usually either 50% or 90% reduction compared with growth in the absence of the drug). This is the reference method for AFST. MIC breakpoints are MICs at which an organism should be considered susceptible, intermediate, or resistant in relation to a certain antifungal (14). Two organizations, the European

Committee on Antibiotic Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI), have standardized methods to perform AFST and developed breakpoints of some antifungals to *Candida* spp.

1.2.2. Mechanisms of antifungal resistance

Various mechanisms of antifungal resistance have been identified and for the most part they are involved with reduced intracellular drug accumulation, counter action of the drug effect and decreased target affinity. The presence of these mechanisms depends on the mode of action of the antifungals.

1.2.2. Echinocandin resistance mechanisms

Resistance to echinocandins remains relatively low, at <3% (15). The exception is *Candida glabrata*, in which echinocandin resistance is rising and there is cause for alarm as many isolates show cross-resistance to azole antifungal agents (16) (17) (18). In *Candida* spp., resistance mutations occur in two highly conserved hot spot regions of *FKS* gene product, *FKS1* and *FKS2*, the catalytic subunit of (1,3)-β-D-glucan synthase. The amino acid substitutions decrease sensitivity of glucan synthase to drug by 50 to 3000-fold (19) (20), and elevate MIC values 5 to 100-fold (21) (22). The echinocandin resistance level conferred by hot spot mutations in *FKS1* or *FKS2* may also depend on the relative expression of these genes, which can vary more than 20-fold (20) (23).

1.3. Susceptibility of *Candida* spp. in Poland

Little is known on the epidemiology of *Candida* spp. in Poland. Nevertheless, the frequency of infections caused by non-*albicans* species in this country is increasing. The prevalence of non-*albicans* species increased from 12.5% to 70% in ten years. The same report showed that for 118 clinical cases of candidiasis in polish hospitals the mortality rate was 8.5% (24). Regarding the distribution of *Candida* spp. in Poland, a total of 302 cases of candidemia were reported over two years in 20 polish hospitals. *C. albicans* was the most isolated species, accounting for 50.96% of isolates, followed by *C. glabrata* and *C. parapsilosis* with frequencies of 14.10% and 13.14% respectively, and the distribution of *C. tropicalis* and *C. krusei* was at 6.73% and 6.41%. Other *Candida* spp. comprised of 8.65% of the isolates (25).

Regarding echinocandin susceptibility in Poland, there are two reports from 2008 on CSP susceptibility testing performed with Etests on isolates collected in polish hospitals and the examined *Candida* isolates were susceptible to echinocandins (26) (27). Another three reports from 2012 (28), 2014 (29) and 2015 (30) had similar results. In 2015 a study performed in the Medical University of Lublin in Poland presented data that showed that most of the studied clinical isolates (90%) showed sensitivity to MCF, while 10% of isolates (*C. tropicalis* and *C. famata*) were resistant to MCF, with MIC values > 32 mg/L (31).

1.5. Cell wall of *Candida* spp. as a target for antifungal research

The cell wall is an essential and highly dynamic fungal structure that has been implicated in several physiological processes. To better understand at molecular level the organization of the fungal cell once drug resistance has been established, the cell wall represents a first choice as this compartment constitutes the barrier between yeast and host. In addition, its absence in mammalian cells makes it an ideally attractive target in antifungal research (32). Studies for identification of cell wall proteins (CWPs) that change their expression in resistance strains may be useful to determine biological markers associated to drug resistance. A suitable strategy to study modulation of cell wall proteins in resistant strains and clinical isolates is the analysis of gene expression that can give a better picture of the differential expressed proteins between sensitive and resistant strains.

1.5.1. *FKS* gene and upregulation of genes encoding cell wall proteins

As previously mentioned, *FKS1* and *FKS2* encode catalytic subunits of the glucan synthases that are responsible for synthesis of (1,3)-β-glucan in the cell wall. Reports describe that in Saccharomyces cerevisiae the deletion mutant *fks1Δ* reduces the glucan content of the cell wall and this results in an increase in the chitin content (also described for *Candida* spp. (33)), upregulation of the *PST1* gene and activation of the expression of *CWP1*, which encodes glycosylphosphatidylinositol (GPI)-dependent cell wall protein (34) (35). The induction of transcription of *CWP2* gene occurs around the same time as *CWP1*, and might therefore be brought about by similar mechanisms (36). These cellular responses have been regarded as compensating for cell wall damage in order to maintain cell wall integrity.

2. Materials and Methods

2.1. Strains

A total of 78 *Candida* strains of six different species (48 *C. albicans*, 18 *C. glabrata*, 5. *C. palmioleophila*, 3 *C. krusei*, 3 *C. parapsilosis*, 1 *C. inconspicua*) were used during the set of experiments. These strains were isolated, between the years of 2008 to 2012, from patients of four polish hospitals: Children's Memorial Health Institute in Warsaw (CZD), Medical University of Gdansk, Pomeranian Medical University in Szczecin and Wrocław Medical University. The isolates originated from a variety of clinical specimens, isolated from swabs of the mouth, throat, faeces, urine, blood, and bronchopulmonary lavage fluid.

2.2. Antifungal susceptibility assays in *Candida* spp.

A total of 30 *Candida* spp. (21 *C. albicans*, 2 *C. glabrata*, 1 C. *inconspicua*, 1 *C. krusei*, 5 *C. palmioleophila*) were submitted to ASFT. Broth microdilution testing was performed using RPMI 1640 medium, inoculum of 2.5 x 10^5 CFU/mL and incubation at 35°C. MIC values were determined visually after 24 h of incubation as the lowest concentration of drug that caused a complete growth inhibition. Control strains were used, *C. albicans* ATCC 90028 and *C. krusei* ATCC 6258.

2.3. Genotyping and PCR fingerprinting

PCR fingerprinting was performed to distinguish between clinical isolates of related species (37). A single primer was used in PCR to amplify DNA sequences from 70 isolates (40 *C. albicans*, 18 *C. glabrata*, 5 *C. palmioleophila*, 3. *C. parapsilosis*, 3 *C. krusei*, 1 *C. inconspicua*). The primer used encoded for the FKS gene and the primer sequence was 5' TTGACTTTGTCTTTAAGATCC 3'. The goal was that each species could be identified by a distinct species-specific multilocus pattern, allowing species identification for all clinical isolates.

2.3.1. DNA isolation

DNA extraction was performed by taking a small fragment of mycelium and resuspending it in 100 µl of extraction buffer (60 mM $NaHCO_3$, 250 mM KCl and 50 mM Tris buffer, pH 9.5), followed by 10 min incubation at 95°C. Next, 100 µl of neutralization buffer was added (2% bovine serum albumin). After vortex mixing DNA-containing solution was stored at 4°C for subsequent analysis (38).

2.3.2. PCR reaction

The components needed to perform the PCR reaction were the following: 10 µL of PCR Mix (A&A Biotechnology, Poland), 0.2 µL of primer, 7.8 µL of sterile water and 2 µL of DNA. PCR Mix is an optimized ready to use standard PCR mixture containing High Fidelity Taq DNA polymerase, PCR buffer, $MgCl_2$ and dNTPs. Mix also contains

red dye and loading buffer. These additives enable direct loading of PCR products on agarose gel upon completing the PCR.

Table 1 – Stages of PCR and respective temperature (°C), hold (s), number of cycles and brief description.

Stages	Temperature (°C)	Hold (s)	Cycles
Initialization	95	300	-
Denaturation	95	45	35
Annealing	40	120	
Extension	72	120	
Final Elongation	72	600	-

PCR products were detected on 1.5 % agarose gel stained with ethidium bromide, a DNA-binding dye. Optimal conditions for electrophoresis were 110 V during 1 h. DNA fragments of different sizes formed bands on the gel which was visible under UV light.

To better understand the relation between these patterns and the phylogenetic relations between species, it was necessary to clone and sequence the interest bands. The bands were cut from the gel and the DNA material isolated using the Gel-Out kit (A&A Biotechnology, Poland). After, NeqSSB polymerase was used to remove AAA adducts from the ends of the PCR product. The DNA was then purified, using the Clean Up kit (A&A Biotechnology, Poland). The cloning vector chosen to carry the genes was pUC19. It is a small, high-copy number *Escherichia coli* plasmid cloning vector with multiple cloning sites.

2.3.3. Medium

Medium's used on this experiment were lysogeny broth (LB), also known as LB medium and LB agar (LA) which is LB broth that contains agar.

2.3.4. Ligation and transformation

The cloning vector chosen to carry the genes was pUC19. It is a small, high-copy number Escherichia coli plasmid cloning vector with multiple cloning sites. The microorganism used to obtain the plasmid was *E. coli* TOP10. The cells were inoculated on 3 mL of LB for 24 h at 37°C. After, 1 mL of the overnight culture was inoculated with 50 mL LB at 37°C until an optical density at 600 nm of 0.2 (OD_{600}=0.2) was reached. The mixture was centrifuged at 4000 rpm for 10 min and the resultant cell pellet was resuspended in 50 mL of 100 mM of a solution of $CaCl_2$ and incubated on ice for 1 h. A volume of 1 µL of pUC19 solution was taken and added to 100 µL of competent cells of *E. coli* TOP10. The plasmid-cell mixture was heated (heat shock) at 37°C for 10 min, allowing the plasmid to enter the cell through the disrupted membrane. The heated mixture was then placed back on ice for 2 min to retain the plasmids inside the bacteria. LB medium was then added, 1 mL, and the mixture was incubated for 1 h at 37°C. Finally, 100 µL of the mixture was spread onto plates containing LA medium with ampicillin (AMP), isopropyl-β-D-thiogalactoside (IPTG) and X-gal. The cells were regrown by inoculation of 3 mL of LB containing AMP during 24 h at 37°C. The mixture was centrifugated and the plasmid isolated and purified using the Plasmid Mini kit (A&A Biotechnology, Poland). The plasmid was then digested by taking 20 µL of the purified plasmid solution (50 ng/µL) and adding 1 µL of the restriction enzyme SmaI, 3 µL of Tango buffer and 6 µL of sterile water. The mixture was incubated for 1 h at 37°C. The ligation between the plasmid and gene is one of the crucial steps for a successful cloning. In a tube it was added 20 µL of the digested plasmid, 20 µL of PCR product, 1 µL of ligase, 5 µL of buffer and 5 µL of ATP. The mixture was incubated at 17°C for 1 h. New competent *E. coli* cells were prepared to proceed with the plasmid transformation. The procedure was to inoculate 20 mL of LB containing tetracycline (TET) with *E. coli* TOP10 F' cells and incubated it for 24 h. 2 mL of this culture were incubated for 2 hours in a total volume of 50 mL of LB. After, the mixture was centrifuged twice. $CaCl_2$ was added, and the mixture was left to incubate on ice for 1 h. For the transformation of the plasmid containing the gene into the *E. coli* competent cells, 50 µL of pUC19 with insert solution was taken and added to 100 µL of *E. coli* competent cells. The cells were incubated with the ligation mixture for 1 h on ice. A heat shock followed with a duration of 10 min and at a temperature of 37°C. The tube was put on ice for 2 min. 1 mL of LB medium was added and the mixture was incubated for 1 h at 37°C.

2.3.5. Cloning and sequencing

In plates with LA medium, AMP, TET, IPTG and X-gal, 150 µL of the bacterial culture was spread and incubated for 24 h at 37°C. For control purposes there was also positive and negative control plates. The negative control was composed of only competent cells with no plasmid to make sure there is no contamination and the positive control contained competent cells with the pUC19 plasmid but with no insert, after growing in LB medium. The blue-white screen is a screening technique that allows for the detection of successful ligations in vector-based gene cloning. The competent cells were grown in the presence of X-gal. If the ligation was successful, the bacterial colony will be white; if not, the colony will be blue. This technique allows for the quick and easy detection of successful ligation. After the selection of the white colonies these were re-plated using the line technique, in LA medium, and left to grow overnight. LB medium was then prepared with 100 mL of TET and AMP. A sample of the colonies was mixed with 5 mL of LB and left to grow overnight. The overnight culture was subjected to centrifugation at 4000 rpm and for 10 min. The Plasmid Midi kit (A&A Biotechnology, Poland) was used to obtain the plasmid with the insert. Sequencing was carried out by Macrogen (The Netherlands). The analysis of sequences was performed on the basic local alignment search tool, BLAST (National Centre for Biotechnology Information).

2.4. Quantitative Real Time RT-PCR

Quantitative real-time reverse transcription PCR (RT-qPCR) is a method that uses the phenomenon of fluorescence resonance energy transfer using a DNA-binding dye. The expression level analysis was performed for 4 different genes associated with the cell wall and possible alterations in the *FKS1* gene: *CHS1*, *PST1*, *CWP1* and *CWP2*. The expression level was verified for 8 isolates (4 *C. albicans*, 3 *C. palmioleophila* and 1 *C. krusei*). The isolates were grown on Sabouraud agar plates for 18 – 20 hours at 30°C. Small amounts of biomass from single colonies of each tested strain were suspended in a volume of 4 mL of Sabouraud broth and incubated with continuous shaking for about 5h at 30°C to achieve optical density OD_{660}=0.6.

2.4.1. RNA isolation

The yeast cells were harvested by centrifugation and mRNA was isolated using the Total RNA Mini Plus Concentrator kit (A&A Biotechnology, Poland) with the acid phenol method (manufacturer's protocol). The isolated RNA was purified with DNase. The reaction mixture composed of 10 µl of sterile RNase-free water, 7 µl of isolated RNA solution, 2 µl of 10 X reaction buffer and 1 µl of DNase (10 U/µl) (A&A Biotechnology, Poland). Incubation followed at 30°C for 30 min and after the mixture was purified with the Total RNA Mini Plus Concentrator kit (A&A Biotechnology, Poland).

2.4.2. Reverse transcription of mRNA to cDNA

Immediately after purifying mRNA the reverse transcription reaction was carried out using TranScriba kit (A&A Biotechnology, Poland) for the synthesis of the first strand of cDNA from the mRNA template.

2.4.3. Real time PCR

Quantitative analysis of expression of the *CHS1*, *PST1*, *CWP1*, *CWP2* and reference *ACT1* genes was performed by real-time PCR with the LightCycler Nano PCR Real-Time System (Roche, Switzerland). The reaction solution was a mixture of 10 µl of RealTime 2 X HS-PCR Master Mix Probe (Taq DNA polymerase 0.1 U/µl , 2 X reaction buffer, $MgCl_2$ 10 mM, dNTPs 0.5 mM, A&A Biotechnology, Poland), 1 µL of each primer solution (10 µM), 0.5 µl of probe solution (10 µM), 1 µL of total cDNA sample, and distilled water up to the final volume of 20 µl.

Table 2 – Stages of real time RT-qPCR and respective temperature (°C), hold (s), change in temperature from one PCR step to another over time (ramp, °C/s) and number of cycles.

Stage	Temperature (°C)	Hold (s)	Ramp (°C/s)	Cycles
Hold	95	300	5	-
3-Step Amplification	95	10	5	35
	56	15	4	
	72	15	5	
Hold	72	300	5	-
Melting	60	20	4	
	95	20	0.1	
Hold	40	600	5	

2.4.4. Relative quantification: Livak's method

Quantitative analysis of the relative level of expression of the investigated genes was carried out by using the $2^{-\Delta\Delta C_T}$ method, also called the Livak's method (39).

The method allows determination of the relative differences in the expression level of analysed target genes and a reference gene. In order to normalize the results, the expression level of a calibrator is used. For this research, the *C. albicans* 2023 strain isolated at Wrocław Medical University was selected as a calibrator. The level of expression of target genes in each strain was determined based on comparison of C_T values of amplification of gene of interest and the reference gene – internal control, in this case, *ACT1*. Equation 3 shows the final form of the $2^{-\Delta\Delta C_T}$ equation (39). This form of the equation may be used to compare the gene expression in two different samples. To reach Equation 3 it is needed to have the expression of the gene of interest in a given yeast (C_T gene of interest), expression of the same gene but in the strain used as calibrator (C_T calibrator) and the expression of an internal control gene (C_T reference gene).

$$\Delta C_T \text{ reference} = C_T \text{ gene of interest} - C_T \text{ reference gene} \quad (1)$$

$$\Delta C_T \text{ calibrator} = C_T \text{ gene of interest} - C_T \text{ calibrator} \quad (2)$$

$$2^{-\Delta\Delta C_T} = \Delta C_T \text{ reference} - \Delta C_T \text{ calibrator} \quad (3)$$

$$R = 2^{-\Delta\Delta C_T} \quad (4)$$

$$\text{Expression level} = \log_2(R) \quad (5)$$

The Livak's equation value of parameter R = 1 indicates that the level of the target gene expression in the investigated sample (strain) and internal control gene are the same. A value greater than 1 indicates a higher level of expression of the tested gene in the cells of the investigated strain in comparison to the cells of the calibrator, whereas a significant increase in the level of the gene expression is considered to have occurred when the value of the parameter R is higher than 2.

3. Results and Discussion

3.1. Susceptibility of *Candida* spp.

The results presented are a subset of a larger collection. The detailed procedure, results and discussion of the complete collection is described by the work of Martyna Mroczyńska and Anna Brillowska-Dąbrowska (2019) (40).

Table 3 – Minimum inhibitory concentration (MIC) distributions for anidulafungin (AND), caspofungin (CSP) and micafungin (MCF) for 21 *C. albicans*, 2 *C. glabrata*, 1 *C. insconspicua*, 1 *C. krusei* and 5 *C. palmioleophila* isolates. The CLSI breakpoint classification is attributed to each respective MIC and isolate (S: Susceptible; R: Resistant).

Species	Strain	MIC value (mg/L)			CLSI classification		
		AND	CSP	MCF	AND	CSP	MCF
C. albicans	26	0.125	0.125	0.063	S	S	S
C. albicans	40	0.031	0.063	0.016	S	S	S
C. albicans	49	0.125	0.125	0.063	S	S	S
C. albicans	114	0.063	0.063	0.063	S	S	S
C. albicans	125	0.031	0.016	0.016	S	S	S
C. albicans	185	0.063	0.016	0.063	S	S	S
C. albicans	266	0.008	0.016	0.016	S	S	S
C. albicans	286	0.063	0.250	0.031	S	S	S
C. albicans	299	0.008	0.008	0.016	S	S	S
C. albicans	374	0.008	0.031	0.008	S	S	S
C. albicans	378	0.008	0.031	0.008	S	S	S
C. albicans	380	0.008	0.031	0.008	S	S	S
C. albicans	387	0.008	0.008	0.008	S	S	S
C. albicans	389	0.008	0.031	0.008	S	S	S
C. albicans	395	0.008	0.031	0.008	S	S	S
C. albicans	1010	0.008	0.031	0.016	S	S	S
C. albicans	1296	0.008	0.016	0.016	S	S	S
C. albicans	1768	0.008	0.031	0.016	S	S	S
C. albicans	2023	0.008	0.016	0.016	S	S	S

C. albicans	2029	0.008	0.031	0.016	S	S	S
C. albicans	2608	0.008	0.031	0.008	S	S	S
C. glabrata	373	0.031	0.063	0.016	S	S	S
C. glabrata	468	0.500	0.500	1.000	R	R	R
C. inconspicua	1444	4.000	4.000	4.000	-	-	-
C. krusei	102	4.000	4.000	4.000	R	R	R
C. palmioleophila	4	4.000	4.000	4.000	-	-	-
C. palmioleophila	368	0.500	1.000	1.000	-	-	-
C. palmioleophila	370	0.008	0.016	0.008	-	-	-
C. palmioleophila	377	0.008	0.016	0.008	-	-	-
C. palmioleophila	405	0.500	4.000	0.500	-	-	-

All isolates of *C. albicans* analysed were considered susceptible to all echinocandins. *C. krusei* 102 was classified as resistant. There are reports of low susceptibility of *C. krusei* to echinocandins (41) (42) and mutations in resistant isolates of *C. krusei* were reported in the HS1 region of the *FKS1* gene (43). *C. glabrata* 468 was classified as resistant. This species has also been reported as echinocandin resistant and its resistance mechanisms are related with mutations in the *FKS1* and *FKS2* genes. Contrary to *C. albicans* and other *Candida* spp., in *C. glabrata* resistance to echinocandins is more severe, common and often presents as multidrug resistance (44) (45). There are no echinocandin breakpoints established for the rare species. The high MIC value of 4 mg/L was observed for one of the isolates of *C. palmioleophila*, strain 4. Two strains, 368 and 370, had MIC values ≤ 0.016 mg/L. The other two isolates had a different MIC value depending on the examined echinocandin. Strain 405 presented a very high MIC value for CSP of 4mg/L. A 2011 study that analysed the susceptibility profile of *C. palmioleophila* indicated that the clinical isolates of the isolates were highly susceptible to echinocandins (AND and MCF with MICs ≤0.03 mg/L) (46). *C. inconspicua* has been described as susceptible to echinocandins with values between 0.002 – 0.25 mg/L (47). The identified isolate of *C. inconspicua*, for all three echinocandins, had a MIC value of 4 mg/L. The emergence of strains with much higher MIC values than what has been previously recorded might mean that these *Candida* spp. are developing resistant mechanisms against echinocandins. These results have shown that echinocandin resistance of *Candida* isolates is an ongoing problem, especially in Poland and within non-*albicans* species given the low echinocandin susceptibility for isolates of *C. palmioleophila*, *C. inconspicua, C. krusei* and *C. glabrata*.

3.2. Identification PCR patterns and sequencing of discriminatory bands

3.2.1. Patterns obtained for tested Candida spp.

The results for PCR fingerprinting are shown in Figures 1 to 6.

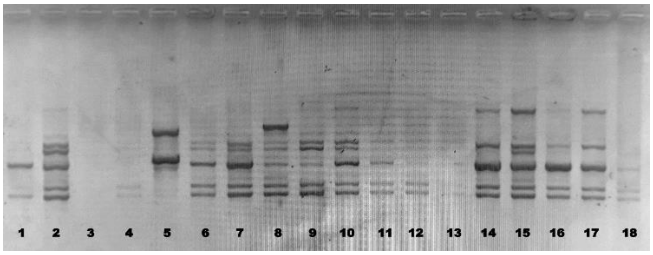

Figure 1 – Agarose gel electrophoresis (1.5% agarose) of PCR amplified products using species-specific PCR primer D. Lanes: 1 – *C. albicans* 40; 2 – *C. albicans* 72; 3 – *C. glabrata* 127; 4 – *C. parapsilosis* 101; 5 – *C. glabrata* 2235; 6 – *C. albicans* 142; 7 – *C. albicans* 26; 8 – *C. albicans* 79; 9 – *C. albicans* 49; 10 – *C. albicans* 117; 11 – *C. albicans* 51; 12 – *C. albicans* 444 ; 13 – *C. albicans* 17; 14 – *C. albicans* 16; 15 – *C. albicans* 34; 16 – *C. albicans* 33; 17 – *C. albicans* 24; 18 – *C. albicans* 38.

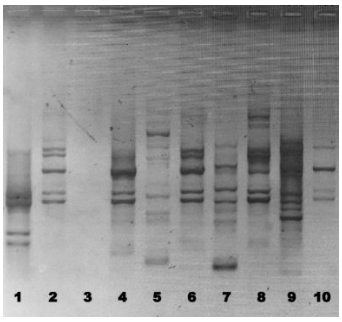

Figure 2 – Agarose gel electrophoresis (1.5% agarose) of PCR amplified products using species-specific PCR primer D. Lanes: 1 – *C. albicans* 114; 2 – *C. inconspicua* 1444; 3 – *C. palmioleophila* 377; 4 – *C. albicans* 2208; 5 – *C. krusei* 9; 6 – *C. palmioleophila* 4; 7 – *C. albicans* 266; 8 – *C. palmioleophila* 368; 9 – *C. albicans* 2029; 10 – *C. albicans* 286.

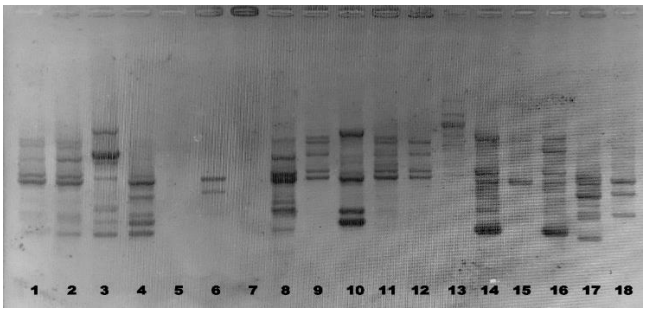

Figure 3 – Agarose gel electrophoresis (1.5% agarose) of PCR amplified products using species-specific PCR primer D. Lanes: 1 – *C. albicans* 472; 2 – *C. albicans* 2048; 3 – *C. glabrata* 1104; 4 – *C. albicans* 185; 5 – *C. glabrata* 82; 6 – *C. glabrata* 118; 7 – *C. glabrata* 81; 8 – *C. albicans* 109; 9 – *C. albicans* 2023; 10 – *C. albicans* 99; 11 – *C. albicans* 561; 12 – *C. albicans* 52; 13 – *C. krusei* 268; 14 – *C. albicans* 388; 15 – *C. albicans* 2204; 16 – *C. parapsilosis* 441; 17 – *C. glabrata* 468; 18 – *C. glabrata* 1150.

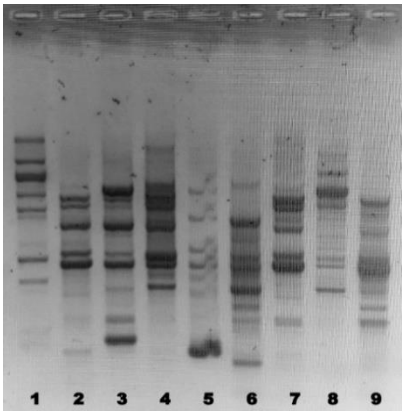

Figure 4 – Agarose gel electrophoresis (1.5% agarose) of PCR amplified products using species-specific PCR primer D. Lanes: 1 – *C. krusei* 9; 2 – *C. palmioleophila* 4; 3 – *C. albicans* 1296; 4 – *C. palmioleophila* 405; 5 – *C. albicans* 1027; 6 – *C. albicans* 1010; 7 – *C. palmioleophila* 368; 8 – *C. krusei* 102; 9 – *C. albicans* 125.

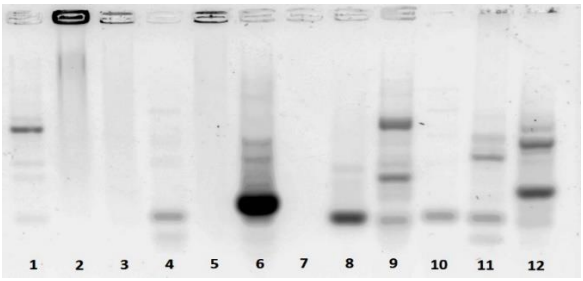

Figure 5 – Agarose gel electrophoresis (1.5% agarose) of PCR amplified products using species-specific PCR primer D. Lanes: 1 – *C. glabrata* 260; 2 – *C. glabrata* 273; 3 – *C. glabrata* 513; 4 – *C. albicans* 299; 5 – *C. glabrata* 316; 6 – *C. glabrata* 365; 7 – *C. albicans* 366; 8 – *C. palmioleophila* 370; 9 – *C. glabrata* 373; 10 – *C. palmioleophila* 377; 11 – *C. parapsilosis* 381; 12 – *C. albicans* 387.

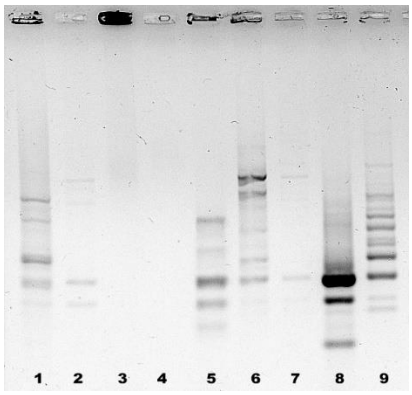

Figure 6 – Agarose gel electrophoresis (1.5% agarose) of PCR amplified products using species-specific PCR primer D. Lanes: 1 – *C. glabrata* 276; 2 – *C. glabrata* 240; 3 – *C. albicans* 89; 4 – *C. albicans* 71; 5 – *C. albicans* 50; 6 – *C. albicans* 395; 7 – *C. glabrata* 2181; 8 – *C. glabrata* 31; 9 – *C. albicans* 391.

The primer used was designed for the *FKS* gene, a gene that is directly related to echinocandin resistance for *Candida* spp. It is possible that strains with low susceptibility to echinocandins have disruptions on this gene. This would be consistent with the fact that the patterns would vary according the existence of these disturbances. The MIC values can provide further information if disruptions on the *FKS* gene can be identified through PCR and predict susceptibility. Unpublished MIC information was used only for consulting and was not included in the previous in section 3.1 because they are part of a previously mentioned study by Martyna Mroczyńska and Anna Brillowska-Dąbrowska (2019).

C. palmioleophila strains presented a few differences between them. It was possible to obtain discernible patterns for strains 4, 368 and 405. This was not the case for strains 370 and 377. There is a clear difference between these two sets, and it could mean that there is a difference between the *FKS* genes of the strains of the two sets. Given the MIC information strains 370 and 377 have the lowest MIC values of all *C. palmioleophila* (≤0.016 mg/L), much lower than strain 4, 368 and 405 (≥0.5 mg/L) (Table 3). Regarding *C. albicans* patterns, the most common and distinguishable pattern is shown for 20 strains. Other patterns associated with this species included: many bands (11 strains) and only one band (3 strains). Other patterns/no pattern at all were also verified (6 strains). Table 4 shows the different *C. albicans* strains and correspondent patterns. When compared to the results from section 3.1 there was no clear relation between susceptibility and patterns. When it comes to the common band pattern, MICs range between 0.008 – 0.25 mg/L for AND, 0.016 – 0.25 mg/L for CSP and 0.016 – 0.063 mg/L for MCF. For the many bands pattern, MICs range between 0.008 – 1 mg/L for AND, 0.016 – 4 mg/L for CSP and 0.008 – 1 mg/L for MCF. For the only one band pattern, MICs range between 0.008 – 0.031 mg/L for AND, 0.008 – 0.250 mg/L for CSP and are all 0.016 mg/L for MCF. Finally, for other patterns, MICs range between 0.008 – 0.063 mg/L for AND, 0.008 – 0.125 mg/L for CSP and 0.008 – 0.063 mg/L for MCF. The ranges presented include unpublished results. It is interesting to notice that the only two resistant strains from this species, 388 and 391 (unpublished results), both presented a lot more bands than the common pattern.

Table 4 – Types of patterns obtained for different strains of *C. albicans* following PCR fingerprinting with a primer encoding for FKS gene.

Pattern	Strains
Common	16, 17, 24, 26, 33, 34, 38, 49, 51, 52, 72, 79, 117, 142, 286, 444, 472, 561, 2023, 2048
Many bands	50, 99, 109, 125, 185, 388, 391, 395, 1010, 1027, 1296, 2029
One band	40, 299, 2204
Others	71, 89, 114, 366, 387, 2208

Regarding *C. krusei*, patterns for strain 9 and 268 are similar but strain 102 presents some identifiable differences. Strains 9 and 268 have a pattern with individual and spaced bands while strain 102 displays more bands and more compressed. These differences may relate to susceptibility given that strain 102 is resistant to all three echinocandins (Table 3) and strains 9 and 268 are resistant to only CSP (unpublished results). For *C. inconspicua* 1444 the result of genotyping did not present a distinctive characteristic pattern when compared to *C. albicans* strains, in fact the pattern presented is similar to *C. albicans* common pattern. This can have implications such as, the strain has been misidentified as *C. inconspicua* or *C. albicans* and *C. inconspicua* are phylogenetically close. It is not possible though to take conclusions from this given that only one strain was analysed. Also *C. glabrata* and *C. parapsilosis,* unfortunately, did not display a common pattern or any perceivable relation between the MIC values of the strains and the obtained patterns. This does not mean that there can't be a relation between them, to verify it a bigger sample would be needed. Some of the isolates didn't display PCR product at all. This could be due to unsuccessful DNA isolation, contamination of the colonies or the PCR mixture/product, or the amount of DNA sample/primer used. Major discriminatory bands were identified for *C. palmioleophila* and *C. krusei*.

3.2.2. Discriminatory band sequencing

After visual analysis the of gels it was decided to proceed with sequencing of the bands marked in Figure 7. Two bands from *C. palmioleophila* 4 and 368 and *C. krusei* 9 and 102 were chosen to proceed with cloning and sequencing. For *C. inconspicua* 1444 one band was also selected, even given the fact that its pattern did didn't seem to be any different than the *C. albicans* most common pattern. It was, although, decided, since this is a rare yeast, to sequence a very discernible band that was displayed in order to try to get possible novel information.

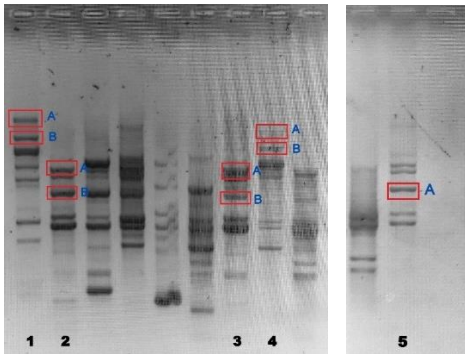

Figure 7 – Boxed bands in red represent the genes of interest chosen that were used in the cloning process from Figures 2 and 4. Lanes: 1 – *C. krusei* 9 and interest bands A and B; 2 – *C. palmioleophila* 4 and interest bands A and B; 3 – *C. palmioleophila* 368 and interest bands A and B; 4 – *C. krusei* 102 and interest bands A and B; 5 – *C. inconspicua* 1444 and interest band A.

The blue/white colony screening revealed white colonies for all the selected bands. Before sending the final product for sequencing a preliminary test was made. Using the plasmids with the insert and the plasmid without the insert electrophoresis was performed. The goal of this test was to determinate if, when compared to the plasmid without the insert, the plasmids with the insert were heavier, meaning they probably contained the gene of interest. From the 38 plasmids obtained by cloning only 7 were sent for sequencing. Unfortunately, the sequencing results did not identify any novel or known genes because most of the results did not have discernible sequences or the sequences belonged to the plasmid. The cloning process was not successful.

3.3. Expression level analysis for *CHS1*, *PST1*, *CWP1* and *CWP2* genes

Real time RT-qPCR was performed for genes *CHS1*, *PST1*, *CWP1* and *CWP2* to check their expression levels on 8 *Candida* spp. isolates (4 *C. albicans*, 1 *C. krusei*, 3 *C. palmioleophila*). Figure 8 display the expression level of the four genes for each species and strains.

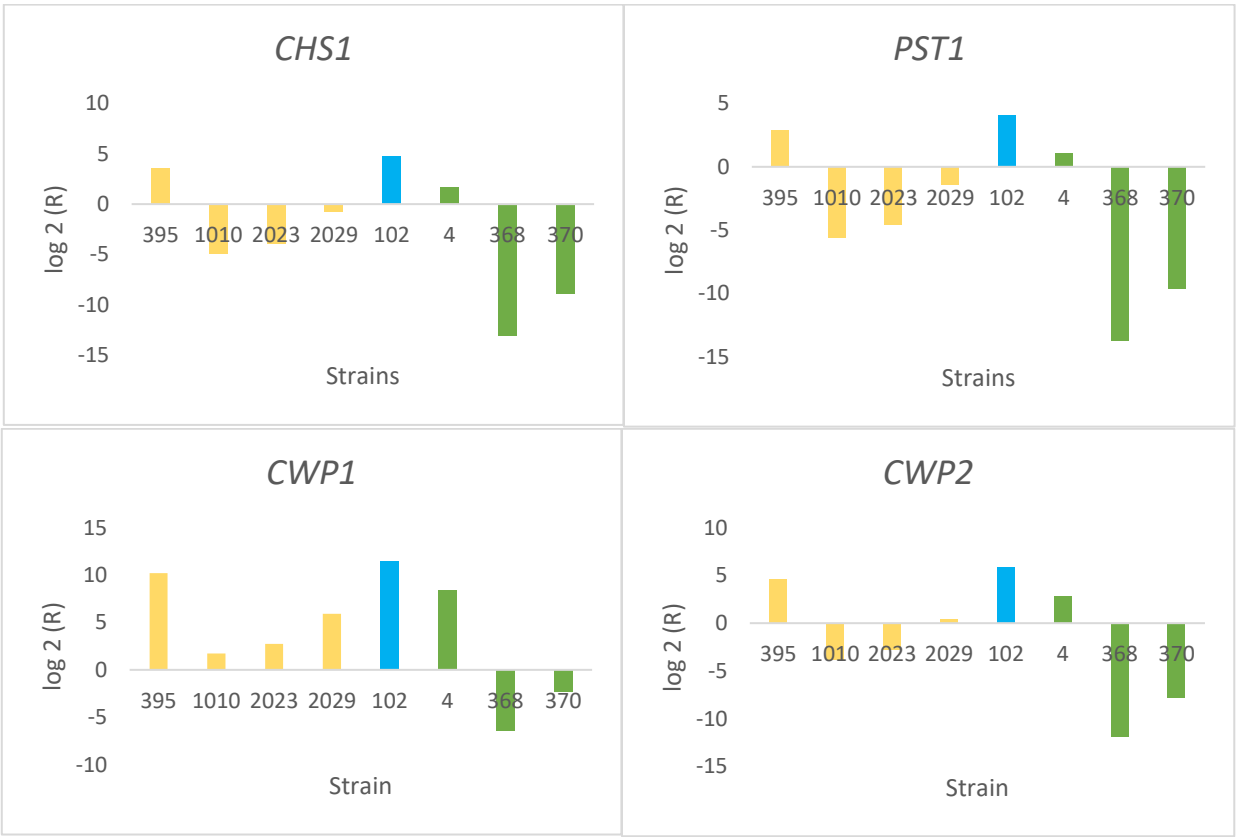

Figure 8 – Expression level of *CHS1*, *PST1*, *CWP1* and *CWP2* genes regarding 8 isolates: *C. albicans* 395, 1010, 2023 and 2029 (yellow); *C. krusei* 102 (blue) and *C. palmioleophila* 4, 368 and 370 (green), in relation to a calibrator assigned as *C. albicans* 2023.

C. palmioleophila 4 and *C. krusei* 102 have similar and high expression levels for all genes. It means the genes are upregulated in these isolates. This is an interesting comparison given that the MIC values for these two strains are the same (Table 3). *C. krusei* 102 has been considered resistant and even though that there are no breakpoints for *C. palmioleophila* the correspondent MIC value is quite high. Less susceptible strains possibly have, besides point mutations, the *FKS1* gene altered and therefore the tested genes were upregulated. On the other hand, some evidence that might support the fact that these genes are connected to alterations in *FKS1* and that they are connected to the resistance mechanism in *C. palmioleophila* is that for the other two strains, 368 and 370, their MIC values are low compared to strain 4, meaning that strains 368 and 370 have higher susceptibility to echinocandins. The genes are also downregulated in these strains, contrary to strain 4 where they are upregulated.

C. albicans 1010 and 2023 share resemblance between their MIC values and expression levels. *CHS1*, *PST1* and *CWP2* show low expression levels and *CWP1* high expression levels. For *C. albicans* 2029 though, *CWP2* also shows high expression levels. *C. albicans* 395, has all genes with high expression levels. It seems that there is not a consensus between the *C. albicans* strains and their level of gene expression. The MIC values of the 4 *C. albicans* isolates are the same for AND. For CSP, strain 2023 is the only strain presenting a lower MIC value and for MCF the MIC value for strain 395 is lower than the others. All the strains, as mentioned before, are classified as susceptible to echinocandins (Table 3). Given this analysis and the fact that the expression levels for strain 395 are much higher than of the other *C. albicans* strains, it seems that there is not an

evident connection between the studied genes and *C. albicans* resistance to echinocandins. It could mean though that the *FKS1* gene is altered in this strain, but it does not present the point mutations that confer resistance to echinocandins. However, since there are no representatives of resistant *C. albicans* strains in this study, it is not possible to draw definite conclusions.

4. Conclusions and Perspectives

The results from the AFST showed that frequency of non-albicans Candida spp. is increasing and so are their less susceptible strains. The fact that there are still no breakpoints for rare yeasts such as *C. palmioleophila* and *C. inconspicua* does not allow the classification of strains with high MIC levels as resistant. However, the elevated MIC values and previous information from literature suggests that resistance mechanisms have been evolving in these species. *C. krusei* and *C. glabrata* strains were classified as resistant to all three echinocandins. Resistant strains and their mechanisms of resistance for these species have been described before but this is not common for *C. krusei*. In terms of epidemiology in Poland though these results are important and highly informative given that they are the first to describe the emergence of non-*albicans Candida* with low susceptibility to echinocandins in the country.

PCR fingerprinting using the primer encoding the *FKS* gene, is proposed as a simple, reliable and highly reproducible diagnosis tool that allows to visualize and identify the different species of *Candida*. It is also fast and cost-effective. *C. albicans* displayed a unique pattern for most of its strains that allowed for a quick visual identification. There were no evident connections between patterns and susceptibility, within the studied sample of isolates but it is worth noting that resistant strains (unpublished results) seemed to fit in within the many band pattern. In the future, it would be interesting to analyse the patterns and ASFT results of a larger collection containing susceptible and resistant strains. Results were particularly interesting for *C. palmioleophila*. The strains of this species displayed an exclusive pattern with discriminatory bands and the patterns seemed to differ according to the susceptibility of the strains regarding echinocandins. This is important information given that the primer used was designed for the *FKS* gene. Unfortunately for *C. inconspicua* it was not possible to find a distinctive pattern since it displayed a pattern similar to the common pattern of *C. albicans*. Two possibilities are that *C. inconspicua* was misidentified or this species and *C. albicans* are phylogenetically close. The expected result was that the pattern would be more like *C. krusei* given that *C. inconspicua* is more closely related to this species. *C. krusei* strains displayed an exclusive pattern with major discriminatory bands. There seems to be a connection between patterns and susceptibility given that the pattern belonging to the resistant strain had a lot more bands than the other two strains resistant to only CSP (unpublished results). This was however a very small sample of this species. More strains should be added in the future to confirm this connection. *C. glabrata* and *C. parapsilosis*, unfortunately, did not display an identifiable pattern or any correlation with the ASFT. Once again, the sample and susceptibility range for this species was limited, so in future assays it would be necessary to widen these variables to extract more significant results. In this study it was not possible to conclude the genotyping process and demonstrate if PCR fingerprinting data reflects phylogenetic relationships between a given set of isolates because it was not possible to successfully clone the discriminatory bands.

The results from real time RT-qPCR show that the relation between possible alterations on the *FKS1* gene and the upregulation of *CHS1*, *PST1*, *CWP1* and *CWP2* is verified for *C. palmioleophila* 4 and *C. krusei* 102, two isolates with low echinocandin susceptibility – meaning that the *FKS1* gene is probably altered in these isolates, besides possible point mutations that confer echinocandin resistance. Since there are no breakpoints for *C. palmioleophila* there is also the possibility though that the levels of expression might relate to stress adaptation responses resulting in high MIC values. It can mean that strains have developed drug tolerance which is nothing less than an intermediate stage for development of resistance and just as worrisome. On the other hand, given the upregulation of the genes in *C. albicans* 395 it is possible that the *FKS1* gene is altered in this isolate. *C. albicans* 395 is classified as susceptible but the levels of expression of all genes match the ones for *C. palmioleophila* 4 and *C. krusei* 9, which means that besides the *FKS1* gene being altered in this isolate there are no point mutations that confer echinocandin resistance. It would be necessary to repeat the study and add a resistant strain of *C. albicans* to reach conclusions regarding this species.

The results of these set of experiments have shown that amongst *Candida* spp., there are important connections between cell wall dynamics and resistance to antifungal drugs. It may not be surprising that such a connection exists, given that most antifungal agents target the fungal membrane or cell wall. It has also shown that *Candida* spp. are developing resistance mechanisms that decrease their susceptibility, creating a higher risk for clinical infections. The impact that these strains may have in the clinical setting is an ever-growing concern. This could be associated with poorer clinical outcomes for patients and breakthrough infections during antifungal treatment and prophylaxis, and increased healthcare costs. This is particularly important when it comes to echinocandins that, as previously mentioned, are currently on the front line of antifungal treatment. Echinocandin resistance among *Candida* spp. is uncommon, except with *C. glabrata* where high level resistance is reported (often associated with azole resistance). The rise in resistant strains against these antifungals is worrisome given the limited treatment options for *Candida* infections. It is important to continue to try to understand how the mechanisms of resistance and the cellular responses to maintain cell wall integrity work in order to develop new strategies to fight these infections.

5. Acknowledgments

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