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Regular paper

First report on echinocandin resistant Polish *Candida* isolates

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Purpose*: Candida* spp. are ranked as one of the four major causative agents of fungal infections. The number of infections caused by *Candida* species resistant to fluconazole, which is applied as the first line drug in candidiasis treatment, increases every year. In such cases the application of echinocandin is necessary. Echinocandin susceptibility testing has become a routine laboratory practice in many countries due to the increasing frequency of clinical failures during treatment with these drugs. Methods: We performed anidulafungin, micafungin and caspofungin susceptibility testing according to the microdilution broth method on 240 *Candida* isolates collected in Polish hospitals. Results: We identified 12 isolates resistant to all echinocandins within 240 examined isolates. Moreover, 6 of the examined samples were identified as rare *Candida* species and among them we observed very high echinocandin MIC values. Conclusion: Our research proves that in Poland there is a problem of echinocandin resistance. Moreover, we identified two species of *Candida* which are rare causative agents of human infections, and there was no reported incidence of such infections in Poland until now.

Key words: *Candida* infections, echinocandin resistance, minimal inhibitory concentration, *C. palmioleophila*, *C. inconspicua*

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Abbreviations: AND, anidulafungin; *C.*, *Candida*; CLSI, Clinical and Laboratory Standards Institute procedure; CSP, caspofungin, GRASO, CHROMagar Candida; ITS, Internal Transcribed Spacer; MCF, micafungin; MIC, Minimal Inhibitory Concentration

INTRODUCTION

Candida spp. are ranked as the fourth leading causative agent of fungal infections in intensive care units (Sanguinetti *et al.*, 2015). About 90% of these infections are caused by *Candida (C.) albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* (Sanguinetti *et al.*, 2015). So far, the most prevalent pathogen during candidaemia that was isolated has been *C. albicans*. According to the clinical practice guidelines, fluconazole and echinocandin are the first line drugs in empiric therapy in case of *Candida* infections (Pappas *et al.*, 2015). The echinocandin group consists of three compounds: anidulafungin (AND), caspofungin (CSP) and micafungin (MCF). The choice of the appropriate antimycotics is related to the patient`s condition, as well as the type of infection. However, an increase in the number of fungal infections caused by non-*albicans* species, such as *C. glabrata* or *C. krusei*, showing natural resistance to fluconazole (Choi *et al.*, 2009), is the reason for the application of echinocandins. Infections caused by *C. glabrata* are now the second most common cause of candidaemia in North America and Europe (Pappas *et al.*, 2015), and result in increased mortality rates in patients with candidaemia (Cornely *et al.*, 2014). The frequency of echinocandin resistance among *Candida* spp. differs depending on the species, the region of infection and the patient (Grossman *et al.*, 2014). Studies conducted in different countries have shown a variety of *C. albicans* resistant to echinocandin. According to Castanheira *et al.*'s research, echinocandin resistance among *C. albicans* is at approximately 3% (Castanheira *et al.*, 2010). However, echinocandin resistance among *C. glabrata* seems to be a serious problem. Studies conducted from 2001 to 2010 had shown an increase in resistance from 2-3% to more than 13% among the *C. glabrata* strains (Perlin, 2015).

A report from 2015 made in Italy in accordance with the Clinical and Laboratory Standards Institute procedure (CLSI) has shown the resistance to AND (2.7%), CSP (16.2%) and MCF (13.5%) among *C. glabrata* isolates (Montagna *et al.*, 2015). So far, there has been no information about clinical isolates being resistant to echinocandin in Poland. The frequency of non-*albicans* infections in Poland is increasing. The mortality of patients with candidiasis was 8.5%, in 118 clinical cases of infections in Polish hospitals (Dzierzanowska-Fangrat *et al.*, 2014). Research conducted in 2013 at 20 Polish hospitals based on a two years period, reported 302 cases of candidaemia. The highest number of infections was found in intensive care (30.8%) and surgical (29.5%) units, whereas hematological units reached 15.9%, and the lowest number of infections was seen in neonatological units (4.6%). The most frequent isolated species was *C. albicans* (50.96%). The frequency of *C. krusei* and *C. tropicalis* was at 24% and 18%, respectively, in the hematology units. The distribution of *C. glabrata* and *C. parapsilosis* was at 14.1% and 13.1%, and there was no statistically significant differences between the departments (Nawrot *et al.*, 2013). The results, published in 2008, 2012, 2014 and 2017, had shown that according to the results of E-tests there were no any non-*Candida* isolates resistant to caspofungin and micafungin (Szymankiewicz & Dancewicz, 2008; Wieczorek *et al.*, 2008; Kurnatowska *et al.*, 2012; Golaś *et al.*, 2014; Sulik-Tyszka *et al.*, 2017).

MATERIALS

In this study we identified and examined AND, CSP and MCF susceptibility of 240 *Candida* isolates, collected in four Polish hospitals in Gdansk, Szczecin, Warsaw and Wroclaw, between the years of 2008 to 2012. The isolates originated from a variety of clinical specimens, for example isolated from swabs of the mouth, throat, faeces, urine, blood, and bronchopulmonary lavage fluid.

Table 1. *In vitro* echinocandin susceptibility test results of 240 isolates of *Candida* spp.

Cumulative no. of isolates susceptible at a MIC [mg/l] of:													
MIC breakpoint[18] [mg/l]				**137 isolates of *C. albicans***									
	S	I	R	≤**0.008**	0.016	0.031	0.063	0.125	0.25	0.5	1	2	≥**4**
AND	≤0.25	0.5	≥1	79	23	14	11	4	1	2	3	–	–
MCF	≤0.25	0.5	≥1	28	69	22	9	3	–	3	3	–	–
CSP	≤0.25	0.5	≥1	2	24	34	28	33	7	3	5	–	1
MIC breakpoint[18] [mg/l]				**72 isolates of *C. glabrata***									
	S	I	R	≤**0.008**	0.016	0.031	0.063	0.125	0.25	0.5	1	2	≥**4**
AND	≤0.12	0.25	≥0.5	3	10	32	13	5	–	4	4	1	–
MCF	≤0.12	0.25	≥0.5	7	31	19	3	3	1	–	7	1	–
CSP	≤0.06	0.12	≥0.25	–	2	7	22	22	10	2	5	–	2
MIC breakpoint[18] [mg/l]				**17 isolates of *C. krusei***									
	S	I	R	≤**0.008**	0.016	0.031	0.063	0.125	0.25	0.5	1	2	≥**4**
AND	≤0.25	0.5	≥1	–	2	3	11	–	–	–	–	–	1
MCF	≤0.25	0.5	≥1	–	1	–	–	12	3	–	–	–	1
CSP	≤0.25	0.5	≥1	–	–	–	–	–	1	2	13	–	1
MIC breakpoint[18] [mg/l]				**8 isolates of *C. parapsilosis***									
	S	I	R	≤**0.008**	0.016	0.031	0.063	0.125	0.25	0.5	1	2	4	8
AND	≤2	4	≥8	–	–	–	–	1	–	2	4	–	–	1
MCF	≤2	4	≥8	–	–	–	–	1	–	–	6	–	–	1
CSP	≤2	4	≥8	–	–	–	–	1	–	2	1	1	2	1
				6 other isolates (5 *C. palmioleophila* and 1 *C. inconspicua*)									
Lack of MIC breakpoint				0.008	0.016	0.031	0.063	0.125	0.25	0.5	1	2	≥**4**
				2	–	–	–	–	–	2	–	–	2
				2	–	–	–	–	–	1	1	–	2
				–	2	–	–	–	–	-	1	–	3

METHODS

All isolates were cultured on CHROMagar Candida (GRASO) medium and incubated for 48 h at 35°C. For the species identification, ITS1, 5.8S RNA, ITS4 (White *et al.*, 1990) regions was amplified and then sequenced. DNA extractions were performed according to an earlier described procedure (Brillowska-Dąbrowska *et al.*, 2013). 2x Master Mix HighGC (A&A Biotechnology) was applied for all of the PCR assays performed. PCR products were purified (Clean-up, A&A Biotechnology) and sequenced (Macrogen).

Table 2. *In vitro* echinocandin susceptibility test results of 6 rare isolates of *Candida* spp.

Species	Number of isolates	Place of isolation	MIC value [mg/l] of:		
			AND	MCF	CSP
C. inconspicua	1444 W	–	4	4	4
C. palmioleophila	4 W	–	4	4	4
C. palmioleophila	368 S	sputum	1	0.5	1
C. palmioleophila	370 G	blood	0.008	0.008	0.016
C. palmioleophila	377 G	liver cysts	0.008	0.008	0.016
C. palmioleophila	405 G	mouth	0.5	0.5	4

[1]W, clinical sample isolated from a patient at Wroclaw hospital; [2]S, clinical sample isolated from a patient at Szczecin hospital; [3]G, clinical sample isolated from a patient at Gdansk hospital

Sequence analysis was performed with VectorNTI (Informax).

Minimal Inhibitory Concentrations (MIC) were determined by broth microdilution and the results were read visually following 24 h incubation, as the lowest concentration of the drug that caused a complete growth inhibition. Also, *Candida albicans* ATCC 90028 and *Candida krusei* ATCC 6258 strains were used as controls. All tests were performed in triplicates and in case of discrepancies they were repeated. AND (Pfizer), CSP (Sigma-Aldrich), MCF (Astellas) were obtained as a standard powder.

RESULTS

Among 240 *Candida* samples, by sequencing an rRNA fragment we identified: 137 *C. albicans,* 72 *C. glabrata* 17 *C. krusei,* 8 *C. parapsilosis* and 6 strains belonging to two rare *Candida* species: 5 *C. palmioleophila* and 1 *C. inconspicua* strain. CHROMagar Candida correctly identified 93.4% *C. albicans*, 97.2% *C. glabrata*, 80% *C. krusei* strains. *C. palmioleophila* developed a turquoise color on CHROMagar, while *C. inconspicua* colonies were pink to violet.

Results of three echinocandins susceptibility examination tests are presented in Table 1. Among 137 *C. albicans* isolates, as many as 3 had shown a significant decrease in susceptibility to AND, 6 to CSP and 3 to MCF (minimal inhibitory concentration value for all echinocandins ≥1 mg/L); 2 isolates were intermediately resistant to AND, 3 to CSP, and 3 to MCF. In general, only 3/137 (2.2%) isolates of *C. albicans* were resistant to all echinocandins.

Out of 72 *C. glabrata* isolates, as many as 9 had shown a significant decrease in susceptibility to AND, 19 to CSP and 8 to MCF (MIC values: ≥0.5 mg/l, ≥0.5 mg/l, ≥0.25 mg/l, respectively). Only 1 isolate was intermediately resistant to MCF and 22 to CSP, (MIC value ≥0.125 mg/l; ≥0.25 mg/l). Only 7 isolates were resistant to all three echinocandins.

In the case of *C. krusei* we observed a decrease in CSP susceptibility of 14/17 isolates. However, these isolates were sensitive to AND and MCF. According to the echinocandin mechanism of action and well known technical problems with establishing MIC for CSP, it is unlikely that such a large percentage of isolates would show resistance only to one antibiotic from this group. Thus, these *C. krusei* isolates were probably not resistant to echinocandins because they were neither resistant to AND nor MCF. We identified only 1 isolate which was resistant to three echinocandins (MICs value ≥4 mg/L for all echinocandins).

Among 8 *C. parapsilosis* we identified one resistant isolate to all echinocandins (MIC values ≥8 mg/l).

The MIC values of rare species of *Candida* were very high, but there is no echinocandin breakpoint established for these species (probably due to the low frequency of occurrence). The MIC value ≥4 was observed for one isolates of *C. palmioleophila*, and the same MIC value for the three echinocandin is exhibited by *C. inconspicua*. Two isolates of *C. palmioleophila* had MIC values ≤0.016 mg/l. The two isolates had a different MIC value depending on the examined antimycotics. The results of echinocandin susceptibility testing of these rare *Candida* isolates are listed in Table 2.

DISCUSSION

Epidemiological studies on *Candida* infections are conducted in many countries (Choi *et al.*, 2009). Various data are available on the prevalence of resistance to echinocandins among fungi of the *Candida* genus. These studies report that the occurrence of resistant isolates varies depending on the site of infection and the patient population. Previous epidemiological studies on resistance of *Candida* spp. in Poland are an insufficient source of data. There are two reports (Szymankiewicz & Dancewicz, 2008; Wieczorek *et al.*, 2008) from 2008 on caspofungin susceptibility testing performed with E-tests on isolates collected in the Polish hospitals. All of the 29 and 93 examined *Candida* isolates were susceptible to echinocandins. Another two reports from 2012 and 2014 had shown that there were no resistant *Candida* isolates within the 10 and 150 specimens collected in the Polish hospitals (Kurnatowska *et al.*, 2012; Gołaś *et al.*, 2014). The latest echinocandin susceptibility testing was performed with E-tests in 2017. Only 46 isolates were examined and echinocandin resistance was not found (Sulik-Tyszka *et al.*, 2017).

Our research has shown that the echinocandin resistance of *Candida* isolates is a problem in Poland, especially within non-albicans species – 9.7% *C. glabrata* isolates were echinocandins resistant (7/72). Echinocandins susceptibility testing had shown that out of all the 240 isolates of *Candida* spp., 14 (5.8%) were resistant to AND; 40 (16.6%) to CSP, and 13 (5.4%) to MCF.

What is very interesting, we isolated 6 isolates belonging to two species that are rarely identified as a cause of human infections. *C. inconspicua* is described in the

literature as a fluconazole resistant and amphotericin B susceptible and is isolated from immunocompromised patients (Baily *et al.*, 1997; Sugita *et al.*, 2004; Guitard *et al.*, 2013; Majoros *et al.,* 2005). We identified one isolate of *C. inconspicua* which was characterized by very high echinocandins MIC.

Out of 5 *C. palmioleophila* isolates, 3 were characterized by high echinocandins MIC value. According to a variety of data, *C. palmioleophila* could be resistant to fluconazole and susceptible to other antimycotics, e.g. echinocandins (Liu *et al.*, 2017; Meletiadis *et al.*, 2016), but there is also some information about elevated caspofungin MIC of *C. palmioleophila* (Brilhante *et al.*, 2017). *C. palmioleophila* were found in animal microflora (Sokół *et al.*, 2018) and there are only a few data available on *C. palmioleophila* as an etiological agent of human infections (Trouvé *et al.,* 2017).

It should be emphasized that data on previous echinocandins exposure (type and duration of antifungal therapy of patients) of the isolates examined in our study are not available. However, this does not change the fact that we indicate the problem of echinocandin resistance in Poland. Moreover, as the number of infections caused by *Candida* species resistant to fluconazole which is applied as the first line drug in candidiasis treatment in Poland increases, the occurrence of echinocandins resistance within *Candida* isolates should be examined.

Declaration of interest

The authors report no conflicts of interest.

Ethics approval

This study was exempt for ethics board approval as patient-specific public health information was not collected.

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