ORIGINAL PAPER

Prevalence and Antifungal Susceptibility of the Emerging Fungal Species, *Candida nivariensis*, Isolated in a Teaching Hospital in Poland

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A b s t r a c t

The data on susceptibility to antifungals of new species within *Candida glabrata* complex are limited. Our study was to enrich a global knowledge of yeast epidemiology and drug resistance. The study was focused on the identification of species within clinical isolates of the *C. glabrata* complex and on the determination of their resistance to antifungals. Four hundred forty-five clinical *C. glabrata sensu lato* strains were isolated from different clinical samples at routine mycological exams at the Infant Jesus Teaching Hospital in Warsaw. The identification of the most of tested isolates to species complex level was performed using the ID 32 C system. The identification of *C. nivariensis* and *C. bracarensis* species within the *C. glabrata* complex was performed by DNA sequencing. The MICs of amphotericin B, fluconazole, itraconazole, posaconazole, voriconazole, caspofungin, anidulafungin, and micafungin were determined by E-test. Twenty-four isolates did not have an ITS-1 region, characteristic of *C. glabrata sensu stricto* and their D1/D2 regions of the 26S rRNA were 99% homologous to *C. nivariensis* 26S rRNA. No strains of *C. bracarensis* were recovered. *C. nivariensis* strains were very susceptible to amphotericin B, anidulafungin, micafungin, and caspofungin. Ninety-two percent of *C. nivariensis* were resistant to itraconazole. The halves of the strains was resistant to posaconazole. Eighty-three percent of *C. nivariensis* were susceptible to voriconazole. None of the tested strains were susceptible to fluconazole. In the present study, none of the *C. nivariensis* strains were simultaneously resistant to azoles and echinocandins. *C. nivariensis* should be recognized as an emerging pathogen, resistant to azoles.

Key words: *Candida glabrata* complex, *Candida nivariensis*, emerging pathogen, resistance to azoles

Introduction

Non-*albicans Candida* (NAC) yeast-like fungi play a more active role in fungal infections. *Candida glabrata*, one of the most important yeast-like fungi of this group, is the second most common cause of candidiasis.

Distribution of *C. glabrata* varies depending on the geographical area. Relatively high incidence of *C. glabrata* was observed in the northern part of Europe and in the USA in contrary to southern countries of Europe and Latin America where *C. parapsilosis* infections are more often found.

The global prevalence of *C. albicans* is decreasing, in contrary to *C. glabrata* and *C. krusei*, which remain stable. The incidence of *C. parapsilosis* and *C. tropicalis* is increasing. It was also demonstrated that *C. glabrata* is more often isolated from elderly patients (Falagas et al. 2010; Alexander et al. 2013; Guinea 2014).

The reason for the change in the profile of *Candida* infection remains unknown, however, an increase in NAC infections, especially *C. glabrata*, seems to be attributable to unreasonable antifungal prevention (Basetti et al. 2009; Gołaś et al. 2014). *C. glabrata* candidemias are on the increase (Quindós 2014). In ten years

(since 1989) *C. glabrata* candidemias have increased from the fourth to the second most common cause of ICU infections (Lockhart et al. 2012).

Nowadays, the *C. glabrata* species complex has been identified including three closely related new species: *C. glabrata sensu stricto*, *C. nivariensis*, and *C. bracarensis* (Alcoba-Flórez et al. 2005; Miranda-Zapico et al. 2011).

C. nivariensis and *C. bracarensis* represented less than 5% within the *C. glabrata* complex (Bishop et al. 2008; Lockhart et al. 2009; Sharma et al. 2013). *C. nivariensis* was more frequently isolated than *C. bracarensis*. These species could become the new sources of opportunistic infections. However, the mycological routine diagnostics cannot differentiate between *C. glabrata sensu stricto*, *C. bracarensis*, and *C. nivariensis*. It can only classify these species as a part of the *C. glabrata* species complex. A complete species differentiation within the complex may be achieved with molecular biology techniques, which can identify closely related yeast-like fungi (Angoulvant et al. 2016). In order to assess the clinical impact of the new species within the *C. glabrata* species complex, it is important to determine their virulence factors. Virulence factors enable yeast-like fungi to begin and carry out the subsequent stages of an infection. Amongst the most important pathogenicity determinants of *C. glabrata sensu lato* is their ability to adhere to abiotic and biotic surfaces, to form biofilm on both surfaces, and to secrete phospho- and lipases, hemolysins, and other cytotoxic enzymes. (Tamura et al. 2007; Silva et al. 2012; Rodrugues et al. 2014).

Little is known about the drug resistance of the *C. glabrata* species complex. Some studies suggest that the new species within the complex may be more resistant to antifungals than *C. glabrata sensu stricto* (Tamura et al. 2007; Silva et al. 2012; Li et al. 2014, Rodrugues et al. 2014; Angoulvant et al. 2016). *C. nivariensis* and *C. bracarensis*, two new species recently discovered, exhibit many traits common to *C. glabrata sensu stricto*, but are isolated less frequently. Despite being detected more commonly, these two species are still unknown and require further investigation.

This study was to identify the clinical species within the *C. glabrata* complex and to determine their resistance to antifungals.

Experimental

Materials and Methods

Four hundred forty-five clinical *C. glabrata sensu lato* strains were isolated from different clinical samples (urine – 154, tracheal aspirate – 63, throat swab – 44, sputum – 34, feces – 32, peritoneal fluid – 27, skin ulcer – 26, vagina – 18, post-surgical wound drainage – 12,

bile – 11, blood – 7, bronchoalveolar lavage – 7, other – 10) at routine mycological exams at the Infant Jesus Teaching Hospital in Warsaw in the years from 2014 to 2016. All samples were collected from adult patients. The samples were cultured following routine microbiological diagnostic guidelines on Sabouraud agar and incubated at 30°C for 24–72 h until representative single colonies were formed. The ID 32 C yeast identification system (bioMérieux, France) was used for species identification within the complexes.

Species identification within *C. glabrata* complex. The isolation of genomic DNA of the *C. glabrata* complex isolates was performed using the Gene MATRIX Bacterial and Yeast Genomic DNA Purification Kit (EurX, Poland) following the manufacturer's guidelines.

***C. nivariensis* and *C. bracarensis* identification within the *C. glabrata* complex.** There were two stages of species identification within the *Candida glabrata* complex. In the first stage, the internal transcribed spacer 1 (ITS-1), characteristics of *C. glabrata sensu stricto* was identified using PCR. The NL-1 (5'-GCAT ATCAATAAGCGGAGGAAAAG') and NL-4 (5'-GGT CCGTGTTTCAAGACGG') primers were used for the amplification. The amplification were performed at following conditions: initial denaturation 94°C for 10 min, followed by 30 cycles of denaturation at 94°C for 30 sec., annealing at 50°C for 1 min, elongation at 72°C, 30 sec., and then final elongation at 72°C for 10 min. In the second stage the DNA was sequenced. Strains without the ITS-1 amplicon specific for *C. glabrata* had the D1/D2 region of the 26S rRNA sequenced. The NL-2A (5'-CTTGTTCGCTATCGGTCTC') and NL-3A (5'-GAGACCGATAGCGAACAAG') primers were used for sequencing (Kurtzman et al. 2003). The sequencing results were analyzed with the BLAST (the Basic Local Alignment Search Tool) software to compare the nucleotide sequences obtained with the reference sequence databases and calculate the statistical significance.

The resistance of *C. nivariensis* to antifungals. E-test (bioMérieux, France) was used on RPMI agar; the minimal inhibitory concentrations (MIC [μg/ml]) for amphotericin B (AMB), fluconazole (FLU), itraconazole (ITC), posaconazole (POS), voriconazole (VOR), caspofungin (CAS), anidulafungin (ANF), and micafungin (MCF) were measured. MIC results (S – susceptible and R – resistant) of FLU, AMB, CAS, MCF, and ANF were interpreted following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (EUCAST 2018). In details, the interpretation for antifungals MICs was as follows: AMB ≤ 1 susceptible, > 1 resistant, FLU ≤ 0,002 susceptible, MCF ≤ 0,032 susceptible, > 0,032 resistant, ANF ≤ 0,064 susceptible, > 0,064 resistant, CAS – isolates that are susceptible to anidulafungin as well as

micafungin should be considered susceptible to caspofungin. MIC results (S – susceptible and R – resistant) of ITC, POS, and VOR the CLSI guidelines were used (CLSI 2008). In details, the interpretation for antifungals MICs was as follows: ITC ≤ 0,125 susceptible, > 1 resistant, POS ≤ 1 susceptible, > 4 resistant, VOR ≤ 1 susceptible, > 1 resistant.

Results

Species identification within the new *C. glabrata* complex. Four hundred forty-five strains were identified as *C. glabrata* and analyzed using PCR. For twenty-four isolates (5.4% of all strains) the ITS-1 amplicon characteristic of *C. glabrata sensu stricto* was not observed and their D1/D2 regions of the 26S rRNA were sequenced. They contained sequences 98% homologous to *C. nivariensis* 26S rRNA. A representative BLAST analysis of the sequenced D1/D2 regions of the 26S rRNA is shown in Fig. 1. Based on the sequencing results, no strains of *C. bracarensis* were identified. Table I presents the source of isolation of *C. nivariensis* and the drug susceptibility results.

Drug susceptibility of *C. nivariensis*. The strains of the new species were isolated from various clinical samples, including primarily sterile specimens. Their sensitivity to antifungals varied.

C. nivariensis strains were all susceptible to amphotericin B, anidulafungin, micafungin, and caspofungin. The MIC50 and MIC90 for amphotericin B were 0.19 mg/l and 0.5 mg/l respectively, and the MIC50 and MIC90 for caspofungin were 0.19 mg/l and 0.19 mg/l respectively. The $MIC_{50} = 0.012$ mg/l, $MIC_{90} = 0.016$ mg/l, and micafungin $MIC_{50} = 0.008$ mg/l, $MIC_{90} = 0.023$ mg/l strains also had low anidulafungin.

Forty-one percent of *C. nivariensis* were resistant to itraconazole with the MIC in the range of 1.5–32 mg/l. The half of the strains (50%) was resistant to posaconazole with the MIC of 1.5–32 mg/l. Eighty-three percent of *C. nivariensis* were susceptible to voriconazole (the MIC in the range of 0.008–2.0 mg/l). All of the strains tested were intermediate-susceptible or resistant to fluconazole with the MIC in the range from 0.25 to 256 mg/l.

Discussion

C. glabrata candidemias are on the increase, as it has been suggested in the literature due to the overuse of fluconazole treatments (Tapia et al. 2012; Colombo et al. 2013; Quindós 2014). There is little information on the isolation of *C. nivariensis* and *C. bracarensis*. These are estimated to constitute 0.2–4.0% of the *C. glabrata* complex (Bishop et al. 2008; Lockhart et al. 2009, Sharma et al. 2013). In the present study, *C. nivariensis* was more frequently isolated and made up for 5.4% (24 strains). Sharma et al. (2013)assessed 100 *C. glabrata* isolates and found five *C. nivariensis* strains. Chowdhary et al. (2010) analyzed 366 *C. glabrata* complex strains and established that two of them were *C. nivariensis*. Li et al. (2014) conducted a study on 301 isolates of *C. glabrata* complex cultured from vulvovaginal candidiasis cases. They found seven isolates of *C. nivariensis*. Similarly, the drug resistance of the new species within the *C. glabrata* complex is not well known. As there are very few species in the world identified as *C. nivariensis* or *C. bracarensis,* the information about their drug resistance profile is very scarce. Li et al. (2014) showed that all *C. nivariensis* isolates were susceptible to nystatin and susceptible or susceptible dose-dependent to fluconazole, itraconazole, miconazole, and clotrimazole. The authors did not provide the MICs values of the antifungals tested.

Both species were susceptible to amphotericin B; their MIC was not higher than 1.0 μg/ml. The international data on *C. nivariensis* and *C. bracarensis* resistance to azoles is different. The MIC values for azole (especially fluconazole) was high against some strains what may suggest that they could have the same resistance mechanisms as *C. glabrata sensu stricto*. Previous studies suggested that the MIC values for echinocandin against *C. nivariensis* and *C. bracarensis* were low. None of these strains was proved resistant to this medication (Angoulvant et al. 2016).

C. glabrata sensu lato may nowadays be resistant to amphotericin B. In 1993 Pfaller et al. discovered 25% of *C. glabrata sensu stricto* with MIC > 1 μg/ml (Pfaller et al. 2004). More and more often the MIC value for amphotericin B against *C. nivariensis* was ≥ 0.5 μg/ml (Angoulvant et al. 2016). In the present study, both *C. glabrata sensu stricto* and *C. nivariensis* were susceptible to amphotericin B.

The *C. nivariensis* strains evaluated in the present study were resistant to fluconazole (100%), itraconazole (41.7%), posaconazole (50%), and voriconazole (17%). One of the few studies of the British Reference Laboratory conducted by Borman et al. (2008) performed on 16 clinical *C. nivariensis* isolates has reported similar results regarding *C. nivariensis in vitro* sensitivity to azoles. However, in contrary to their findings on the resistance to voriconazole and posaconazole (MIC_{50} at 4 mg/l and 1 mg/l, respectively), in the present study only 17% of the strains were resistant to voriconazole and 50% to posaconazole (MIC_{50} at 0.125 mg/l and 0.75 mg/l, respectively).

Other studies also suggested high value of the MIC for fluconazole (between 16–128 mg/l) against *C. nivariensis* (Fujita et al. 2007; Borman et al. 2008;

Sharma et al. 2013). In the present study, the MIC for fluconazole ranged from 0.25 to 256 mg/l.

In our opinion, all strains that had the MIC above 0.002 mg/l should be considered as the intermediate susceptible to fluconazole since there are no established interpretive breakpoint criteria to designate the *C. nivariensis* strain as either susceptible or resistant to fluconazole. Our opinion is supported by the EUCAST guidelines on the susceptibility to fluconazole of *C. glabrata*, which classify all stains above this value as the intermediate susceptible *in-vitro*.

In contrary to the findings of the present study, Li et al. (2014) proved that *C. nivariensis* was susceptible to fluconazole and itraconazole. Also Tay et al. (2014) established that *C. nivariensis* was susceptible to fluconazole and voriconazole.

Our study results also differ from the results presented by Huo et al. (2017) who studied 12 *C. nivariensis* strains and one *C. bracarensis strain* isolated from ten Chinese hospitals, and found that all strains were susceptible to azoles but not to fluconazole. Based on these findings we believe that susceptibility to azoles may vary geographically and can be attributed to the divergent use of azole in various locations. It is worth to mention that five of our isolates with the MIC value of 256 mg/l against fluconazole showed also the highest MICs values for itraconazole, posaconazole, and voriconazole. This finding may suggest cross-resistance among azoles, similar to the one described for *C. glabrata* (Panackal et al. 2006). This has to be elucidated for *C. nivariensis*.

Echinocandins, i.e. caspofungin, anidulafungin, and micafungin, are the newest available antifungal medication. However, the number of *C. glabrata sensu lato* strains with a lowered sensitivity to echinocandins have been described in several publications (Katiyar et al. 2006; Cleary et al. 2008; Thompson et al. 2008; Garcia-Effron et al. 2009; Arendrup et al. 2013).

Pfaller et al. (2011) evaluated 215 *C. glabrata sensu lato* isolates and established that 16.7% of them were resistant to echinocandins. Low values of the MIC for echinocandin against *C. nivariensis* have already been reported. In the present study, all strains *C. nivariensis* were susceptible to echinocandins.

Our data are in concordance with the data published by Morales-López et al. (2017) regarding the strains isolated during 30 years in Argentina. They tested resistance to echinocandins of five *C. nivariensis* strains isolated from a collection of 122 *C. glabrata* complex strains. All *C. nivariensis* strains tested were resistant to echinocandins with the MICs ranging from 0.015 to 0.03 mg/l and from 0.06 to 0.13 mg/l for anidulafungin and caspofungin, respectively. Since the relatively low number of *C. nivariensis* has already been examined worldwide it is difficult to estimate the real resistance rate to echinocandins. *C. glabrata sensu*

stricto simultaneous resistance to azoles and echinocandins was observed in the past years and it is an alarming phenomenon (Pfaller et al. 2011; Alexander et al. 2013; Morales-López et al. 2017). Cleveland et al. (2015) observed a simultaneously increased resistance to azoles and echinocandins, from 1.8% to 2.6% within five years of observation. In the present study, any *C. nivariensis* strain was simultaneously resistant to azoles and echinocandins.

Since data on the epidemiology and susceptibility to antifungals of *C. nivariensis* are limited, our study may enrich the global knowledge about its epidemiology and drug resistance. Moreover, it indicates the need for proper microbiological analysis with the use of molecular methods or with the updated spectra of the matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). *C. nivariensis* should be recognized as an emerging, azoles-resistant pathogen.

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Conflict of interest
The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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