RESEARCH ARTICLE

Antifungal susceptibility and virulence factors of *Candida* spp. isolated from the genital tract of outpatients

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ABSTRACT

Vaginal yeast infections are one of the most widespread infection diseases among women. The present study is focused on the taxonomic composition, antifungal resistance, and some virulence factors of *Candida* strains isolated from samples of outpatients. During one year period from April 2016 to March 2017 the 97 *Candida* spp. strains were collected from vaginal, cervical, and urethral secrets of outpatients at IMDL "Chronolab" Plovdiv. The majority of isolates were identified as *C. albicans* (84%), followed by *Candida glabrata* (7%), *Candida krusei* (4%), *Candida parapsilosis* (3%), and *Candida tropicalis* (2%) and the most affected age group is women between 21 and 40 years old. Antifungal resistance was low and mainly associated with *C. glabrata*, with total susceptibility to the tested antifungal drugs over 95%. Analysis of the hydrolytic enzyme activities and the biofilm formation abilities showed that only 8% of the strains produced gelatinase and phospholipase, 6% produced caseinase, and 5% esterase. Seven of the tested *Candida* strains (7.2%) formed stable biofilm after 24 h cultivation in Sabouraud dextrose broth supplemented with 6% glucose. This study revealed no significant correlation between the antifungal susceptibility and the studied virulence factors of *Candida spp*. isolates from the genital tract of outpatients.

Key words: *Candida;* antifungal susceptibility; hydrolytic enzymes; biofilm formation

Introduction

Vaginal yeast infection is highly problematic for many women, especially in its recurrent form. About 40-50% of women experience at least one additional episode and 5-6% suffers from recurrent vulvovaginal candidiasis, caused by fungal opportunistic pathogens of the genus *Candida* (Swidsinski et al., 2019). *Candida* is part of the normal microbiota in many women and its presence is often asymptomatic. *C. albicans* is the most frequently isolated fungal pathogen and in certain conditions as in immunocompromised patients or after antibiotic therapy can cause superficial infections such as oral and vaginal candidiasis. Other non-albicans species like *C. glabrata*, *C. tropicalis* and *C. krusei* induce vaginitis and are more often resistant to conventional therapy (Spampinato & Leonardi, 2013). The vulvovaginal candidiasis is characterized by a replacement of vaginal microbiota with dominant species (*lactobacilli*), with species normally found in women`s genital tract such as *Gardnerella vaginalis*, *Bacteroides* spp., *Peptostreptococcus* spp., *Mobiluncus* spp. or with species that are part of natural microbiota of the vagina and gastrointestinal tract as *E. coli*, *Streptococcus spp*., *Enterococcus spp.*, etc. Candidiasis affects mainly women in reproductive age and at least one time in their life (Hedayati et al., 2015).

Symptomatic candidal vulvovaginitis and superficial infections are treated successfully with topical or oral antifungal agents such as itraconazole, ketoconazole, fluconazole, etc. The first drug of choice is mainly from the azole family due to the favorable oral bioactivity (> 90%) and the approval of the European Medicines Agency (EMEA) and the US Food and Drug Administration (FDA) for their safety profile. *Candida albicans* is highly susceptible to fluconazole. After a single oral dose of 150 mg drug, 90% of *Candida* cells are inhibit for less than 72-96 hours (Spampinato & Leonardi, 2013).

Virulence factors can be defined as all traits produced by infecting *Candida* spp. strain to establish the process of infection in the host. These virulence traits directly interact with host cells and lead to tissue damage (Haynes, 2001). Set of virulence factors such as adhesins, synthesis of hydrolytic enzymes and biofilm formation contribute to the pathogenesis of *Candida* spp. The virulence profile depends on the infecting

species, site of infection, and host reaction (Mayer et al., 2013).

Biofilms are surface-associated microbial communities and the cells are in extracellular polymeric substance (EPS) containing carbohydrates, proteins, phosphorus, and hexosamines. In *in vitro* conditions, the early phase of biofilm formation takes 11 hours and the yeast cells form microcolonies. The intermediate phase (12-30 hours) has characterized by ESP production and forming bilayer. The next phase is the maturation of the biofilm (about 38-72 hours) includes the development of a thick layer of ESP and the final phase is the dispersion of daughter yeast cells and spread the colonization/infection (Cavalheiro & Teixeira, 2018). Biofilm protects yeast cells from antifungal agents and host defense mechanisms, especially from phagocytosis of neutrophils (Mayer et al., 2013). Antifungal resistance is higher in biofilm-forming strains. For example, the minimal inhibitory concentration for fluconazole is 5 to 8 times higher in biofilm-forming cells of *C. albicans* than planktonic cells (Chandra and Mukherjee, 2015).

The aim of this study was to analyze species and age distribution, drug susceptibility, and virulence factors such as the production of hydrolases and biofilm formation abilities of fungal isolates from samples of outpatients with infections of the genital tract.

Materials and Methods

Fungal strains

The tested strains were collected from vaginal, cervical and urethral samples of outpatients at IMDL „Chronolab“, Plovdiv, Bulgaria for one year in 2016 from April 2016 to March 2017. They were isolated after cultivation on Candida Chrome Agar (HiMedia, India) and subsequently frozen at -80°C in skim milk (Merck, Germany). The phenotypic characterization of the isolates was analyzed on MICRONAUT-*Candida* panel using Multiscan FC microplate reader (ThermoScientific, China) and MICRONAUT software (MERLIN Diagnostika GmbH, Germany). *Candida albicans* ATCC 10231, *C. glabrata* ATCC 2001, *C. tropicalis* ATCC 1396 and *C. parapsilosis* ATCC 22019 were used as controls for all further analysis.

Antifungal susceptibility

The tests were performed on Mueller-Hinton agar supplemented with 2% dextrose with 0.5 µg/mL methylene blue (Giri & Kindo, 2014). In brief, 100 µL inoculum (0.5 McFarland) from each strain cultivated on Sabouraud Dextrose Agar (HiMedia, India) for 24h were inoculated by spread plate technique and disks with amphotericin B (10µg), itraconazole (10µg), fluconazole (25µg) and voriconazole (1µl) (HiMedia, India), were placed on the agar surface. After 48 hours, the zones of inhibition were measured, with *C. albicans* ATCC 10231 used as a control. The results were interpreted according to CLSI guidelines (M44-A) (CLSI, 2009).

Production of hydrolytic enzymes

The strains were tested for caseinase, gelatinase, phospholipase, and esterase activity, as described by Figueiredo-Carvalho et al. (2017). Briefly, for caseinase analysis strains were inoculated by stabbing in Sabouraud Dextrose Agar (HiMedia, India) supplemented with 1% casein. The caseinase activity was determined by measuring the diameter of the transparent halo formed around the colony as a result of the casein hydrolysis. The gelatinase activity was assessed after inoculation of gelatin deeps (Sabouraud Dextrose Broth 30 g/l, gelatin – 120 g/l; pH 6.8). After 7 days of incubation at 37°C, the test tubes were cooled to 4°C. The liquefaction of the medium is considered as a positive result. Phospholipase and esterase activities were determined after the inoculation by stabbing on egg yolk agar plate (Sabouraud Dextrose Broth 30 g/l, 0.1M $CaCl_2$, 1,5% agar and 2% fresh egg yolk) and Tween agar plate (Sabouraud Dextrose Broth 30 g/l, 1.5% agar, 1% Tween 80, 0.01% $CaCl_2$), respectively. The positive result is observed as an esterification zone (phospholipase) or halo of precipitation (esterase) around the colonies. Phospholipase, caseinase, and esterase activities were expressed as a ratio (Pz) between the diameter of the colony and the diameter of the precipitation zone. The Pz value was divided into four categories: 1.0 no enzyme activity; 0.990 to 0.700 – weak producers; 0.699 to 0.400 – good producers and lower than 0.399 – excellent producers.

Biofilm formation

Biofilm formation *in vitro* was determined as described by Deorukhkar et al. (2014). Flat bottomed microplates (Costar) were pretreated with 50% fetal bovine serum for at least 30 min at room temperature. The wells were loaded with 100µl Sabouraud Dextrose Broth (HiMedia, India) with 6% glucose, inoculated with 10 µl overnight cultures of *Candida spp*. and incubated for 24 h at 37°C. After incubation, the samples were transferred in new microplates and plankton growth was measured at 620nm. The original plates were washed three times with 0.89% saline solution and stained with 0.1% crystal violet for 15 min. The plates were washed three times with saline again, each well was loaded with 110µl 95% ethanol for 20 min and OD_{620} was measured. The cut-off value was determined by the average OD value in 4 wells with sterile medium ± SD. OD value higher than the cut-off value was considered positive.

Statistical analysis

Statistical analyses and graphical representation of the results were performed with Statistica v. 10 (StatSoft).

Results

Taxonomic composition

Ninety-seven strains *Candida* were isolated from the genital tracts of outpatients for one year from April 2016 to March 2017 – 95 vaginal isolates, 1 cervical and 1 urethral. The majority of tested strains were from outpatients of reproductive age (Figure 1). Twenty-four percent of the samples were from pregnant women and eight samples (8%) were with coinfections. In six cases coinfections were with strains *E. coli* and *Klebsiella pneumoniae* – three samples each. The other two coinfections in female samples were with *Enterococcus faecalis* and *Streptococcus agalactiae*. In male outpatients in addition to *Candida*, a strain of *Enterococcus faecalis* was isolated. The phenotypic characterization of the tested isolates confirmed their affiliation to genus *Candida* with 99.2-99.9% reliability. Taxonomic distribution is presented in Figure 2.

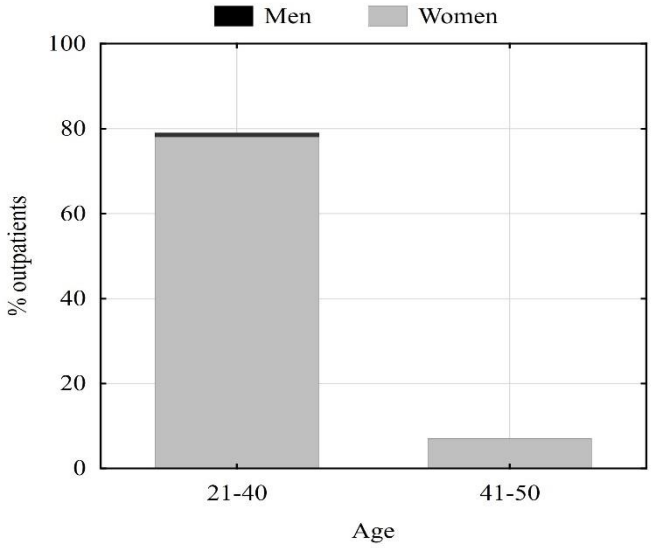

Figure 1. *Age distribution of the outpatients.*

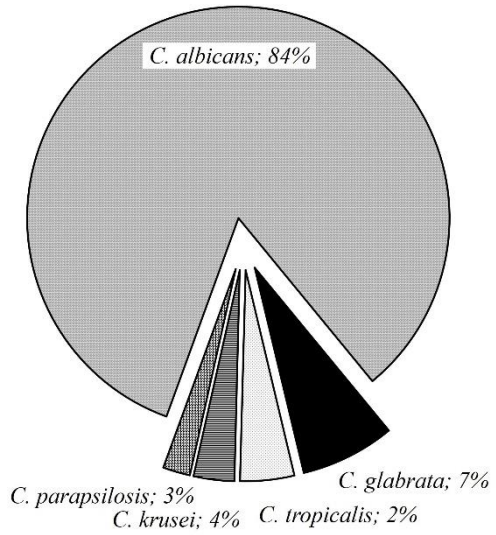

Figure 2. *Species distribution of the fungal isolates.*

Antifungal susceptibility

All strains *C. albicans* except one showed high sensitivity to amphotericin B, fluconazole and voriconazole. Only one strain *C. albicans* was susceptible-dose dependent (S-DD) to itraconazole. S-DD susceptibility to itraconazole was found in four of seven strains (57%) *Candida glabrata*, and two to voriconazole (29%). Three of *C. glabrata* strains were resistant to amphotericin B (43%) and four strains (57%) were resistant to fluconazole. None of the strains *Candida krusei*, *Candida parapsilosis*, and *Candida tropicalis* was found to be resistant to the tested antifungal agents.

Hydrolytic activities

Six of the tested 81 strains *C. albicans* (7%) could hydrolyze gelatin and casein at the same time for less than 48 hours. Two of them were good producers (Pz value < 0.699) and four were weak producers (Pz value > 0.700). Only two strains of *Candida parapsilosis* showed low gelatinase activity but could not hydrolyze casein. Only 10% of *C. albicans* strains showed phospholipase activity. Two of the tested strains showed activity with Pz value < 0.399 and were defined as excellent producers. Esterase activity was observed in five strains *C. albicans* (5/81 6%) with Pz value > 0.700 which defined them as weak producers. None of *C. glabrata*, *C. krusei* and *C. tropicalis* strains showed any of the tested hydrolytic exoenzymes activities.

Biofilm formation

Only seven strains formed biofilms (Figure 3). Weak biofilm formation was observed for capabilities showed *C. albicans* G 143 and *C. tropicalis* G 224 ($OD_{620nm} \leq 0.200$). *C. glabrata* G 227 and *C. albicans* G 102 formed good biofilm ($OD_{620nm} \leq 0.400$). The two of excellent biofilm-forming strains *C. albicans* G 98.2 and *C. albicans* US 12.2 ($OD_{620nm} \geq 0.401$) were isolated from patients with co-infections – the first with *E. coli* and the second with *E. faecalis*. Both coinfecting non-*Candida* strains were able to form weak biofilm *in vitro* as well.

We used cluster analysis and the results showed antifungal susceptibility was in separate cluster from the virulence factor associated with *Candida* spp. The antifungal drugs are divided in two small groups – fluconazole and intraconazole which are triazoles with common mechanism of action. The biofilm-forming had negative correlation with extracellular enzymes. The cluster analysis showed that antifungal susceptibility did not correlate with the investigated virulence factors (Figure 4).

Discussion

Candidiasis is generally caused by opportunistic yeast pathogens forming the normal genital microbiota. Our study showed that yeast infections of the urogenital tract of outpatients are caused generally by the genus *Candida*, with

Candida albicans being the most frequently isolated species (84%) followed by non-*albicans* species (NAC) (16%). The high *C. albicans* percentage is in agreement the findings for the European countries (Corsello et al., 2003; De Vos et al., 2005; Ahmad & Khan, 2009), while Hedayati et al. (2015), reports a predominance of NAC – *C. glabrata* (22%), *C. dubliniensis* (16.4%), *C. kefyr* (8.2%), *C. pintolopesii* (8.2%) and *C. guilliermondii* (2.7%) over the *C. albicans* in Asian countries. The number of NAC isolates in our study was significantly lower compared to the last decade of statistics (Ahmad & Khan, 2009). The study confirms that candidal infections are more typical for women and are rarely established in male samples. Female outpatients in childbirth age were the most affected age group (69%) with the rate of infection two-fold higher in pregnant vs. non-pregnant women. Such infections pose a health risk for the newborns, as they may cause preterm delivery and low birth weight (Rasti et al., 2014).

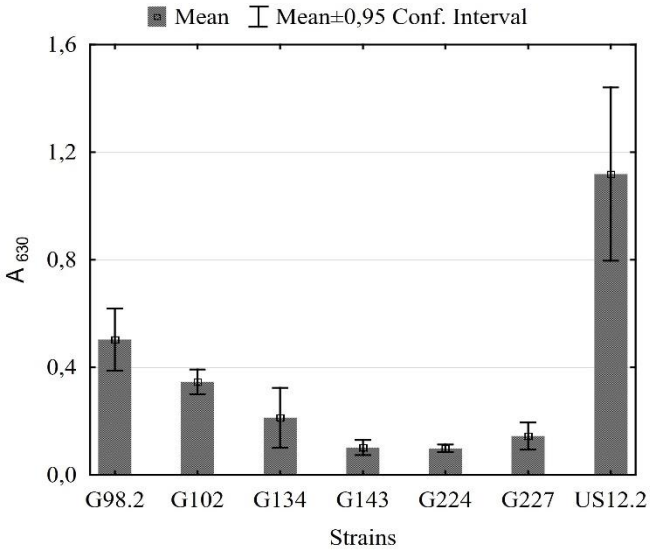

Figure 3. *Biofilm-forming capacity of Candida strains.*
Legend: *Candida albicans* – G 98.2, G 134, G143, G 227 and US 12.2; *Candida glabrata* – G 102; *Candida tropicalis* – G 224.

The antifungal susceptibility patterns showed that *C. albicans* is highly susceptible to all tested drugs (≥ 99%). Berkow and Lockhart (2017) report that *Candida* strains resistant to a drug in the azole family are common in HIV patients and less in patients with candidal vulvovaginitis. In the current study, 57% of *C. glabrata* and none of *C. albicans* were resistant to fluconazole. *C. glabrata* have the highest resistance among *Candida* species. It has been frequently isolated from patients after azole treatment (Whaley et al., 2017). Cross-resistance between the drug agents in the azole group is observed for all fluconazole-resistant *C. glabrata* and these results correlate with other studies (Müller et al., 2000; Panackal et al., 2006). Resistance to amphotericin B is rare in clinical practice, but *C. glabrata* is often considered as an intermediate resistant or S-DD to the agent. Its resistance to azoles in our study correlated with the resistance to amphotericin B, which was as high as 57%. A similar connection has been established (Ellis, 2002), who suggests that if the strain is resistant to the agents from the azole group, the MIC to amphotericin B will be high, too.

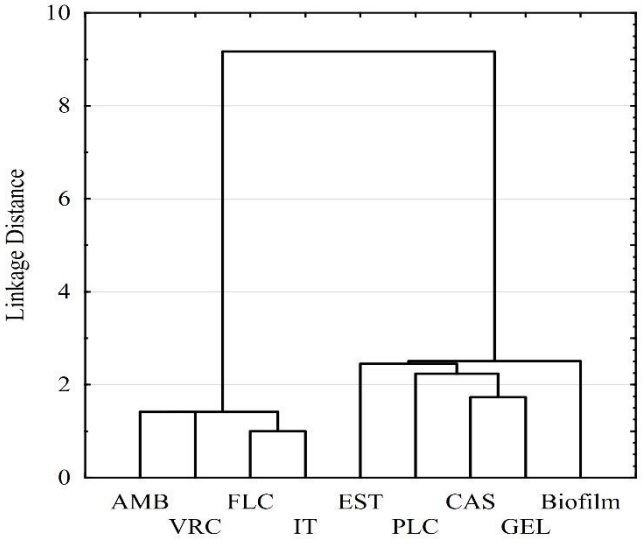

Figure 4. *Cluster analysis based on Wards linkage of the similarities between antifungal susceptibility and different virulence factors.*

The common virulence factors of *Candida spp*. include synthesis of extracellular enzymes and biofilm formation. The ability of the yeast to attached and damaged the tissue with different classes proteinases play an important role in the successful invasion of the host. Despite the results from other authors where the percentage is quite high (Deorukhkar et al., 2014; Figueiredo-Carvalho et al., 2017), we observed low proteinase activity (8% for gelatinase and 6% for caseinase production). Only two strains *C. parapsilosis* can hydrolysis gelatin but cannot casein whereas *C. albicans* isolates hydrolyzed both substrates. The lysis of the host cell is due mainly to phospholipases and these enzymes are one of the most investigated virulence factors. We found that 8% of *C. albicans* strains produced phospholipase and 6% of them were low producers of esterase. Many studies proved that the presence of these exoenzymes is not always connected with the pathogenesis of the species. For example, strains isolated from healthy volunteers have shown high phospholipase and proteinase activities, which are more likely related to the normal commensal fungi-host interactions, than to the strain pathogenesis (Oksuz et al., 2007).

Biofilm formation is considered one of the most important virulence factors because of the ability of the yeast cells to attach on a different surface, to evade the host immune

responses, and to resist to antifungal therapy (Marak & Dhanashree, 2018). In the present study, biofilm formation was found only for 7% of all *Candida* spp. Other scientists reported a higher percentage of biofilm-forming strains and prevalence for non-*albicans* isolates (Deorukhkar et al., 2014; Sariguzel et al., 2015; Tulasidas et al., 2018).

Conclusions

This study confirmed that *C. albicans* is the dominant fungal species and *C. glabrata* is the most frequent non-*albicans* isolate from vulvovaginitis. Antifungal resistance was rare and mainly associated with *C. glabrata* whereas the secretion of exoenzymes was found primarily in *C. albicans* isolates. Hydrolytic activities of the all tested strains were poorly spread and they were not related to the antifungal susceptibility. All biofilm forming strains were sensitive to antifungal drugs and could not produce any hydrolytic enzymes. In general, the tested strains demonstrate predominantly commensal characteristics. Further studies are needed to confirm the hypothesis that the fungal infection is more a result of the change in the balance of the normal microbiota. The results suggest that the success in pathogenesis depends on multiple factors combining the strain's virulence profile, the host normal microbiota composition, and the immune status of outpatients. This shapes up the uniqueness of each case of fungal infection and makes obligate to take into account potential causes for a change in the composition of the normal microbiota and to study the antifungal sensitivity of isolates before the onset of therapy avoiding empirical prescribing of antifungals.

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