

Prevalence of Closely Related *Candida albicans* Species among Patients with Vulvovaginal Candidiasis in Southern Poland Based on the *hwp1* Gene Amplification

KAROLINA KLESIEWICZ^{1*}, PAULINA MROWIEC¹, KATARZYNA KANIA¹, IWONA SKIBA-KUREK¹,
 JOANNA BIAŁECKA^{1,2}, MAGDALENA NAMYSŁ³ and MARIANNA MAŁEK¹

¹Department of Pharmaceutical Microbiology, Jagiellonian University Medical College, Cracow, Poland

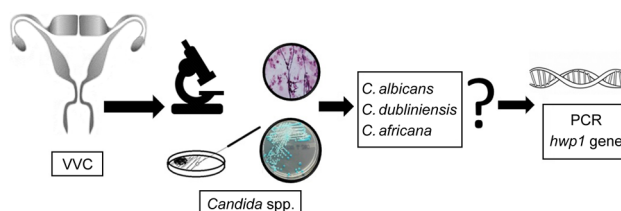
²Centre of Microbiological Research and Autovaccines, Cracow, Poland

³University Hospital, Cracow, Poland

Submitted 21 December 2022, accepted 13 February 2023, published online 24 March 2023

Abstract

Candida albicans remains the most common species isolated from women with vulvovaginal candidiasis. However, closely related species such as *Candida africana* and *Candida dubliniensis* may also occur, although they are often misidentified. The aim of the study was to confirm the phenotypic identification of *C. albicans* and its closely related species isolated from women with genital tract infections by amplification of the *hwp1* (hyphal wall protein 1) gene in a PCR assay. We report a detailed molecular identification of *C. albicans* and its closely related species among 326 patients in the Małopolska region, Poland. Initial phenotypic identifications were confirmed by amplification of the *hwp1* gene. Based on molecular analysis, we revealed 307 strains (94.17%) as *C. albicans* and 17



as *C. dubliniensis* (5.22%). No strain of *C. africana* was detected. Two patients had co-infection with *C. albicans* and *C. dubliniensis* (0.61%). A PCR assay targeting the *hwp1* gene was reliable for correctly identifying species among the *C. albicans* complex.

Key words: *Candida albicans*, *Candida africana*, *Candida dubliniensis*, vaginitis, vulvovaginal candidiasis

Introduction

Despite advances in the treatment of fungal infections, vulvovaginal candidiasis (VVC) remains a common problem among women of childbearing age worldwide (Sustr et al. 2020). This disease affects women to varying degrees, regardless of ethnic origin, and social or living conditions. This ailment can be induced by various factors ranging from host factors such as age, estrogen levels, sexual activity, exposure to stress, allergic diseases, or medications (Farr et al. 2021). However, most cases do not have an identifiable trigger. It is estimated that 70–75% of women will be diagnosed with vulvovaginal candidiasis at least once in their lifetime

(Hedayati et al. 2015), and 5–8% of adult women have recurrent vulvovaginal candidiasis (RVVC), which is defined as four or more episodes per year (Sobel 1992). VVC affects approximately 138 million women annually (in the range of 103 to 172 million), with a global annual prevalence of 3,871 per 100,000 women; 372 million women are affected by RVVC during their lifetime (Denning et al. 2018). More than 40 species of the *Candida* genus are capable of causing infection (Pfaffer et al. 2014), but *Candida albicans* remain a significant cause of VVC (Sobel and Sobel 2018). The incidence of *C. albicans* vulvovaginitis is 70% to 90%.

The taxonomy of some *Candida* species has undergone significant changes due to the development of

* Corresponding author: K. Klesiewicz, Department of Pharmaceutical Microbiology, Jagiellonian University Medical College, Cracow, Poland; e-mail: karolina.klesiewicz@uj.edu.pl

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identification techniques, primarily molecular biology. Due to difficulties in identification, the newly described related species were grouped into complexes and named cryptic yeasts. The *C. albicans* complex includes three closely related species *C. albicans* sensu stricto, *Candida dubliniensis*, and *Candida africana* (Sullivan et al. 1995; Tietz et al. 2001). *C. dubliniensis* and *C. albicans* have many phenotypic resemblances, such as the capacity to form pseudohyphae, chlamydospores on cornmeal agar, and germ tubes in serum (Moran et al. 2012). Phylogenetic studies indicate that *C. dubliniensis* is most closely related to *C. albicans* (McManus et al. 2008), and it is often difficult to discriminate between these two species in clinical samples. The second species from the *C. albicans* complex, *C. africana*, had previously been considered an atypical *C. albicans* strain. However, in 2001 it was proposed to isolate a separate species of *C. africana* that produces pseudohyphae but not chlamydospores (Tietz et al. 2001). Several other features, such as the inability to grow in 42°C, the incapacity to assimilate the aminosugars N-acetyl glucosamine and glucosamine, as well as the disaccharide trehalose, and the organic acid DL-lactate, could be helpful in proper identification, but during routine diagnostics misidentification often occurs in mycological laboratories (Sullivan et al. 2004). To avoid these drawbacks of classical techniques, molecular analyses have been applied in multiple studies to identify and distinguish between *Candida* species (Byadarahally Raju and Rajappa 2011; Neppelenbroek et al. 2014). A singleplex PCR assay using a single primer pair targeting the hyphal wall protein 1 (*hwp1*) gene encoding a mannoprotein specific for the filamentous form of *C. albicans*, has been proposed to distinguish *C. albicans* sensu stricto, *C. dubliniensis*, and *C. africana* according to the distinct size of the amplicon (Romeo and Criseo 2008; 2009).

The available clinical and epidemiological data show that *C. africana* and *C. dubliniensis* are isolated from patients all over the world; however, there are scant data on the frequency of occurrence of these species in Poland, identified as the cause of VVC. Due to limited knowledge concerning its prevalence, this study aims to confirm the phenotypic identification of *C. albicans* and its closely related species isolated from the genital tract by amplifying the *hwp1* (hyphal wall protein 1) gene in the PCR assay.

Experimental

Materials and Methods

Materials. A present study covered 326 vaginal yeast isolates, presumptively identified as *C. albicans*, by phenotypic methods. All isolates were obtained from

female patients (median 33, from 17 to 65 years) with vaginitis, diagnosed at the Centre of Microbiological Research and Autovaccines in Cracow, Poland.

The including criteria were as follows: *C. albicans* or *C. dubliniensis* in a phenotypic screening assay. Species other than *C. albicans* or *C. dubliniensis* were excluded from the analysis in this paper.

Phenotypic assay. Phenotypic analysis based on culturing methods was performed as part of routine clinical practice at the Centre of Microbiological Research and Autovaccines in Cracow, Poland.

Two (2) vaginal swabs were taken from the posterior vaginal vault, using a sterile speculum. The first vaginal swab was used for Gram staining with PREVI® Color Gram stainer (bioMérieux, France).

The second swab was used to perform cultures of aerobic bacteria (TSA with 5% sheep blood; GRASO, Poland), *Gardnerella vaginalis* (Agar Gardnerella; bioMérieux, France), and yeast-like fungi (Sabouraud dextrose agar with chloramphenicol; GRASO, Poland and chromID® *Candida*; bioMérieux, France). The media were incubated for 24 h or 48 h at 35°C under appropriate conditions. The identification of species was conducted on VITEK®2 (bioMérieux, France).

Vulvovaginal candidiasis (VVC) was diagnosed by positive *Candida* spp. cultures and microscopic observation of yeast-like cells and/or hyphae in the Gram-stained vaginal smears evaluated according to the scale developed by Kuczyńska, modified by Kasproicz. The scale assessed the presence of leukocytes, clue cells, lactobacilli, other bacteria, and fungi based on the abundance scale ranging from 0–3 in oil-immersion microscope fields (1,000×) (Kasproicz and Białecka 2012).

Initial species identification of strains was carried out based on the appearance of the colonial morphology on chromogenic medium (chromID®, bioMérieux, France). As a control of chromogenic agar, the reference strains were included: *C. albicans* ATCC® 90028™, *Candida glabrata* ATCC® 2001™, *Candida tropicalis* ATCC® 9968™ (Fig. 1A), *C. albicans* ATCC® 90028™, *C. dubliniensis* NCPF 3949 and *C. africana* MYA 2669™ (Fig. 1B).

Afterward, further identification was performed by an automatic biochemical method using the YST Card, VITEK®2 compact (bioMérieux, France) according to the manufacturer's instruction. The results were approved when the acceptable score was generated using the database system, covering 27 *Candida* genus species (with the exception of *C. africana*). The results were interpreted by the VITEK®2 database at different confidence levels as excellent identification (probability range from 96% to 99%), very good (93–95%), good (89–92%), acceptable (85–88%), low discrimination (<85%), and unidentified microorganisms. The results of yeast identification were considered acceptable when the confidence level was ≥85% probability.

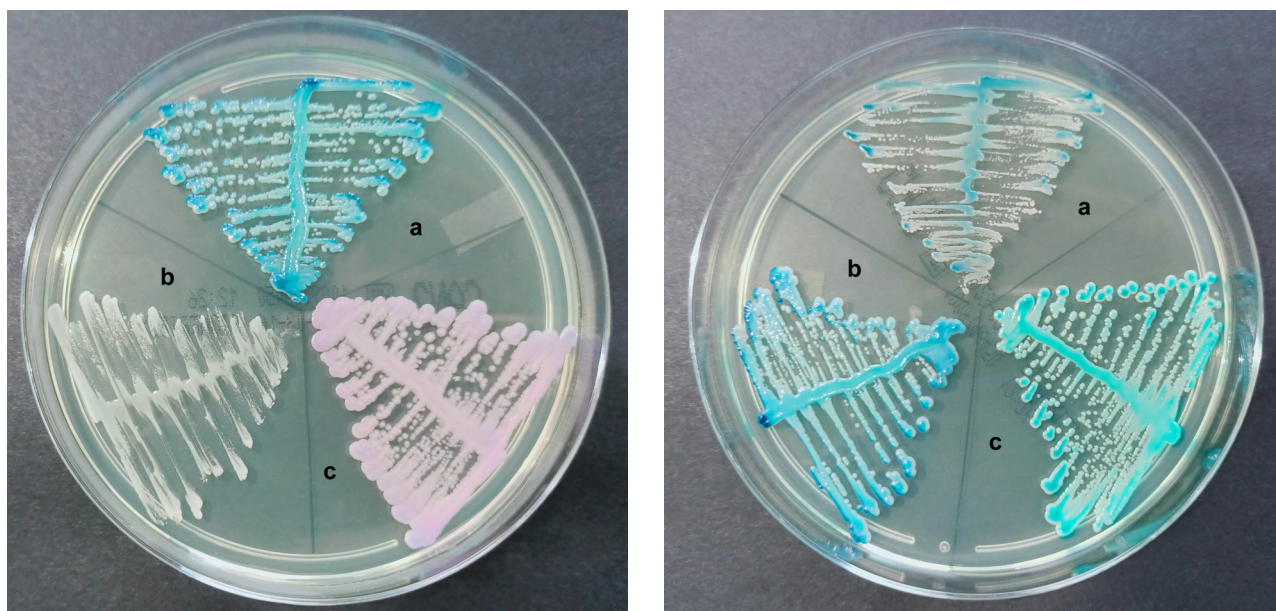


Fig. 1. Morphology of reference strains of yeasts colonies after 48 h incubation in 37°C on chromID® *Candida* (bioMérieux, France).

- A. a) *Candida albicans* ATCC® 90028™ (dark blue colonies), b) *Candida glabrata* ATCC® 2001™ (white colonies),
c) *Candida tropicalis* ATCC® 9968™ (pink colonies).
B. a) *Candida africana* ATCC® MYA 2669™ (small, light blue colonies), b) *Candida albicans* ATCC® 90028™ (dark blue colonies),
c) *Candida dubliniensis* clinical strain (turquoise colonies).

Molecular identification. The molecular tests were conducted at the Department of Pharmaceutical Microbiology of the Jagiellonian University Medical College, Cracow, Poland. Fungal DNA was extracted with the Yeast Mini isolation kit (A&A Biotechnology, Poland), according to the manufacturer's instructions.

The species identification of *Candida* isolates was performed using PCR with CR-F (5'-GCTACCACTTCAGAATCATCATC-3') and CR-R (5'-GCACCTTCAGCTGTAGAGACG-3') primers that targeted the *hwp1* (hyphal wall protein 1) gene, encoding a manoprotein specific for the filamentous form of *C. albicans*, as previously described (Romeo and Criseo 2008).

The PCR conditions were as follows: initial denaturation at 94°C for 2 min, 30 cycles of denaturation at 94°C for 15 s, annealing at 63°C for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min, in a T-Personal Thermal Cycler (Biomtra, Germany). The reaction mixture of the final volume 25 µl contained: 5 µl of GoTaq® Flexi Buffer, 0.5 µl of PCR Nucleotide Mix (10 mM each) (Promega, USA), 1 µl of each primer (Genomed, Poland), 2 µl of genomic DNA, 2 µl of MgCl₂ (25 mM), 0.125 µl of GoTaq® DNA Polymerase (5 u/µl) (Promega, USA) and Nuclease-Free Water (Promega, USA). The PCR products of the size of 941 bp, 569 bp, and 700 bp for *C. albicans*, *C. dubliniensis*, and *C. africana* respectively, were expected.

The PCR amplicons were separated by 2% agarose gel electrophoresis and visualized with ethidium bromide staining (Sigma-Aldrich Chemie, Germany).

Reference strains included *C. albicans* ATCC® 90028™, *C. dubliniensis* NCPF 3949, and *C. africana* MYA 2669™.

MALDI TOF assay. Simultaneously with the molecular assay, MALDI-TOF MS (Matrix-assisted laser desorption ionization-time of flight mass spectrometry) identification of the selected strains of *Candida* (including isolates identified by VITEK®2 at a low discrimination level) was performed using the VITEK®2 MS Biotyper (bioMérieux, France) at the University Hospital in Cracow, Poland. The identification accuracy was determined by a confidence level of > 80%, according to the manufacturer's recommendation.

In vitro antifungal susceptibility assay. A commercial assay for *in vitro* antifungal susceptibility testing Fungitest™ (BioRad, France), was used to determine the efficiency of miconazole at the concentration of 0.5 and 8 µg/ml, ketoconazole at the concentration of 0.5 and 4 µg/ml, itraconazole at the concentration of 0.5 and 4 µg/ml, and fluconazole at the concentration of 8 and 64 µg/ml. The colorimetric analysis and interpretation of the results were performed according to the manufacturer's recommendations. The reference strain *Candida parapsilosis* ATCC® 90018™ was used as a quality control to establish the reproducibility of the susceptibility testing results.

According to the manufacturer's recommendation of Fungitest™ (BioRad, France), we use the following criteria of drug susceptibility: susceptible (S), intermediate (I) (referring to "susceptible, increased exposure" according to the EUCAST recommendations (EUCAST 2020), and resistant (R).

Ethics approval. Our report is a retrospective *in vitro* study without the participation of human or animal subjects. The study materials were yeast strains collected by routine laboratory practice, not for scientific purposes, and stored in glycerol tubes at -80°C as a biobank collection. All human data associated with strains were anonymized before the researcher gained access to it. Following the guidelines of the Jagiellonian University Medical College Bioethics Committee, Cracow, Poland, this type of research does not meet the criteria of a medical experiment and is not subject to ethics review.

Results

Results of phenotypic analysis. According to a phenotypic analysis based on culture on chromogenic media, all 326 *Candida* strains included in the study were obtained from patients diagnosed with vulvovaginal candidiasis. In addition, the presence of aerobic bacteria in vaginal swabs was confirmed, such as: Gram-positive cocci (*Staphylococcus* spp.) in one patient, streptococci (group B *Streptococcus* – GBS) in 55 patients, Gram-negative bacilli (*Escherichia coli* in 13, and *Klebsiella* spp. in one patient) and colonization by anaerobic bacteria (*G. vaginalis*) in 73 patients (Table I). All women with co-occurrence of *Candida* spp. with bacteria presented reduced *Lactobacillus* spp.

Results of molecular analysis. Based on the PCR assay targeting the *hwp1* gene, preceded by MALDI TOF analysis, we confirmed the initial phenotypic identification in 307 (94.17%) samples as *C. albicans*, while 17 (5.22%) strains belonged to the *C. dubliniensis* species (Table II). Moreover, molecular identification demonstrated two co-infections (0.61%) by *C. albicans* and *C. dubliniensis*. No strain of *C. africana* was detected (Fig. 2).

Table I
Prevalence of single VVC and co-occurrence with bacteria.

Type of co-occurrence	No. (%) n = 326
Single VVC	197 (60.43)
VVC + bacteria:	129 (39.57)
<i>Gardnerella vaginalis</i>	60 (18.40)
<i>Streptococcus agalactiae</i>	41 (12.58)
<i>Gardnerella vaginalis</i> + <i>Streptococcus agalactiae</i>	11 (3.37)
<i>Escherichia coli</i>	9 (2.76)
<i>Escherichia coli</i> + <i>Streptococcus agalactiae</i>	3 (0.92)
<i>Gardnerella vaginalis</i> + <i>Escherichia coli</i>	2 (0.61)
Methicillin susceptible <i>Staphylococcus aureus</i>	1 (0.31)
<i>Streptococcus pyogenes</i>	1 (0.31)
<i>Klebsiella pneumoniae</i> + <i>Streptococcus agalactiae</i>	1 (0.31)

VVC – vulvovaginal candidiasis

Table II
Molecular discrimination among *Candida albicans* complex.

Species	No. (%) n = 326
<i>Candida albicans</i>	307 (94.17)
<i>Candida dubliniensis</i>	17 (5.21)
<i>Candida albicans</i> + <i>Candida dubliniensis</i>	2 (0.61)

Comparison of *C. albicans* complex identification results from VITEK®2, MALDI-TOF MS, and PCR assays. Phenotypic analysis based on chromogenic medium (chromID® *Candida*; bioMérieux, France) identified all clinical isolates as *C. albicans* strains. Despite slight differences in the appearance and colors of the yeast colonies (Fig. 1B), no distinction between *C. albicans* sensu stricto, *C. dubliniensis*, and *C. africana* species was possible according to the manufacturer’s recommendations.

The reference method for this study found as “a gold standard”, was molecular identification based on the *hwp1* gene amplification, which confirmed that all

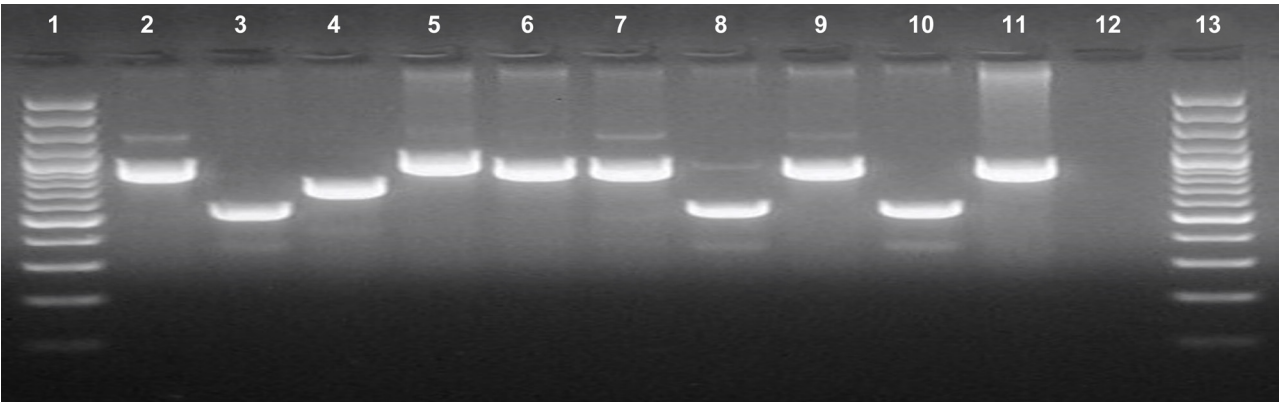


Fig. 2. Representative samples of the *hwp1* gene PCR assay visualised in electrophoresis.
Line 1 and 13 – molecular weight marker (O’GeneRuler 50 bp DNA Ladder; Thermo Scientific, USA)
Line 2 – positive control of *Candida albicans* ATCC® 90028™ (product size 941 bp). Line 3 – positive control of *Candida dubliniensis* NCPF 3949 (product size 569 bp). Line 4 – positive control of *Candida africana* ATCC® MYA 2669™ (product size 700 bp)
Line 5, 6, 7, 9, 11 – clinical *Candida albicans* strains. Line 8, 10 – clinical *Candida dubliniensis* strains. Line 12 – negative control

Table III
Percentage of compatible results obtained by the methods used to discriminate species among *Candida albicans* complex (VITEK®2 system and MALDI-TOF MS) with the *hwp1* gene amplification with PCR.

	The compliance with PCR assay No. (%) n = 326	
VITEK 2	319 (97.85)	7 (2.15)
MALDI-TOF MS	323 (99.08)	3 (0.92)

reference strains comprised the *C. albicans* complex: *C. albicans* sensu stricto (ATCC® 90028™), *C. dubliniensis* (NCPF 3949), and *C. africana* MYA 2669™.

The VITEK®2 system (bioMérieux, France) showed agreement with the molecular assay in 97.85% of clinical strains. Most *C. albicans* sensu stricto were identified at an excellent and very good discrimination level. All the incorrect identification results (2.15%) referred to *C. dubliniensis* strains (Table III, IV), which were finally discriminated by PCR assay. The VITEK®2 system did not allow identification of *C. africana*. The reference strain of *C. africana* MYA 2669™ was identified as *C. dubliniensis*/*C. parapsilosis* at the low discrimination level (Table IV).

The protein analysis by MALDI-TOF MS (VITEK®2 MS Biotyper; bioMérieux, France) did not require

lengthy biochemical reactions. It was more accurate method than the biochemical analysis with the VITEK®2 system. Based on MALDI-TOF MS, 99.08% of clinical isolates were correctly identified as *C. albicans* or *C. dubliniensis*. Only 0.92% of the identification results differed from the results obtained with PCR (Table III). However, MALDI-TOF MS failed to detect the *C. africana* reference strain, and neither did the VITEK®2 system (Table IV).

Results of antifungal susceptibility testing. Antifungal susceptibility testing covered susceptibility to azoles, which are recommended for VVC. The susceptibility to amphotericin B and 5-flucytosine was found clinically unsuitable for VVC treatment (Workowski et al. 2021).

In total, 100% of *C. albicans* isolates were susceptible to fluconazole and ketoconazole, while 99.35% (305/307) of them were susceptible to miconazole, and 96.74% (297/307) to itraconazole. The ratio of *C. albicans* intermediate to itraconazole (referring to “susceptible, increased exposure” according to the EUCAST recommendations (EUCAST 2020)) amounted to 3,26% (10/307). Only 0.65% (2/307) were intermediate resistant to miconazole (Fig. 3). All *C. dubliniensis* strains were susceptible to all antifungals tested.

Table IV
Discrepancies between species identification among *Candida albicans* complex obtained by the Vitek®2 system, MALDI-TOF MS and the *hwp1* gene amplification with PCR.

Isolate No.		VITEK 2		MALDI-TOF MS		PCR assay based on the <i>hwp1</i> gene amplification	
		Species identification	Confidence level (probability)	Species identification	Confidence level	Species identification	Product size (bp)
CA67	clinical strain	<i>Candida albicans</i> / <i>Candida dubliniensis</i>	low (<85%)	<i>Candida dubliniensis</i>	99.90%	<i>Candida dubliniensis</i>	569
CA95	clinical strain	<i>Candida albicans</i>	excellent (96%)	<i>Candida albicans</i>	99.90%	<i>Candida albicans</i> + <i>Candida dubliniensis</i>	941 + 569
CA98	clinical strain	not identified	–	<i>Candida dubliniensis</i>	99.90%	<i>Candida dubliniensis</i>	569
CA125	clinical strain	<i>Candida ciferrii</i>	acceptable (86%)	<i>Candida dubliniensis</i>	99.90%	<i>Candida dubliniensis</i>	569
CA13	clinical strain	<i>Candida albicans</i> / <i>Candida dubliniensis</i>	low (<85%)	<i>Candida dubliniensis</i>	99.90%	<i>Candida dubliniensis</i>	569
CA137	clinical strain	<i>Candida albicans</i>	good (91%)	<i>Candida albicans</i>	99.90%	<i>Candida dubliniensis</i>	569
CA313	clinical strain	<i>Candida albicans</i>	very good (95%)	<i>Candida albicans</i>	99.90%	<i>Candida albicans</i> + <i>Candida dubliniensis</i>	941 + 569
<i>Candida albicans</i> ATCC® 90028™	reference strain	<i>Candida albicans</i>	excellent (98%)	<i>Candida albicans</i>	99.90%	<i>Candida albicans</i>	941
<i>Candida dubliniensis</i> NCPF 3949	reference strain	<i>Candida dubliniensis</i>	very good (93%)	<i>Candida dubliniensis</i>	99.90%	<i>Candida dubliniensis</i>	569
<i>Candida africana</i> MYA 2669™	reference strain	<i>Candida dubliniensis</i> / <i>Candida parapsilosis</i>	low (<85%)	<i>Candida albicans</i>	99.90%	<i>Candida africana</i>	700

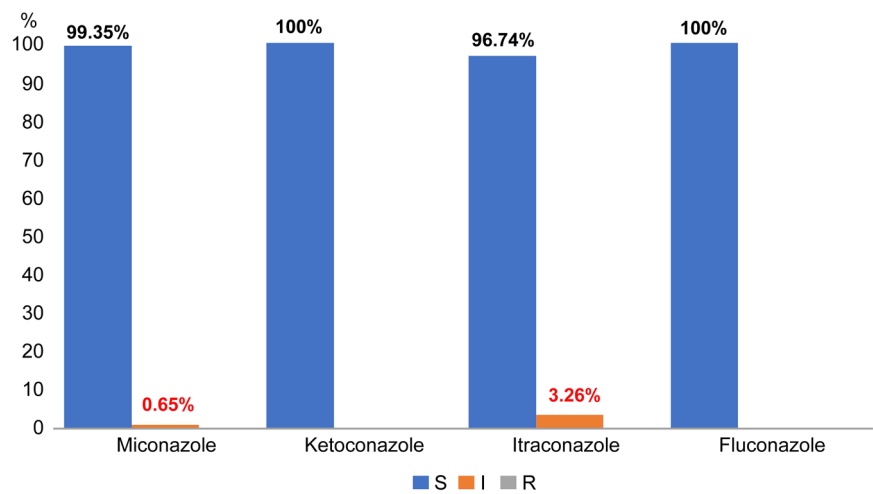


Fig. 3. Susceptibility of 309 *Candida albicans* clinical isolates caused VVC obtained by Fungitest™ (BioRad, France).
S – susceptible, I – intermediate (referring to “susceptible, increased exposure” according to the EUCAST recommendations), R – resistant.

Discussion

Vulvovaginal candidiasis (VVC) remains a threatening problem among women (Sustr et al. 2020). All 326 *Candida* strains included in the study were obtained from women with confirmed VVC. Furthermore, in 128 cases (~39%), the coincidence of yeast and aerobic and/or anaerobic bacteria was revealed by phenotypic methods. We are in line with the other observations that the occurrence of yeasts in the genital tract disrupted the natural bacterial microbiota, especially the number of *Lactobacillus* spp., which is generally considered to constitute a major part of the normal vaginal microbiota and are natural competitor of *Candida* spp. (Zangl et al. 2019).

C. albicans is the most common isolated species among women with VVC; however, there are still problems with discriminating closely related species among *C. albicans* complex; thus, their prevalence and clinical importance remains misestimated. Despite the phenotypic and biochemical differences (e.g., production chlamydospores and ability to assimilate N-acetylglucosamine and glucosamine), the identification of *C. africana* remains challenging with the use of currently existing methods in routine laboratory practice (Tietz et al. 2001; Criseo et al. 2015).

One of the most commonly utilized, rapid, and cost-effective tests are chromogenic media, which are frequently used to detect and identify yeasts of the *Candida* genus. However, culture-based methods may still need to be improved as they are often influenced by various culture conditions (Tietz et al. 2001; Criseo et al. 2015; Scharmann et al. 2020). Despite subtle differences in the *C. albicans* sensu stricto, *C. dubliniensis*, and *C. africana* colonies morphology on the ChromID® medium used in this study, no distinction of these species is possible according to the manufacturer’s recommendations (Fig. 1B).

The other method commonly used in routine laboratory practice for yeast identification is the VITEK®2 system. This automated system is based on kinetic analysis detecting metabolic changes and continuous colorimetric-based monitoring of reactions which provides reliable identification of 27 medically relevant species of *Candida* genus, including both *C. albicans* and *C. dubliniensis*. To this day, there is no possibility of *C. africana* identification by VITEK®2, ID YST Card. Identifying newly described species, species of modified systematic names, and rare ones is problematic for most commercial systems because the databases do not include a representative set of profiles, resulting in misidentifying closely related microorganisms.

Due to the limited ability of phenotypic approaches, such as chromogenic media and the VITEK®2 system, to identify rare species and to confirm the identification of *C. africana* isolates, phenotypic tests have to be supplemented with molecular methods.

According to the performed molecular assay based on the *hwp1* gene amplification, we revealed that the diagnosis of 326 strains initially identified by phenotypic methods as *C. albicans* was confirmed in 94.17% (307/326), while 5.21% (17/326) strains were identified as *C. dubliniensis*. Moreover, molecular identification allowed the detecting of two cases of co-infection with *C. albicans* and *C. dubliniensis*. Although *C. africana* is isolated from vaginal specimens worldwide, we could not isolate any species of this organism from the presumptive vaginal isolates of *C. albicans*.

The meta-analysis provided by Ghareghbolagh et al. (2020), showed that the distribution of *C. albicans* closely related species varies among the world’s regions, and the global prevalence of *C. africana* among *C. albicans* complex reached 1.67%. Our results align with those from Turkey, Malaysia, and China, where 195, 98, and 87 *Candida* isolates from vulvovaginitis

cases were detected (Gumral et al. 2011; Yazdanpanah and Khaithir 2014; Hu et al. 2015). Furthermore, a single study from Iran has confirmed the lack of *C. africana* in vaginal specimens, even though the overall prevalence of *C. africana* in this country was one of the highest (~3%) (Pakshir et al. 2017). It confirms that variations in the prevalence of this species could be observed among and across continents and even within one country.

Epidemiological data indicated that the incidence of *C. dubliniensis* in vaginal samples has varied within the tested population from 0 to even 29.52% (Jamilian et al. 2007; Shan et al. 2014). Several studies demonstrated the very low prevalence of *C. dubliniensis* in VVC/RVVC patients (Theill et al. 2016; Hazirolan et al. 2017; Mucci et al. 2017). Our results based on molecular discrimination confirmed that 5.22% of the VVC cases were caused by *C. dubliniensis* and approximated those reported by Borman et al. (2013) (6%). Furthermore, we revealed two patients (0.61%) who presented with concurrent co-infection by *C. albicans* and *C. dubliniensis*. Although *C. dubliniensis* has been frequently associated with oral infections in HIV-positive patients, it has also demonstrated significant levels of adhesion to vaginal epithelial cells (average adherence 85.6%), suggesting that this species is well adapted, in terms of adhesion capability, to the vaginal environment (Vidotto et al. 2003).

Multiple global studies have shown that *C. albicans* and the related species isolated from VVC patients are usually susceptible to antifungal agents (Theill et al. 2016). The results of the Fungitest™ assay used in this study confirmed these findings. Whereas only single strains of *C. albicans* have shown reduced susceptibility to itraconazole and miconazole, all *C. dubliniensis* strains were susceptible to all antifungals tested. However, *C. dubliniensis* is known to develop rapid resistance to fluconazole, and consequently, patients do not respond to standard doses of fluconazole (Kolekar et al. 2019). Furthermore, global overuse of azole antifungal agents without prescription for the treatment of *Candida* vaginal infection and repeated exposure to antifungal agents can lead to the induction of azole resistance in strains (Naeimi et al. 2018; Mohammadi et al. 2021). In our study, we did not observe any strains resistant to fluconazole. At the same time, resistance to this antifungal was frequently reported among *C. albicans* isolates from VVC patients, with a resistance rate ranging from 14.7% to even 53.7% (Mohammadi et al. 2021). The microbroth dilution method is the reference method for antifungal susceptibility testing of glucose-fermenting yeasts (EUCAST 2020; CLSI 2022); however, the commercial kits (e.g., Fungitest™; BioRad, France) are still in use as a screening test for the detection of antifungal efficacy (Cuenca-Estrella et al. 2005). The microdilution method recommended by EUCAST

is demanding and rarely used in routine diagnostics of *C. albicans* strains isolated from women with VVC. Methods such as E-test strips or automatic VITEK®2 YST08 methods are expensive and are not applicable in the routine diagnosis of genital tract infections. In addition, the VITEK®2 system and the available antibiogram charts provide data for fluconazole, only one of the drugs used in VVC treatment. Moreover, the interpretation criteria according to EUCAST for drugs used in the treatment of VVC apply only to fluconazole and itraconazole. There are currently no other recommendations regarding topical antifungal drugs (e.g., miconazole, econazole, clotrimazole).

According to the Centers for Disease Control and Prevention recommendations (CDC), treatment of uncomplicated VVC (which constitutes the majority of VVC) is based on empirical therapy. Culture and antifungal susceptibility testing should be considered only for patients who remain symptomatic after failure of the empirical treatment (Workowski et al. 2021). However, according to the demands reported by the physicians, susceptibility tests are performed. Currently, there are few commercial tests available that can be used to assess the susceptibility testing of drugs applicable in the treatment of VVC. Fungitest™ (BioRad, France) was the only method used to test antifungal susceptibility in this study and is still routinely used in many microbiological laboratories in Poland. However, the concentrations of drugs available in this kit do not meet the current European (EUCAST 2020) and American criteria (CLSI 2022). According to the manufacturer's instructions, the interpretation of drug susceptibility is based only on scientific reports. Moreover, this semi-quantitative method has many advantages: it does not require complex handling and is cost-effective. Furthermore, the agreement with the European Committee on Antibiotic Susceptibility Testing (EUCAST) data for Fungitest™ was high, with correlation indices of 0.95 ($p < 0.01$) (Cuenca-Estrella et al. 2005).

Conclusions

C. albicans sensu stricto remains the major factor of vulvovaginal candidiasis, while *C. dubliniensis*, and *C. africana* are a minor species within the *C. albicans* complex (Gharehbolagh et al. 2020). Our results based on molecular discrimination confirmed that the most common species causing vulvovaginal candidiasis in Southern Poland is *C. albicans* sensu stricto (94.17%). However, we argue that the reported incidence of *C. dubliniensis* and *C. africana* suffers from certain limitations, such as a low number of studies from each country and the unavailability of data for the majority of countries. While it has been estimated that *C. albicans*

is responsible for 78.3% of genital tract candidiasis in Poland (Wójkowska-Mach et al. 2021), the regional occurrence of closely related *C. albicans* species such as *C. dubliniensis* and *C. africana* is unknown. As far as we know, this is the first study concerning the prevalence of *C. albicans* and its closely related species isolated from the genital tract in our country.

The global prevalence of *C. albicans* may be overestimated due to a lack of routinely used tests aimed at the discrimination of closely-related species; therefore, *C. dubliniensis* and *C. africana* isolates continue to be misidentified as *C. albicans* (Shokoohi et al. 2021). Due to minor phenotypic differences between closely-related species belonging to the *C. albicans* complex, it is not easy to recognize them properly by routine diagnostic methods (e.g., biochemical tests, chromogenic media).

A PCR assay targeting the *hwp1* gene was reliable in identifying species among the *C. albicans* complex. Its simplicity, rapidity, and selectivity make this method helpful in investigating *C. albicans* and its closely related species epidemiology.

ORCID

Karolina Klesiewicz <https://orcid.org/0000-0003-2756-3340>

Paulina Mrowiec <https://orcid.org/0000-0002-5404-0388>

Katarzyna Kania <https://orcid.org/0000-0001-5033-5368>

Iwona Skiba-Kurek <https://orcid.org/0000-0002-0533-2130>

Marianna Małek <https://orcid.org/0000-0001-8940-6514>

Acknowledgement

The research project was founded by Jagiellonian University Medical College's statutory funds (K/DSC/002884; N42/DBS/000295).

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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