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ANTIFUNGAL SUSCEPTIBILITY PATTERNS AND VIRULENCE FACTORS OF CLINICAL *CANDIDA SPP.* ISOLATES

Olga Burduniuc, PhD, Associate professor, Head of Microbiological laboratory
Public Health Laboratory Diagnostic Department, National Agency of Public Health, State University of Medicine and Pharmacy "Nicolae Testemițanu", Chișinău, Republic of Moldova

Abstract
Objective. The global emergence of antifungal resistance among *Candida spp.* is a growing threat to public health. Candida infections may resist antifungal drugs actions resulting in their therapeutic deficiency. Considering the increase rate of Candida infections and resistance to antifungal agents in the last decades, this study was conducted to evaluate the *in vitro* expression of different virulence factors among clinical isolates of Candida species, as well as to assay their susceptibility patterns against antifungal agents.
Material and methods. Chromogenic media was used for Candida isolation. A total of 127 Candida isolates were identified by conventional methods, Vitek 2 system. The antifungal susceptibility test was carried out by Vitek-2 system. Candida species were tested for virulence factors such as extracellular enzymes and biofilm formation by the standard phenotypic methods.
Results. *C. albicans* continues to remain one of the most common opportunistic pathogenic fungi in humans. The authors highlighted the importance of research on understanding the mechanisms of antifungal resistance and its pathogenicity. The study demonstrates that *C. albicans* isolates were the most virulent and produce the highest number of extracellular enzymes: hemolysin, phospholipase, protease, esterase and catalase. The study showed the most effective antifungal agent was fucitozin (98.4%), while 29.4% isolates were resistant to fluconazole and 11.8% to voriconazole.
Conclusion. *C. albicans* were the most virulent and produce the highest number of extracellular enzymes compared to non-*albicans Candida* isolates. The study showed the most effective antifungal agent was fucitozin. In conclusion, more locally relevant studies should be carried out to monitoring of the distribution and susceptibility profile.
Key words: antifungal susceptibility, virulence factors, *Candida spp.*

Introduction

Candida species are part of the normal human microbiota and are commonly found in skin, gastrointestinal tract, and genitourinary system, but can also cause various infections in susceptible patients that includes elderly, hospitalized, or immunosuppressed patients [1].

Therefore the incidence of Candida infections is on the rise with the increase in number of immunocompromised patients due to excessive use of immunosuppressive drugs as well as the use of medical and surgical interventions [2].

Although, *Candida albicans* is the most prevalent species, an epidemiological shift in Candida pathogens has been recently noted by the increasing number of infections caused by non-*albicans Candida* species (NAC) like *Candida tropicalis* [3, 4].

The evolution of the antifungal resistance may be due to the use of selective therapies with inadequate doses or to the drug's frequent use in the fungal infection prophylaxis, both in human and animals, which may affect the selective clinical resistance [5, 6].

The increased species diversity and incidence of Candida infections observed in recent decades have resulted in the need for an accurate, rapid identification of Candida isolates and antifungals susceptibility, important aspects for proper patient management and prevention of emergence of drug resistance [1, 2, 7].

Patients with risk factors (treatment with broad spectrum antibiotics, prolonged use of central venous catheters, persistent neutropenia, the administration of corticosteroids, HIV infection, diabetes and others) are predisposed to develop deep-seated or mucosal Candida infections [8].

Expression of virulence factors like germ tube formation, adhesins, phenotypic switching, thigmotropism, and biofilm formation and the production of hydrolytic enzymes contribute to the pathogenesis of candidiasis. Analyzed studies showed that the expression of these factors among *Candida spp.* may vary depending on the infecting species, geographical origin, type of infection, the site and stage of infection, and host reaction [9, 10].

Secretory proteinases, as Candida virulence factors, can improve the potential of fungal organisms to colonize and penetrate into the host tissue and disrupt the immune system [11].

Phospholipase production is another major virulence factor of *C. albicans*, which binds the fungus to the target tissue and generates a pathway to enter the tissue following the hydrolysis of phospholipids and degradation of cell membranes [12].

In addition, haemolysin, as another extracellular enzyme, contributes to the invasion of yeasts through absorption of

iron [13].

The esterase is an enzyme responsible for the hydrolysis of an ester group and is also an important virulence factor which degrades the ester bonds and accentuates its tissue invasion [14].

Candida infections may resist antifungal drugs, making them difficult to treat. Most published studies have demonstrated that *C. albicans* is the most common cause of severe infections. At the same time, antifungal resistance is most common in other species, especially, *Candida auris*, *C. glabrata* and *C. parapsilosis* [15].

Biofilm formation, which is another virulence factor of *C. albicans*, plays a pivotal role in the pathogenesis of fungi through the mass production of pseudohyphae [16, 17, 18].

Biofilm contains dense yeast cells and hyphae, which can lead to antifungal resistance, as well as frequent recurrence of Candida infections. Furthermore, long-term treatment and excessive exposure to antifungals lead to variable levels of resistance in Candida species [19, 20].

The purpose of this study was to assay the antifungal susceptibility patterns of Candida species and to evaluate the in vitro expression of different virulence factors among clinical isolates.

Materials and Methods

The study included 127 *Candida spp.* isolated from various human biosubstrates. The collected samples were processed in the Microbiology laboratory, National Agency for Public Health, Republic of Moldova. The virulence factors studied were exoenzymatic activity (phospholipase, lipase, caseinase, gelatinase and proteinase), biofilm formation, and haemolysin production.

Sample Collection and growth on culture media

The collected swab specimens were inoculated on Sabouraud dextrose agar (SDA) with 0.01% chloramphenicol. All plates were incubated at 37°C for 5 days with daily growth assessment. The purified growing colonies and macroscopic characteristics were determined.

Identification and antifungal susceptibility testing. The isolated colonies were identified by germ tube test, colony color on Brilliance Candida (Oxoid) media and by Vitek-2 YST cards (bioMérieux).

The antifungal susceptibility was performed automatically by Vitek-2 Compact system. Inoculum suspensions for Vitek-2 cards were obtained from the overnight cultures, with the turbidity being adjusted to a 1.8-2.2 McFarland standard according to the manufacturer's recommendations, using the DensiCheck (bioMérieux). A standardized inoculum suspension was placed into a Vitek-2 cassette, along with a sterile polystyrene test tube and an antifungal susceptibility test card for each organism. Each suspension was diluted by transferring 280µl to a tube containing 3ml of saline solution. After inserting the card with the yeast suspension, it was incubated and read automatically. The incubation time varied from 9.1 to 27.1 h based on the rate of growth in the drug-free control well. *Candida parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as control isolates. The results were expressed as minimum inhibitory concentrations (MICs) after incubation for 48 h at 37°C. MIC results were interpreted according to species-specific clinical breakpoints (established by European Committee on Antimicrobial Susceptibility Testing (EUCAST) [21].

Enzymatic Activity

Phospholipase activity. The phospholipase activity was assayed by egg yolk agar plate method. The medium was slightly modified by enriching SDA with egg yolk as substrate. Egg yolk was added to equal amounts of saline and 5 ml of this was added to the medium. The inoculated plates were incubated at 37°C for 48 h. Hydrolysis of lipid substrates present in the egg-yolk results in the formation of a calcium complex with fatty acids released by the action of the secreted enzymes, resulting in a precipitation zone around the colony. Phospholipase activity was measured according to the method described by Price et al. [22]

Protease activity. Determination of protease production of tested isolates was conducted by using yeast carbon base (YCB) medium (1.17%) supplemented with 0.2% bovine serum albumin (BSA). The YCB-BSA medium was adjusted to pH 5.0, sterilized by filtration and added to previously autoclaved agar (2%). 23 Aliquots (10µl) of 48h-old cultured fungal cells (106 yeasts) were inoculated onto the surface of the YCB-BSA medium and incubated at 37°C for seven days. The proteolytic activity results in a clear zone around the colony, which corresponds to the hydrolysis of the BSA present in the medium. The colony diameter (a) and the diameter of colony plus the precipitation zone (b) were measured by a digital paquimetro [23].

Esterase activity. The esterase activity of Candida isolates was evaluated using the Tween 80 opacity test according to the method described by Fatahinia et al. Initially, 10 g of bacteriological peptone, 5 g of sodium chloride, 0.1 g of calcium chloride, and 15 g of agar were dissolved in 1000 ml of distilled water. The medium was autoclaved and gradually cooled to about 50°C. Then, 5 ml of autoclaved Tween 80 was added to the medium and distributed in 8-cm sterile plates. Again, 10 µl of each Candida suspension (10^6 cells/ml) was spot-inoculated on plates and incubated at 30°C and checked on a daily basis for 10 days. All the inoculations were performed in duplicate [24].

Catalase activity. The catalase test was performed as described by Trabulsi and Altherthum. The Candida isolates were transferred to a microscope slide and a drop of 3% hydrogen peroxide was added. The immediate onset of bubbles on the surface of the suspension corresponded to a positive reaction, indicating the conversion of H_2O_2 into water and oxygen [25].

Hemolytic activity. Haemolysin assay for Candida isolates was conducted according to a previous protocol developed and validated by Luo G et al. In short, Sabouraud dextrose agar supplemented with 6% human blood and 3% glucose (pH = 5,6) was used to determine the hemolysin production. Suspension of yeast (10^6 cells/ml) was prepared in saline solution and 10 µl was spot inoculated on human blood agar plates, incubated at 37°C in 5% CO2 for 5 days. Reading and interpretation of hemolysis was performed under a magnifying glass [26].

Biofilm formation. The in vitro biofilm production by *Candida spp.* isolates was quantitatively determined using the microtiter plate method with modification of a crystal violet assay as described by Silva S. et al. Candida isolates were first cultured at 37°C for 24 h on Sabouraud Dextrose Agar Plates (SDA). 200 µl of standardized cell suspensions (containing 10^6 cells/ml) in yeast peptone galactose medium (YPG)) were transferred to each well of 96-well polystyrene microtiter plates and incubated at 37°C on a shaker at 120 rpm/min. As a negative control, there was used a test medium without cells added to three wells of each plates. At 24 h, 50 µl of YPG medium was added. The plates were then incubated for further 48 h. After the adhesion stage, non-adherent cells were removed by washing the wells twice with sterile ultra-pure water. Biofilms were fixed with 250 µl of methanol, which was removed after 15 min of contact. The microtiter plates were dried at room temperature, and 250 µl of 0,1% crystal violet added to each well and incubated for 5 min. The wells were then gently washed with sterile, ultra-pure water and 250 µl of acetic acid added to release and dissolve the stain. The absorbance of the obtained solution was read in triplicate in a microtiter plate reader at 620 nm. Experiments were repeated as part of three independent assays [27].

In order to identify whether biofilm formation of isolates exists or not, the cut-off OD was defined as using the OD (the optical density) of the reference isolate. Therefore, the results were classified as: OD ≤ 2×OD reference – biofilm negative; 2×OD reference < OD < 3×OD reference – weak biofilm production; OD ≥ 3×OD reference – strong biofilm production.

Ethical issues

This study was conducted with the approval of the ethics committee no. 65/12.04.2017 of the State University of Medicine and Pharmacy "Nicolae Testemițanu", Republic of Moldova. The isolates used in this study were obtained from routine clinical samples and did not involve direct contact with the patient, thus no consent was required.

Results

Accurate identification of the infecting strain of Candida is essential for selection of appropriate antifungal drug. In our country, however, most clinical microbiology laboratories use conventional techniques, which are the mainstay of species identification of Candida isolates. Analyzing the diversity of isolates (n=127), it was found that the most frequently recorded were non-*albicans Candida* species (n=68; 53.5%) compared with *C. albicans* (n=59; 46.5%). Of the non-*albicans Candida* species identified in this study, *C. parapsilosis* (n=36; 52.9%) was the most predominant, followed by *C. glabrata* (n=17; 25.0%), *C. krusei* (n=7; 10.3%), *C. fomata* (n=3; 4.4%), *C. kefyr* (n=2; 2.9%), *C. tropicalis* (n=2; 1.6%) and *C. dublinensis* (n=1; 0.8%) (Figure 1).

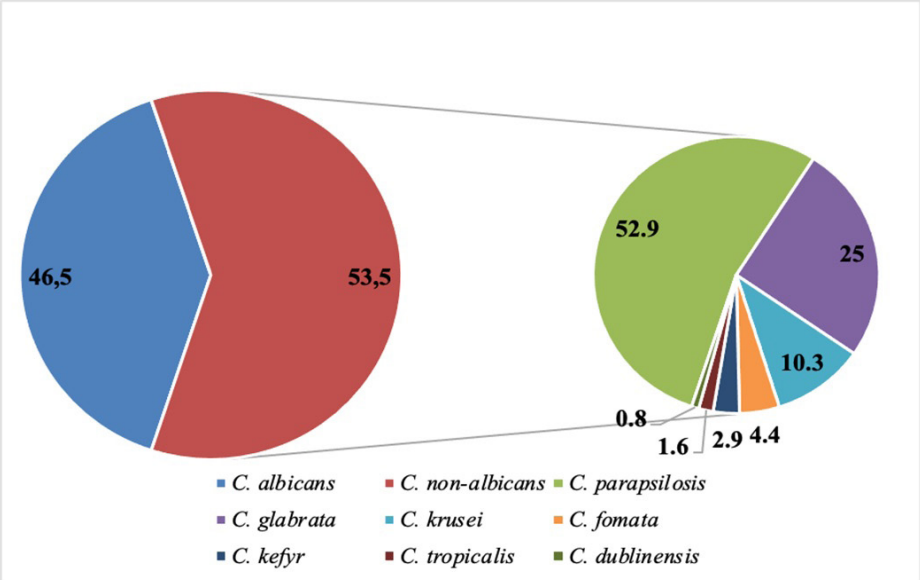

Figure 1. *Diversity of Candida species involved in human pathology*

Antifungal susceptibility patterns

The MIC values obtained for five antifungal agents among the *C. albicans* and Non-*albicans Candida*, isolated from clinical specimens, are presented below. The results demonstrated that the most effective antifungal agent used in this study was fucitozin, (125 isolates, 98.4%), followed by amfotericin B (115 sensitive isolates, 90.5%), micafungin (111 sensitive isolates, 87.4%), voriconazole (109 sensitive isolates, 85.8%) and finally fluconazole (102 sensitive isolates, 80.3%). No resistance was detected to fucitozin for *C. glabrata*, while 5 isolates (29.4%) were resistant to fluconazole, and 2 isolates (11.8%) were resistant to voriconazole.

Enzymatic and hemolytic activity in different Candida species

It was demonstrated that Candida species have the ability to produce various hydrolytic enzymes as virulence factors to contribute to its pathogenicity (proteases, phospholipases, hemolysins, catalases, etc.). Therefore, the assessment of pathogenic enzymatic factors in *C. albicans* isolates were found to be in a higher percentage compared to non-*albicans*

Table 1

In vitro biofilm formation capacity by various Candida species.

Biofilm-forming capacity	*Candida albicans*		*Candida parapsilosis*		*Candida glabrata*		*Candida spp.*		Total	
	abs	%	abs	%	abs	%	abs	%	abs	%
Biofilm negative	28	47.5	1	2.8	6	35.3	5	33.3	40	31.5
Biofilm positive	**31**	**52.5**	**35**	**97.2**	**11**	**64.7**	**10**	**66.7**	**87**	**68.5**
Strong biofilm formers	11	35.5	16	45.7	2	18.2	5	50.0	34	39.1
Moderate biofilm formers	13	41.9	17	48.6	5	45.4	3	30.0	38	43.7
Weak biofilm formers	7	22.6	2	5.7	4	36.4	2	20.0	15	17.2

Candida isolates, except for catalase, that was 72.1% in non-*albicans Candida* and 64.5% in *C. albicans*. Of non-*albicans Candida* species, these factors were the most expressed in *C. glabrata* (82.3%) and *C. parapsilosis* (72.2%) isolates.

The frequency of hydrolytic enzymes, such as phospholipase and proteinase, in *C. albicans* was 91.5% and 81.4%, and in non-*albicans Candida*, 47.1% and 60.3%, respectively.

In this study, we found that 56.7% of the Candida species had esterase activity, whereas the highest esterase activity was found in *C. albicans* (67.8%), followed by *C. glabrata* (47.0%) and *C. parapsilosis* (44.4%).

The discrepancy in results could be due to differences in geographic regions, methods of diagnosis, as well as sample size.

It was also found that most of the *Candida spp.* isolates showed hemolytic activity (α-haemolysis) in 91.3% of cases, which demonstrates the virulence of these isolates. Thus, in *C. albicans*, this enzyme was detected in 96.6% and in non-*albicans Candida* – 86.8%.

The results of the determination of the biofilm formation capacity by the clinical Candida isolates demonstrated that out of the 127 isolates of *Candida spp.* – 87 (68.5%) produced biofilm (OD > 0.112). Regarding the biofilm status, 34 (39.1%) isolates produced strongly adherent biofilm (OD > 0.220), 38 isolates (43.7%) produced moderately adherent biofilm (OD 0.112 - 0.220) and 15 (17.2%) produced poorly adherent biofilm (Table 1).

Based on obtained results, the Candida isolates were categorized as weak, moderate, and strong biofilm formers. Thus, 39.1% of Candida spp. isolates were strong biofilm formers, 43.7 % – moderate and 17.2% – weak biofilm formers.

Discussion

The analyzed studies indicated the importance of *C. albicans* to be involved in human pathology and to cause infections ranging from superficial to systemic and life-threatening. The authors highlighted the necessity of conducting research on virulence factors of Candida species due to their wide range, from dimorphism, biofilm formation, secretion of extracellular enzymes, etc. [28]

Our results are consistent with other research in which *C. albicans* (n=93, 65%) was the most common and *C. glabrata* (n=27, 19%) as the second reported species [29].

Numerous researches demonstrated that for the best management of fungal infection and to prevent the emergence of drug resistant isolates, it is necessary to evaluate the antifungal susceptibility pattern of clinical isolates. The recent study demonstrated that non-*albicans Candida* species (especially, *C. tropicalis*) is the predominant species, and there is a significant proportion of isolates with reduced susceptibility to azole, but not to echinocandin [30].

Another similar study demonstrated that among different species of candida, isolated *C. albicans* was the predominant species (79.33%). And in this study antifungal resistance of tested species of candida was higher to fluconazole and the least resistance was seen with amphotericin-B [31].

At the same time, most researches highlight that antimicrobial resistance in fungi will continue to increase, creating new challenges in the decades to come and more locally relevant studies should be carried out to monitoring of the distribution and susceptibility profile [32].

The results obtained in the study show that of all the hydrolytic enzymes studied in *C. albicans,* phospholipase was most frequently highlighted, which was also found in the analyzed studies [33].

In line with our results, Pakshir et al. reported that esterase activity of *C. albicans* and *C. parapsilosis* isolated from onychomycosis and oral lichen planus lesions were 87.5% and 43.7%, respectively. In the study conducted by Kumar et al. esterase activities of *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. dublinis*, and *C. lipolytica* isolated from clinical samples were 92.2%, 92.3%, 25.6%, 16.6%, and 100%, respectively [34, 35].

Another study highlighted the complexity of the intracellular mechanisms leading to the formation of Candida biofilms, including those controlling adhesion, changes in cell morphology, extracellular polymeric substance (EPS) production and external factors, including the surface where the biofilm forms [36].

Conclusions

1. Our study explored the distribution of the susceptibility profiles and the presence of different virulence factors of

Candida species.

2. The study showed the most effective antifungal agent was fucitozin (98.4%) and 29.4% isolates were resistant to fluconazole and 11.8% to voriconazole.

3. This research has demonstrated that among the Candida species, *C. albicans* was the most virulent and produced the highest number of extracellular enzymes such as hemolysin, phospholipase, protease, esterase and catalase.

4. Better knowledge and experimental evidence of the viru-lence factor for each species and their correlation with each other will help in understanding the pathogenesis of fungal infection.

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Corresponding author: Olga Burduniuc, e-mail: olgaburduniuc3@gmail.com
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