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Research article

Phenotypic and genotypic variations in *Candida albicans* isolates from Romanian patients

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Abstract

Background: During the last two decades a major increase in the proportion of severe fungal infections has been noted due to the excessive use of broad-spectrum antibiotics, catheters, and a growing number of immuno-compromised patients.

Objectives: This is the first investigation providing complete data regarding the phenotypic and genotypic profiles of Candida albicans (C. albicans) isolates in Romanian patients.

Methods: We investigated 301 isolates in terms of genotype determination (G), resistogram (R), phospholipase activity (Pl), haemolysis (Hl), proteinase activity (Pt), and biofilm formation (BF).

Results: The analyzed isolates of C. albicans showed low values for Pt (61.73%), Hl (95.49%), and BF (60.71%), and did not present any Pl activity (92.23%). More than half of the investigated samples were genotype A with 450 bp (52.92%) and the majority (86.19%) were resistant to sodium selenite (A), boric acid (B), sodium periodate (D) and silver nitrate (E), but sensitive to cetrimide (-). One-way ANOVA analysis revealed significant effects of the infection site on biofilm formation (p = 0.0137) and no significant correlation was found between the genotype (A, B, C) and the infection site (p =0.449).

Conclusions: Based on the obtained results it can be concluded that C. albicans isolates in Romanian patients exhibit different genotypic and phenotypic patterns, and no significant correlations between genotype and infection site could be observed.

Keywords: Candida albicans, infection site, genotype, phenotypes, Romania

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Introduction

The fourth source of bloodstream infections as prevalence, *Candida* yeasts remain the most recurrent fungal pathogen in humans [1]. *C. albicans* is the most frequent species causing mortality rates of 50% [2-3]. *C. albicans* is a member of the normal human microbiome, usually being commensal and harmless, but under certain circumstances, it becomes an opportunistic pathogen and produces candidiasis if the defence mechanisms of the host are damaged [4].

Candida infections are a major health issue supplied, paradoxically, by the improvements in medical care, where the most important predisposing factors for the infections are the use of antibiotics, indwelling catheters, immunosuppression, chemotherapy and radiotherapy [5-7]. *C. albicans* produces two major types of infections: superficial infections, such as oral or vaginal candidiasis, and deep-seated life-threatening systemic infections (such as *Candida* endocarditis and acute invasive candidiasis as causes of sepsis) [8].

Several factors that contribute to the pathogenic potential of this fungus have been identified such as biofilm formation, the secretion of hydrolases, contact sensing and thigmotropism and phenotypic switching [9]. Understanding how these mechanisms and factors contribute to infection has significantly increased during the last years, but at the same time, new virulence strategies have recently been observed.

Antifungal therapy is a critical component of the patient management for acute and chronic diseases [10]. The presence of oral *Candida* yeasts is considered a signal indicative of immune system impairment and can be correlated with a progressive disease [11].

Oral candidiasis is one of the most frequent types of *Candida* infection found especially in denture wearers and individuals with severe conditions (e.g. HIV-infected patients), patients under antibiotic or chemotherapy, and patients with systemic diseases such as diabetes [12]. HIV-infected patients are passible to mycoses as cell-mediated immunity decays [12].

In diabetic patients, hyperglycaemia can favour several infectious diseases ranging from superficial candidiasis to deep-seated mycoses [13]. Because of the poor immune system, oral candidiasis development is often faster and more severe [10]. Candidemia incidence is statistically correlated with hyperglycaemia and is a serious mortality cause in patients with diabetes [14].

Candida has the ability to produce various virulence factors that amplify their property to colonize mucosal or synthetic surfaces and, afterwards, to invade the host tissues. Studies regarding isolates from people with candidemia are more numerous and to our knowledge, the present study is the first investigation providing complete data regarding the phenotypic and genotypic profiles of *C. albicans* strains isolated in Romanian patients.

We report a screening of 301 clinical yeast isolates in terms of phenotypic and genotypic profiles: genotype determination (G), resistogram (R), phospholipase activity (Pl), haemolysis (Hl), proteinase activity (Pt) and biofilm formation (BF) in order to investigate the relationship between *C. albicans* strains and their pathogenicity.

Material and methods

Clinical isolate processing

Three-hundred and one *C. albicans* clinical isolates were collected in four tertiary hospitals from different regions of Romania (i.e. Iași, Cluj-Napoca, Timișoara, and Tîrgu Mureș). Samples were recovered from bloodstream infections (BSI), deep-seated mycoses (DEEP) (lower respiratory tract, peritoneal cavity, upper urinary tract in non-catheterized patients, and cerebrospinal fluid) and superficial mycoses (SUP) (female genital tract, oropharyngeal, and

gastro-intestinal tract). The isolates were collected from patients who presented at the same time at least two of the following risk factors: low birth weight (<1500 g), old age (>65 years), insulin-dependent diabetes mellitus, recent major surgery, broad-spectrum antibiotic therapy, central venous catheter, organ transplantation, immunosuppression (HIV infection or other predisposing conditions), total parenteral nutrition or mechanical ventilation.

The isolates were presumptively identified by local hospital laboratories and then submitted to the Laboratory of Antimicrobial Chemotherapy from the Department of Public Health of "Ion Ionescu de la Brad" University, Iași, Romania. The last identification was performed using ID32C strips (bioMérieux, France). Isolates identified as *C. albicans* were also tested using duplex PCR by a method previously described by Romeo & Criseo [15].

***C. albicans* genotype determination (G) by Polymerase Chain Reaction (PCR)**

In order to extract DNA, two colonies of *C. albicans* were taken from Sabouraud Dextrose Agar (SDA) plates and suspended in 200 µl sterile distilled water in a sterile Eppendorf tube. DNA was obtained by lysing the cells at 95°C for 5 min and by immersing in ice. PCR was performed according to the method previously described [16-17] using the primers CA-NT-L (5'-ATAAGGGAAGTCGGCAAAATAGATC-CGTAA-3') and CA-NT-R (5'-CCTTGGCT-GTGGTTTCGCTAGATAGTAGAT-3'). The products were analysed by electrophoresis on 1.5% agarose gels in Tris-borate-EDTA (TBE) buffer at 70 V for 30 min and were subsequently stained in a solution of 0.5 µg of ethidium bromide per mL.

According to the PCR products, the genotypes of *C. albicans* can be classified by the size of DNA amplified into 3 groups: 450 bp for group A, 840 bp for group B, 450 and 840 bp for group C) [17-19].

Resistogram (R). The resistogram typing of the isolates of *C. albicans* was performed according to McCreight and Warnock method [20], with a few modifications [21]. The plates containing chemicals at different concentrations (mg/mL Sabouraud dextrose agar) used for the resistogram typing were: sodium selenite: 0.1, 0.2, 0.3, 0.4 (A); boric acid: 1.15, 1.3, 1.45, 1.6 (B); cetrimide: 0.06, 0.08, 0.1, 0.12 (C); sodium periodate: 0.01, 0.02, 0.03, 0.04 (D); silver nitrate, 0.0075, 0.01, 0.0125, 0.015 (E) [20]. After incubation for 40 h at 37ºC, the growth of each strain was read. For example, the resistogram "AB - - E" signifies that the tested strain was resistant to sodium selenite (A), boric acid (B) and silver nitrate (E), but sensitive to cetrimide (-) and sodium periodate (-) [21].

Phospholipase activity assays (Pl). Extracellular phospholipase activity was evaluated according to the method described by Prince *et al.* [22] and Sanita *et al.* [8]. After incubation the diameter of hyaline zones around the colonies and the diameter of the colonies was measured using Image J software [23]. The phospholipase activity (Pl value) was measured in terms of the ratio of the diameter of the colony to the total diameter of colony plus the precipitation zone. Pl were also evaluated as follows: $Pl < 0.64$ (high), $0.64 \leq Pl < 1$ (low) and $Pl = 1$ (none).

Haemolysis (Hl) was evaluated using a blood plate assay [24-25]. After incubation, the plates were measured using Image J software [23]. The presence of a translucent zone of haemolysis around the inoculum site, viewed with transmitted light, indicated positive haemolytic activity. Haemolysin activity (Hl value) was measured and categorised as follows: $Hl < 0.64$ (high), $0.64 \leq Hl < 1$ (low) and $Hl = 1$ (none).

Proteinase activity (Pt) determination was made by the agar plate method as previously described by Barros *et al.* [26]. The plates were incubated at 37°C for 72 h and the enzymatic activity was determined by the formation of a

halo around the colony, measured in terms of the ratio of the diameter of the colony to the total diameter of colony plus zone of precipitation. The proteinase activity (Pt value) was measured with the aid of Image J software and categorised as follows: Pt< 0.64 (high), $0.64 \leq Pt < 1$ (low) and Pt = 1 (none) [23].

Biofilm formation (BF). The assessment of the biofilm forming capacity of the yeasts was performed following protocols published by Pierce *et al.* [27] with some modifications. The method is based on inducing the formation of biofilms in 96-well microplates and on the metabolic capability of live yeast cells to reduce a tetrazolium salt (XTT) to water soluble coloured formazan compounds [28]. The colour intensity of the formazan solution was measured with a microplate reader.

The *C. albicans* SC5314 strain, which forms abundant biofilms with a complex structure, was used both for quality control and as a unit of measure for comparison. Isolates were classified as low biofilm formers (LBF) or high biofilm formers (HBF) if their biomass absorbance was less than the first quartile (Q_1 OD_{570} = 0.366) or greater than the third quartile (Q_3 OD_{570} = 1.286), respectively. The isolates in between the first and third quartile (Q_1-Q_3) were defined as intermediate biofilm formers (IBF).

Descriptive statistics, chi-square test, one-way ANOVA, and Pearson's correlation coefficient were used to analyze the obtained data.

Results

In terms of *C. albicans* infection site, of the 301 samples, 57.47% were from superficial infections (SUP, n=173) like: vaginal discharge (n=39), vaginal discharge in pregnant women (n=29), pharyngeal exudate (n=4), onycomycosis (n=2), gastrointestinal tract (n=2), scraped lingual (n=5), oral sampling (n=32), oral sampling from patients with diabetes (n=6), oral sampling from pregnant women (n=4), oral sampling from HIV patients (n=26), oral sampling from denture patients (n=3), oral sampling from TBC patients (n=1), nail sample (n=1), balanitis sample (n=1), faeces samples (n=11), faeces samples from HIV patients (n=6), and colecistitis sample (n=1). *C. albicans* was sampled 20.59% from blood culture (BSI, n=62) and 21.92% of strains were from deep-seated life-threatening systemic infections (DEEP, n=66) like: urine (n=3), urine from HIV patients (n=1), respiratory tract (n=3), cerebrospinal fluid (n=1), bronchial aspirate (n=2), bronchial aspirate from TBC patients (n=15), sputum (n=26), sputum from TBC patients (n=8) and drain tube (n=7) (Table 1).

Genotype determination (G). *C. albicans* were classified into three different groups, based on the length of polymerase chain reaction (PCR) amplification product, namely genotypes A with 52.92%, B with 17.85%, respectively C, with 29.20% (Table 1).

Resistogram (R). The 301 strains were grouped into 5 resistotypes (Table 1). The majority were resistant to sodium selenite (A), boric acid (B), sodium periodate (D) and silver nitrate (E), but sensitive to cetrimide (-) (86.19%).

Phospholipase activity (Pl). *C. albicans* isolates presented low phospholipase activity (Pl) with values between 0.61 and 1 (Figure 1). The low activity of phospholipases was observed in 7.76% of the isolates (Table 1).

Haemolysis (Hl). The haemolytic activity of different *C. albicans* strains obtained from a variety of clinical sources (Figure 1) was evaluated. Most of the investigated strains presented low haemolytic activity (95.49%) (Table 1).

Proteinases (Pt). Most of the analyzed strains presented a low level of proteinases (61.73%), and the rest of the strains did not present any Pt (38.27%) (Table 1, Figure 1).

Biofilm formation (BF). Biofilm formation is heterogeneous, with isolates classified as either high or low biofilm formers (LBF and HBF)

Table 1. Statistical analysis of the *C. albicans* phenotypic and genotypic profile

Infection site	
SUP	57.475 %
BSI	20.598 %
DEEP	21.922 %
Genotype	
A	52.922 %
B	17.857 %
C	29.220 %
Resistogram	
AB-DE	86.816 %
AB- -E	0.643 %
ABCDE	1.929 %
-B-DE	5.466 %
A- -DE	5.144 %
Phospholipases activity	
Pl-low	7.766 %
Pl-none	92.233 %
Hemolysis	
Hl-low	95.498 %
Hl-none	4.501 %
Proteinases	
Pt-low	61.736 %
Pt-none	38.263 %
Biofilm formation	
LBF	60.771 %
IBF	37.299 %
HBF	1.929 %

Infection site: SUP-superficial infections, BSC-blood stream infections, DEEP-deep-seated life-threatening systemic infections;
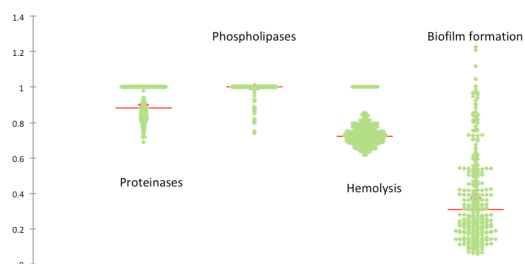**Genotype**: A: 450 bp, B: 840 bp, C: 450&840 bp;
Resistogram: AB-DE: resistant to sodium selenite (A), boric acid (B), sodium periodate (D), silver nitrate (E) and sensitie to cetrimide(C); AB- -E: resistant to sodium selenite (A), boric acid (B), silver nitrate (E) and sensitive to cetrimide (C) and sodium periodate (D); ABCDE: resistant to sodium selenite (A), boric acid (B), cetrimide (C), sodium periodate (D) and silver nitrate (E); -B-DE: resistant to boric acid (B), sodium periodate (D), silver nitrate (E), and sensitive to sodium selenite (A), cetrimide (C); A- -DE: resistant to sodium selenite (A), sodium periodate (D), silver nitrate (E) and sensitive to boric acid (B), cetrimide (C).
Phospholipases: Pl-low: low phospholipase activity, Pl-none: none phospholipase activity.
Haemolytic activity: Hl-low: low haemolytic activity, Hl-none: none haemolytic activity.
Proteinases: Pt-low: low proteinase activity, Pt-none: none proteinase activity.
Biofilm formation: LBF: low biofilm formers, IBF: intermediate biofilm formers, HBF: high biofilm formers.

Fig. 1. The descriptive statistics of *C. albicans* phenotypic properties

(Figure 1). Isolates were categorised as low biofilm formers (LBF) for 60.77% of the strains or high biofilm formers (HBF) for 1.92% if their biomass absorbance was less than the first quartile (Q_1 OD570 = 0.366) or greater than the third quartile (Q_3 OD570 = 1.286), respectively. Those isolates in between the first and third quartile (Q_1-Q_3) were defined as intermediate biofilm formers (37.29%) (Table 1).

Statistical analysis. One-way ANOVA revealed significant effects of prevalence on biofilm formation (p = 0.0137) (Table 2). No other statistical significant effects were found between the analyzed parameters. The results of the chi-square test (Table 2) showed a significant positive association only between prevalence and resistograms (p = 0.0832).

There was a strong correlation between phospholipases and haemolytic activity (r = 0.563) and proteinases (r = 0.548) and also between haemolytic activity and proteinases (r = 0.886) (Table 3). No significant correlation was found between the genotype (A, B, C) and the infection site (p =0.449).

Discussions

Genotype determination (G). *C. albicans* strains converge to be genetically similar, when derived from similar population groups, in correlation with the immune condition, anatomic site or geographical location [28]. It was not

Table 2. Statistical analysis: one-way Anova and chi-square association.

		Proteases	Phospholipases	Haemolysis	Biofilm formation	Resistogram
One-way Anova	Infection site	0.543	0.382	0.313	0.013*	0.432
	Genotype	0.891	0.415	0.116	0.381	0.852
Chi-square	Infection site	0.527	0.265	0.853	0.144	0.083*
	Genotype	0.807	0.109	0.088	0.471	0.252

The mean difference is significant (p≤0.05)

Table 3. Pearson correlation between different phenotypic properties of C. albicans isolates

	Proteinases	Phospholipases	Haemolytic activity	Biofilm formation
Proteinases	-	0.548	0.886*	0.001
Phospholipases	0.548*	-	0.563*	0.392
Haemolytic activity	0.886*	0.563*	-	0.076
Biofilm formation	0.001	0.392	0.076	-

* Statistically significant positive relationship.

proved that the virulence of *C. albicans* is related to genotypes. One study found that genotype A was more prevalent among invasive isolates and that genotype B and C were more common among none-invasive isolates (p<0.05) [29]. Also, Al-Karaawi *et al*. [28] reported that genotype A is the most predominant type in patients with oral *Candida* infections. Interestingly, the obtained results in the current study showed no significant correlation between the genotype (A, B, C) and the infection site (p =0.449, table 1). Moreover, our previous study showed a significant correlation between genotype and isolates from HIV patients (p<0.0001) (unpublished data) concluding that maybe *C. albicans* pathogenicity relies on a series of particular inherent and environmental factors mostly related to the host. Thus, for example, in order to facilitate the hyphae invasion, *Candida* has the ability to produce a variety of hydrolytic enzymes, such as proteases, lipases, phospholipases, esterase and phosphatases [25].

Resistogram (R). A direct comparison between data from the current study and the previously reported ones [20-21] in terms of resistogram methods is difficult due to the chemical con-

centrations of the used compounds, the number of strains and their prevalence. To facilitate data analysis, data from the 301 tested strains were grouped into only 5 resistotypes. The obtained general results revealed that most of the strains (86.816%) were completely resistant to sodium selenite, boric acid, sodium periodate and silver nitrate, but showed an unexpected sensitivity to cetrimide (resistogram pattern AB_DE).

Phospholipase activity (Pl), and secreted aspartyl proteinases (SPA) are determinant of not only commensal colonization, but also for the pathogenic potential of these yeasts [30]. For example, some authors showed that enzymatic secretion vary for *C. albicans* isolates of different strain and origin [31]. *C. albicans* secretes phospholipases A, B and C, which are considered to be virulence factors, being associated with the damage, adherence and penetration of the host cells [32].

Literature reports showed that 30-100% of the oral isolates produced phospholipases, with variable degree of enzymatic activity, due to the origin of the isolates, the phenotypic variability or the variation in the method employed. [23]. Vidotto *et al*. [33] did not find a relationship be-

tween phospholipase activity and genotype, regardless of the body site where the strains were collected. Their results are confirmed by this study.

Proteinase activity (Pt). *C. albicans* has various secreted hydrolytic enzymes of which secreted aspartyl proteinases contribute to the pathogenesis of candidiasis. Their role in pathogenicity is that the enzymes have the ability to degrade a number of important host factors [34]. However, some of the analyzed *C. albicans* strains proved to have a low level of proteinases and the rest of the strains did not present any proteinase activity.

Haemolysis (Hl) is known to facilitate iron acquisition from host erythrocytes, being an important virulence factor that contributes to *Candida ssp.* dissemination [24]. Watanabe *et al.* [35] stipulated that the beta haemolysin in *C. albicans* is a cell wall mannoprotein. Luo *et al.* [25] noticed that 6 of 14 *Candida* species tested did not produce beta-haemolysis although mannoprotein is a universal component of the *Candida* cell wall, maybe due to the variation of the cell wall mannoprotein content in different *Candida* species. It seems that the absence of glucose in the medium altered the sugar moiety of the mannoprotein leading to the loss of beta-haemolytic activities [24]. Even if the previous studies prove that haemolysis plays an important role in *C. albicans* disseminations, most of the investigated strains presented low haemolytic activity (95.49%).

Biofilm formation (BF). The ability of the fungal cells to form biofilms is the main factor that blocks the antifungal activity [36]. Candidaemia is often associated with the ability of *Candida* to form biofilms on indwelling medical devices such as central venous catheters and prosthesis [37]. A biofilm is a structural microbial community attached to a surface and packed with a self-produced extra-cellular matrix [38]. Biofilms are defined by resistance to antibiotics

and higher drug concentrations are required [39-40]. Biofilm formations vary between *Candida* isolates and categorizing them may help to predict how these will behave clinically [28].

There is a fundamental gap in understanding exactly what drives biofilm formation and its clinical implications. We aimed to evaluate and categorize *C. albicans* biofilms into distinct levels of biofilm formation. Our isolates were categorised mostly as low biofilm formers (LBF) for 60.77%, proving that their pathogenicity relies not only on their properties to form biofilms, but maybe on a mixture of factors related to their phenotype and definitely related to the host characteristics.

In conclusion, all the isolated and analyzed strains of *C. albicans* had strain-dependent variable levels of enzymatic activity, and they were not all biofilm producers. There were only a few significant statistical correlations in the studied virulence factors, including genotype and prevalence. Although much progress has been made in understanding the phenotypic and genotypic profile of *C. albicans*, still little is known regarding their interaction with the host.

Acknowledgments

Conflict of interest

The authors declare no conflict of interest.

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