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Antifungal resistance and predominance of virulence determinants among *Candida albicans* isolated from various clinical specimens

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ABSTRACT

Candida albicans remains the most common cause of hospital-acquired fungal infections due to its virulence determinants. Resistance to antifungal therapy has increased dramatically, narrowing the few available therapeutic options due to their potential toxicity. However, the association between *C. albicans* virulence determinants and resistance profiles needs further investigation. *C. albicans* (n=25) isolated from various clinical samples were identified. Antibiogram analysis of the tested isolates against different antifungal agents was performed and their minimum inhibitory concentrations (MICs) were verified. Virulence determinants including extracellular hydrolytic enzymes, biofilm formation, and cell surface hydrophobicity (CSH) were investigated. Correlations between virulence determinants and resistance profiles of the experimented isolates, in addition to their potential association with the source of clinical specimens, were analyzed. All isolates were amphotericin B, nystatin and micafungin sensitive, while 100% were clotrimazole, fluconazole and voriconazole resistant. Extracellular hydrolytic activities were detected in 52, 68 and 100% of the tested isolates for phospholipase, protease, and hemolysin, respectively, while CSH and biofilm production was shown in 24 and 20% of isolates, respectively. CSH had significant ($P<0.05$) positive as well as negative associations with amphotericin B and fluconazole MICs, respectively. Source of clinical isolates showed significant ($P<0.05$) influence on some resistance and virulence patterns.

Keywords: *Candida albicans*; resistance; Virulence; correlation; source.

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1. INTRODUCTION

Candida albicans is part of the normal human microbiome and considered to be a harmless commensal [1]. However, within a few decades,

candidiasis has become more frequent resulting in a wide array of superficial and deep infections [2] that range from those affecting the skin, mouth and vagina to systemic ones especially in

immuno-compromised, HIV, chemotherapy and organ transplant patients [1], which have eventually led to medical and economic public health burdens [3].

In *Candida species*, extracellular hydrolases are essential for nutrient acquisition, adherence, colonization, host tissue penetration, invasion, dissemination and evasion from host immune responses [4]. Moreover, biofilms are associated with soaring rates of resistance to environmental stresses, as well as altered gene expression profiles [5].

Antifungal agents available for candidiasis management include polyenes, azoles, fluoropyrimidines, and the recently developed echinocandins, with the latter being used as an alternative for infections resistant to the previously mentioned antifungal agents [6]. Antifungal drug resistance has emerged as a consequence of the extensive antifungal prescription and the insufficient resources concomitant with the escalating mycotic infections, which occurs through different mechanisms such as upregulation of drug efflux pumps leading to a reduced intracellular antifungal concentration, qualitative or quantitative changes in the drug targets as well as metabolic alterations [7].

This study aimed to appraise the antifungal resistance as well as virulence determinants comprising secreted hydrolases, CSH and biofilm production, as well as to evaluate the association between these phenotypes among *C. albicans* isolated from several clinical samples in Egyptian patients.

2. MATERIAL AND METHODS

2.1. *C. albicans* isolates

In the current study, *C. albicans* (n= 25) were isolated from various clinical samples; urine (n=

11), sputum (n= 9), drain (n= 2), pus (n= 1), wound (n= 1) and blood (n= 1), which were collected from El-Demerdash hospital, Ain Shams University (Cairo, Egypt) during 2015. Identification and maintenance of isolates were carried out as previously described [8], [9, 10].

2.2. Antifungal resistance profile

Antibiogram analysis against nystatin (NYS), amphotericin B (AMB), fluconazole (FLU), voriconazole (VOR), clotrimazole (CLT) and micafungin (MCF) for the tested isolates was carried out by the broth microdilution method and the MICs were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) M27-A3 protocol recommendations [10, 11].

2.3. Phospholipase assay

Production of phospholipase by *C. albicans* was assessed by the plate method described by [10, 12, 13]. The phospholipase index (P_z) was expressed as the ratio of the colony diameter to the precipitation zone diameter. A P_z value of less than 1 refers to phospholipase activity by the tested isolate [13].

2.4. Aspartyl protease assay

Production of secreted aspartyl protease by *C. albicans* was evaluated by the plate method described by [10, 14]. The protease index (Pr_z) was expressed as the ratio of the colony diameter to that of the unstained zone of proteolysis. A Pr_z value of less than 1 refers to aspartyl protease activity by the tested isolate [15].

2.5. Haemolysin assay

Haemolysin production by *C. albicans* was determined by the plate method previously described by [10, 16]. Hemolytic activity (H_z) was expressed as the ratio of colony diameter to that of the hemolytic zone. A H_z value of less than 1 refers to hemolytic activity by the tested isolate [15].

2.6. Cell surface hydrophobicity

The microbial adhesion assay to hydrocarbons (MATH) modified after [10, 17] was used to evaluate the cell surface hydrophobicity (CSH) of the tested isolates using xylene. The hydrophobicity percentage was calculated using the following formula: cell surface hydrophobicity (%) = $[1 - (A_1/A_0)] \times 100$. The hydrophobicity levels were classified as hydrophilic, moderately hydrophobic and strongly hydrophobic [18].

2.7. Biofilm formation

Production of biofilm was assessed using the crystal violet method previously described by [10, 19, 20]. The medium used for the biofilm assay was Sabouraud's dextrose broth (SDB) (Oxoid LTD., U.K) supplemented with glucose (8% w/v) to promote biofilm formation. The OD cut-off value (ODc) for biofilm production was described as three standard deviations above the mean absorbance of the yeast-free negative control and accordingly, the isolates were classified as none, weak, moderate and strong biofilm producers [21].

2.8. Statistical analysis

The current study's statistical analyses were completed with the SPSS version 22.0 statistical software. Non-parametric tests were carried out and the quantitative variables were designated as a minimum, median and maximum. Using the Spearman's rank correlation, the association between median MICs and virulence determinants was conducted and expressed in terms of Spearman's correlation coefficient (r_s). Differences between median values of quantitative variables were checked among the different sources groups using the Kruskal Wallis test [10]. The p -values of less than 0.05 were reckoned statistically significant in all tests.

3. RESULTS

3.1. Identification of *Candida albicans*

Typical *C. albicans* germ tubes were seen under a light microscope after human serum inoculation and incubation for 2-4 h at 37 °C for all the tested isolates (n= 25). *C. albicans* isolates

identification was confirmed using the *Candida* Chromogenic agar, which appeared as green colonies for all the tested isolates (n= 25).

3.2. Antifungal resistance profiles

Results of the antibiogram analysis revealed that *C. albicans* isolates demonstrated high resistance rates to CLT (92%) (MIC ≥ 1 µg/mL), VOR (100%) (MIC ≥ 1 µg/mL) and FLU (100%) (MIC ≥ 8 µg/mL). On the contrary, 100% of the isolates showed susceptibility to MCF (MIC < 0.5 µg/mL), AMB (MIC ≤ 1 µg/mL) and NYS (MIC < 8 µg/mL).

When evaluating the potential association between the source of clinical specimens and the resistance profiles of *C. albicans*, results revealed that isolates recovered from sputum samples showed statistically significant ($p < 0.05$) higher levels of resistance to NYS, CLT, and MCF as well as those in the miscellaneous group to NYS in comparison to those in the urine group samples (Table 1). Furthermore, no statistically significant difference ($p > 0.05$) was observed in the resistance patterns of AMB, FLU, and VOR based on the source of clinical specimens.

3.3. Virulence determinants assays

C. albicans virulence determinants (secreted hydrolytic enzymes, CSH, and biofilm formation) were evaluated in relation to the source of the clinical specimens and results were demonstrated in Fig. 1. In regards to the secreted hydrolases production, 52% of the isolates produced phospholipase with P_z varying from 0.7253 to 0.9524, whereas secreted aspartyl protease was proven in 68% with Pr_z values varying from 0.3889- 0.8889. Moreover, 100% of the isolates had hemolytic activity with H_z varying from 0.4667 to 0.6866. Concerning CSH, the tested isolates showed variable hydrophobicity levels and were accordingly distributed into different categories, where most of them were hydrophilic (76%) and the rest showed strong (16%) and moderate (8%) hydrophobic characters. Moreover, *C. albicans* isolates were grouped according to their ability to produce biofilms where 80% of isolates were non-biofilm producers and the rest demonstrated moderate (8%) and weak (12%) biofilm formation

capacities. It is worthy to note that the majority of the tested isolates showing strong cell surface

hydrophobicity and biofilm forming capacity were recovered from sputum samples.

Table 1. Comparison of median antifungal MICs across the different *Candida albicans* clinical specimens' sources

Source	Median MICs ($\mu\text{g}/\text{mL}$)					
	Amphotericin B	Nystatin	Clotrimazole	Fluconazole	Voriconazole	Micafungin
Urine	0.03 (0.03-0.06)	0.25 (0.006-0.50)	32 (2-64)	128 (64-128)	32 (32-32)	0.08 (0.03-0.125)
Sputum	0.06 (0.03-0.5)	0.5 (0.25-2.00)	64 (32-64)	128 (64-256)	32 (32-64)	0.16 (0.06-0.125)
Miscellaneous	0.03 (0.03-0.125)	0.5 (0.25-4.0)	32 (32-34)	128 (64-128)	32 (32-32)	0.08 (0.06-0.125)
P value	0.212	0.004	0.004	0.619	0.411	0.026

**p*-values of less than 0.05 were considered statistically significant. Minimum and maximum values are presented between brackets. MICs: Minimum inhibitory concentrations

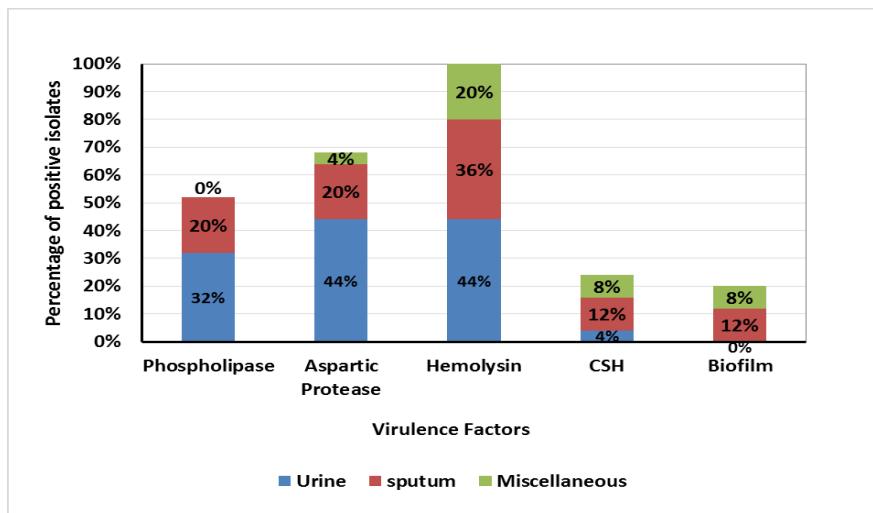


Fig. 1. Percentage of virulence determinants production (phospholipase, an aspartyl protease, hemolysin, cell surface hydrophobicity, and biofilm formation) by *Candida albicans* isolates with respect to the source of clinical specimens

When evaluating the potential relationship between the source of clinical specimens and virulence determinants of the isolates, the results revealed that *C. albicans* isolates recovered from urine samples had a statistically significant ($p <$

0.05) higher level of aspartyl protease production (i.e. lower Prz value), as compared to that recovered from the sputum and miscellaneous group samples (Table 2). However, no statistically significant ($p > 0.05$) difference was

detected in the virulence patterns of phospholipase, hemolysin, CSH, and biofilm relatively to the source of clinical specimens.

3.4. Correlation between antifungal resistance profiles and virulence determinants

Correlation between *C. albicans* antifungal resistance profiles and virulence attributes is shown in **Table 3**. Correlation coefficients highlighted significant ($p < 0.05$) positive ($r_s = 0.414$), as well as negative ($r_s = -0.439$) associations between CSH and AMB and FLU MICs, respectively.

Table 2. Comparison of median values of virulence factors across *Candida albicans* clinical specimens' sources

Virulence Factor	Source of clinical specimen			<i>p</i> value
	Urine (n = 11)	Sputum (n = 9)	Miscellaneous (n = 5)	
Phospholipase (Pz)	0.8889 (0.7326-1.0000)	0.8586 (0.7253-1.0000)	1.0000 (1.0000-1.0000)	0.057
Asp. Protease (Prz)	0.6143 (0.3889-0.8889)	0.8194 (0.4292-1.000)	1.0000 (0.4792-1.000)	0.043
Haemolysin (Hz)	0.5503 (0.4848-0.6458)	0.5556 (0.4667-0.6866)	0.5707 (0.5185-0.6345)	0.624
Cell surface hydrophobicity	9.6296 (2.8700-20.4684)	8.5685 (5.5000-90.7870)	15.6371 (5.0830-86.3283)	0.498
Biofilm (OD ₅₉₀)	0.03733 (0.0155-0.0683)	0.0525 (0.007-0.450)	0.034 (0.009-0.378)	0.832

**p*-values of less than 0.05 were considered statistically significant. Minimum and maximum values are presented between brackets. Pz, Phospholipase index; Prz, Protease index; Hz, Haemolytic activity; OD, Optical density.

Table 3. Correlation coefficients (r_s) between virulence factors and antifungal MICs of *Candida albicans* clinical isolates

Antifungal MICs	Virulence factors, p^* (r_s)				
	Phospholipase	Asp. protease	Haemolysin	CSH	Biofilm
Amphotericin B	0.295 (0.152)	0.329 (0.108)	-0.054 (0.796)	0.414 (0.04)	0.377 (0.063)
Nystatin	0.293 (0.155)	0.210 (0.314)	0.116 (0.581)	0.095 (0.652)	0.037 (0.859)
Clotrimazole	0.139 (0.508)	0.241 (0.245)	-0.025 (0.907)	0.340 (0.096)	0.033 (0.875)
Fluconazole	0.026 (0.900)	-0.315 (0.125)	0.229 (0.271)	-0.439 (0.028)	-0.346 (0.091)
Voriconazole	-0.210 (0.314)	-0.144 (0.493)	0.170 (0.417)	-0.198 (0.342)	-0.326 (0.112)
Micafungin	0.014 (0.948)	0.208 (0.319)	0.199 (0.341)	0.129 (0.538)	-0.166 (0.429)

* p -values of less than 0.05 (**in bold**) were considered statistically significant. MICs: Minimum inhibitory concentrations

4. DISCUSSION

As the most prominent opportunistic fungal pathogen, candidiasis has increased dramatically worldwide, with the species *C. albicans* responsible for most of these infections [22]. The current study was conceived to explore the relationship between the resistance and virulence profiles of *C. albicans* isolated from various clinical samples in Egyptian patients. Antibiogram analysis against six antifungal drugs was performed and the isolates were further evaluated for production of secreted hydrolases, CSH, and biofilm formation.

The antibiogram analysis revealed high susceptibility rates to MCF, AMB, and NYS. These observations are in accordance with some previous studies have reported susceptibility to AMB [23], NYS [24] as well as MCF [25]. However, high resistance rates were detected against CLT, FLU, and VOR. These findings are

in accordance with a previous study in Portugal [23] regarding the FLU resistance, while others reported lower resistance rates for CLT and VOR such as 23% in India [26] and 3.2% in China, respectively. These variations in antifungal resistance profiles can be pertained to the different sources and localities of collections of the clinical specimens in addition to the rational antifungal prescription. Furthermore, some of these studies had their isolates collected from cancer and HIV patients and involved other non-*albicans Candida*. It is noteworthy that azole resistance can be attributed to the overexpression or mutations in ERG11 gene, which encodes the lanosterol 14 α -demethylase enzyme [27] or overexpression of drug efflux pumps, which results in a decreased intracellular drug concentrations [28]. The current observations may also highlight the possible impact of the inappropriate antifungal use on the emergence of such massive azole resistance rates in Egypt, the

fact that could be attributed to providing treatment for patients without performing antifungal susceptibility testing, which is needed to customize an optimum therapeutic plan for each case individually. Moreover, species identification is an essential step to provide appropriate treatment to critically ill patients [29].

The current results reflected significant associations ($p < 0.05$) between the source of clinical specimens and the resistance patterns of the tested isolates. Deorukhkar et al [30] reported that fluconazole resistance was more seen in *Candida tropicalis* isolates recovered from cases of candidemia, oropharyngeal candidiasis, and vulvovaginal candidiasis, while AMB resistance was more common in *C. tropicalis* recovered from blood cultures and oropharyngeal swabs. These observations suggest that the source of clinical specimens can have an impact on the susceptibility of *Candida* species to antifungal therapy. However, a higher number of samples is required to confirm such a conclusion.

In the current study, production of hydrolytic enzymes was evaluated as a major part of *C. albicans* pathogenicity determinants. Haemolysin, an aspartyl protease, and phospholipase were detected in 100, 68 and 52% of the *C. albicans* isolates, respectively. Various production rates of extracellular hydrolases have been reported by other workers as 47.6%, [31] 82.1% [32] and 100% [33] for phospholipase, aspartyl protease, and hemolysin, respectively, which emphasizes the importance of secreted hydrolases as potent *C. albicans* virulence attributes. In addition, the majority of the isolates demonstrated hydrophilic nature, while fewer isolates showed moderate and high hydrophobicity levels. These findings are in agreement with a previous report in Portugal [23], where *C. albicans* was found to have the lowest hydrophobic character among the different *Candida* spp. tested. Furthermore, biofilm

production was evident in 20% of the tested isolates, which reflects similarities with a Swedish study [34], where *C. albicans* isolates showed low biofilm production in comparison to the other *Candida* spp., indicating that biofilm formation may be more essential for non-*albicans* *Candida* infections than the *C. albicans* ones.

The present study revealed significant associations ($p < 0.05$) between the source of clinical specimens and virulence determinants of *C. albicans*. In a reported study by Taylor et al [35], site-specific virulence of *C. albicans* was evident for mucosal sites, although few isolates were virulent systemically. *Candida* spp. have the ability to colonize and virtually infect each organ in the human body, the fact that makes it fundamental for the opportunistic pathogen to acquire a number of similar but independently regulated, functionally distinct hydrolytic enzyme genes expressed under a variety of environmental conditions in order to survive and induce infection at different body sites [36].

In an attempt to explore the potential association between *C. albicans* resistance and virulence profiles, the present study revealed significant positive and negative associations ($p < 0.05$) between CSH and AMB as well as FLU MICs, respectively. In a previous Spanish study [37], *C. tropicalis* isolates, which were AMB-resistant showed enlarged cell walls in comparison to AMB-sensitive ones. These findings could be attributed to higher β -1,3-glucan levels in the cell walls of the resistant *Candida* isolates rendering them more hydrophobic in nature. In another report conducted by Graybill et al [38], they pointed out that compromised pathogenicity of some *C. albicans* isolates was correlated to rising fluconazole resistance, which indicates that these limited-virulence isolates may not prominently induce infection.

5. CONCLUSION

The current study reported the susceptibilities of *C. albicans* isolates to various antifungal agents representing different classes and evaluation of different pathogenicity mechanisms in addition to their potential relationship between them and the source of clinical specimens. According to the current study's findings, the development of novel therapeutic tactics combining drug resistance and virulence mechanisms in candidiasis management would be recommended. Further mechanistic studies are required to explore the multi-factorial profile of gene expression in *C. albicans* clinical isolates. The current study would also encourage the use of other antifungal agents than azoles in the management of candidiasis as well as applying combination therapy in dose regimens to decelerate the escalating azole resistance rates.

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Conflict of interest

The authors declare no conflict of interests.

List of abbreviations

CLSI, Clinical and Laboratory Standards Institute; SDA, Sabouraud's dextrose agar; SAPs, secreted aspartyl proteases; NAC, non- *albicans* *Candida*; BMD, broth microdilution; MOPS, N-morpholinepropanesulfonic acid; P_z, Phospholipase index; BSA, bovine serum albumin; Prz, Protease index; Hz, Haemolytic activity; MATH, microbial adhesion assay to

hydrocarbons; S, susceptible; S-DD, susceptible dose dependent; I, intermediate; R, resistant; MICs, minimum inhibitory concentrations; AMB, amphotericin B; NYS, nystatin; CLT, clotrimazole; FLU, fluconazole; VOR, voriconazole; MCF, micafungin; CSH, cell surface hydrophobicity; OD, optical density; ODc, OD cut-off; CFU, colony forming unit, PBS, phosphate buffered saline.

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