RESEARCH / INVESTIGACIÓN

Role of *Candida glabrata* as nosocomial pathogen and its susceptibility to Fluconazole, Voriconazole, Caspofungin, Micafungin and Amphotericin B

Teeba Hashim Mohammed[1], Mohsen Hashim Risan[1], Mohammed Kadhom[2], Emad Yousif[3*]

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Abstract: Candida has different types that could cause bloodstream infections. A total number of 150 samples were collected from candidemia patients and examined. The *Candida spp.* Species isolated from blood samples were analysed. These were identified by culturing the species using different media, namely the chromogenic agar test. Then, the virulence factors of all samples were tested. *The Candida* glabrata isolates were tested with six commercial antifungal drugs. *C. glabrata* 67 (44.6%), *C. albicans* 34 (22.6%), *C. krusei* 18 (12%), *C. tropicalis* 17 (11.3%), and *C. parasilosis* 14 (9.3%). the production of phospholipase ranged between 0.63-0.99 mm. It was found that 96% of the species showed phospholipase activity in aerobic conditions. The protease activities of *Candida spp.* Isolates were experimentally tested by area of inhibition around the colonies, where 59.3% had the double (++) protease activity, 31.4% with (+) grade, and 9.3% had (–) grade or clear zone around the colony. The hemolytic capacity ranged from 0.69-0.89 in the optimum aerobic environments. Finally, 38.33% of the isolated *Candida spp.* were positive and 61.67% negative for biofilm formation. Out of the total positive *Candida spp.* for biofilm formation, 21.73% were strong biofilm producers, and 78.27% were weak. Minimum fungicidal concentration (MFC) of Fluconazole for *C. glabrata* isolates was not appropriate (NA) due to the occurrence of low inhibition tested for species. Micafungin exhibited the lowest fungicidal activity against *C. glabrata* ranging from 0.03 - 0.125, while Fluconazole showed the highest.

Key words: Candidemia, chromogenic agar, *Candida glabrata*, Antifungal.

Introduction

Candida is a significant genus of Ascomycete fungi, commonly called yeast, consisting of approximately 150 species, more than 20 of them of clinical importance[1]. *Candida spp.* became the fourth most public reason for bloodstream infections (BSI) more than two decades ago[2,3]. The highest occurrence rate was described in the USA[4], where candidemia signifies an essential danger for hospital patients[5]. Hence, *C. albicans* and *C. glabrata* remain the primary cause of aggressive candidiasis, responsible for 50% of all cases followed by other species of *Candida*, such as *C. tropicalis* and *C. parapsilosis*[6,7].

The effect of *Candida* species is presented by interrupting BSI incidents; their phospholipase and protease activity and the sensitivity towards the main antifungal agents were available[8]. Described mortality data comprised the age, period of hospitalization, skin infection, severe renal failure, preservation of the dominant venous line, and mechanical ventilation[9,10]. The germs become active at 37 °C, where protease and phospholipase are produced and facilitated adherence to the surfaces of host cell membranes; these were measured to be significant factors in starting the infection. Proteinase and phospholipase release can prime the membranes dysfunction or uniform rupture that assists the microbe to adhere to the host[11]. The communication in the pathogenesis of aggressive candidiasis, proliferation in fungal colonization is the best significant factor in the invasive candidiasis for pathogenesis. The density and colonized apparent area are responsible for the strength of the infection. Precise identification of *Candida* species helps control the hazards of candidemia infections and transmission from exogenous sources in certain patients[12]. Virulence factors are also associated to a great degree with the antifungal resistance shown by the microorganisms.

Furthermore, the capabilities of *Candida* species to produce drug-resistant virulence factors are significant in their development of human disease[13]. Recognition of *Candida* to the type's level is significant to improve the range of the antifungal agent to be prescribed. On the other hand, essential and developing resistance to azoles characterizes a significant trial for experimental, therapeutic, and prophylactic approaches. The range of candidemia has altered with the appearance of *C.glabrata* species, a strain with the hazard of increased transience and antifungal treatment of drug resistance, particularly in immunocompromised and very ill patients. The situation is very significant to recognize *Candida* to the type's level to improve the range of the antifungal agent[14].

Materials and methods

Collection of samples

Samples were collected from 150 patients with leukaemia and dialysis who were hospitalized at Madinat Al-tib hospital. The collection continued over four months, from September 2019 to January 2020. Processes of blood culturing and yeast segregation were conducted in the fungus laboratory at the College of Biotechnology/ Al-Nahrain University. The blood samples were inoculated on an SDA medium with chloramphenicol. All plates were incubated at 37°C±1 for 24, 48 hrs. Later, samples were examined and biochemical tests were performed to confirm the diagnosis of the species; the approval was obtained to take blood samples from the hospital administration and the discharged patients.

Isolation of *Candida spp*

Samples were inoculated on the appropriate culture medium containing sabouraud dextrose agar with chloramphenicol. All plates were incubated in aerobic conditions of 37 °C for 48 h[15].

[1] College of Biotechnology, Al-Nahrain University, Baghdad, Iraq.
[2] Department of Renewable Energy, College of Energy and Environmental Sciences, Alkarkh University of Science, Baghdad, Iraq.
[3] Department of Chemistry, College of Science, Al-Nahrain University, Baghdad, Iraq

Corresponding author: emad_yousif@hotmail.com

Identification of *Candida spp.* Isolates

First, single colonies were separated from the prime positive cultures; then, they were isolated according to published protocols[16] that involved the following examination. All isolates were grown on sabouraud dextrose agar and incubated at 37 ºC for 48 hrs. Pure isolates of *Candida* colonies were examined to explore their form, size, color, and surface. Chromogenic agar plates were inoculated by streaking. *Candida* colonies using sterile loop then incubated at 37 Cº±1 for 48 hrs. The growth and color of colonies were observed.

The medium was prepared by dissolving 45.9 grams of the standard medium in one liter of purified water, mixed well, and melted by heating with repeated agitation. The mixture was boiled for 2 minutes until the complete dissolution and was not autoclaved[17].

Virulence Factor Tests

The quick preliminary medical identification of *Candida spp.* usually depends on the detection of germ tubes from *Candida* cells, where their width or length is about three to four times the size of the cell. *Candida spp.* cells were added to 1000 µl of human serum with a sterilized loop, and incubated aerobically for three hours at 37 ºC. Then, cells were visualized under a microscope[18].

The egg yolk agar technique is used to test the phospholipase activity[19]. The culture medium contained of 1 L of SDA comprising (1M NaCl, 0.005 M CaCl2, and 10% egg yolk). From prepared yeast suspension, 10 microliters were taken to one McFarland (3×10^8 CFU/ml) inoculated at 37 Cº for 48 hr. in the petri dish, 3 replicates per sample. After that, it was incubated at 37 °C for 3 days in aerobic conditions; the significance of phospholipase action was calculated with this formula: (Pz value= Colony diameter/ (Colony diameter + zone of precipitation)[20]. To test the proteinase activity, bovine-serum albumin agar was used. The prepared agar consisted of 0.1% KH_2PO_4, 0.05% $MgSO_4$, 2% agar, and 1% bovine serum albumin at a final pH of 4.5. Similar inoculation aerobic conditions as above were applied for 48 h. The existence of the proteinase activity test was determined by observing a clear zone around the *Candida spp.* colonies[21]. Regarding the structural haemolytic action, SDA consisted of 7 ml human blood, 3g glucose, and 90 ml of sterile media was prepared using an autoclave with a final pH of 5.6 ± 0.2. 10 microliters were taken to one McFarland (3×108 CFU/ml) inoculated onto a dish; this was replicated 3 times per sample. Then, it was incubated at 37 °C for five days in aerobic conditions. After incubation, a clear/semi-clear zone around the yeast colony was detected as a positive haemolytic activity. The significance of hemolysis activity (Hz) was tested according to the equation: Hz rate = Colony diameter / (Colony diameter + Zone of precipitation) Agreeing to this scheme, activity kinds were recognized allowing to the Hz catalogue: Hz<0.69= very strong (++++), Hz = 0.70- 0.79= strong (+++) , Hz = 0.80- 0.89= slight (++), Hz = 0.90- 0.99=frail (+), and Hz =1 that is to say "Negative" results[22]. Biofilm production was tested using different recognition tests for *Candida spp.*, which needs the use of a specifically prepared brain heart infusion broth (BHI), solid medium supplemented (agar) with glucose and Congo red indicator. The medium contained BHI (37 gm/L), agar no.1 (10 gm/L), glucose (80 gm/L), and Congo red stain (0.8 gm/L) as an indicator[23].

Determination of minimum inhibitory concentration (MIC) of several antifungals for *Candida glabrata*

The MIC of Fluconazole, Voriconazole, Caspofungin, Micafungin, Amphotericin B, and Flucytosine against *Candida glabrata* was determined broth microdilution method established on the Clinical and Laboratory Standards Institute (CLSI) natural resistance + base (bioMerieux, USA). Manufacturers' recommendations were strictly followed to obtain adequate measurements, which confirmed sterility and efficacy of the prepared media and used mixtures. Card type AST-YS07 of a barcode no. 287 (bioMerieuxm, USA) was used with an analysis time of 24 hours[24].

Determination of MFC for *Candida glabrata*

For MFC determination, samples with different concentrations of MIC and *C. glabrata* were subcultured in Petri dishes containing SDA and incubated at 37 °C for 48 hours. The MFC is the minimum product concentration that stops apparent growth; it is the minor concentration capable of killing the yeast[25].

Results

Group's classification

In this study, 150 samples were taken from three groups. The first group comprised 50 infected patients (women and men), as illustrated in Table 1; the second group comprised 50 patients of children with leukemia (Table 2); the third group comprised 50 patients with dialysis (women and men), as listed in Table 3. The first and third groups were divided into five categories, while the second group contained three subcategories. Generally, it was found that the total number of infected patients with *Candida* present in the bloodstream were isolated as follows: 67(44.6%) with *C. glabrata*, 34 (22.6%) with *C. albicans*, 18 (12%) with *C. krusei*, 17 (11.3%) with *C. tropicalis*, and 14 (9.3%) with *C. parapsilosis*.

Determination of *Candida* species by Chromogenic agar Medium

Candida spp was cultivated in a chromogenic agar medium plates and the characteristic dyed colonies were obtained after incubation; the medium supported the development of all isolates. *C. albicans* isolates produced green colonies when incubation for 48 h, while *C. glabrata* formed light pink smooth colonies and *C. krusei* colonies were pink; the distribution graduated from a light surrounding to a dark center. Also, *C. tropicalis* color was dark blue and *C. parapsilosis* formed pale colored colonies[26] as shown in Figure 1.

Determination of Virulence Factors for *Candida* Species

The development of germ tubes was achieved, as long tubes, within 2 hours, and positive results of *Candida albicans* isolates were observed, while no *C. tropicalis* production within that time was detected, as shown in Figure 2.

The germ tubes were grown in an incubator, and a particular diagnostic was used for distinguishing the *C. albicans* by separating it from other yeasts. Other species of yeasts, in general, did not produce germ tubes during this 2-hour incubation period. The Pz value varied between 0.63–0.99 mm when species were measured. It was found that 144 (96%) *Candida spp.* isolates expressed phospholipase activity in aerobic conditions, whereas the rest did not. *Candida spp.* possibly vary depending on the species and basis of isolates; Figure 3 shows the phospholipase activity of different *Candida* types on egg yolk agar medium.

The protease activities of *Candida spp.* isolates were tes-

Age group	No. of infected in women/25	(%)	No. of infected in men/25	(%)
> year 20	4	16	3	**12**
20-30 year	2	8	1	**4**
31-40 year	5	20	8	**32**
41-50 year	7	28	7	**28**
51-75 year	7	28	6	**24**
Total	25	100	25	**100**

Table 1. Age group relationship with the number (and percentage) of infected patients with *Candida spp* from blood samples for adults (women and men).

Age group	No. of infected Children/ 50	(%)
4-8 years	21	**42**
8-10 years	12	**24**
10-12 years	17	**34**
Total	50	**100**

Table 2. Age group relationship with the number (and percentage) of infected patients with *Candida spp* from blood samples for children.

Age group	No. of infected women/25	(%)	No. of infected men/25	(%)
> year 20	-	0	1	**4**
20-30 year	1	4	3	**12**
31-40 year	2	8	4	**16**
41-50 year	5	20	6	**24**
51-75 year	17	68	11	**44**
Total	25	100	25	**100**

Table 3. Age group relationship with the number (and percentage) of infected patients with *Candida spp* from blood samples of patients with dialysis for women and men.

ted for 3 days after an inoculation loop on bovine-serum albumin agar by the inhibition area around the colony. Eighty-nine (59.3%) isolates had the double (++) protease activity, while the grade (+) of protease activity was for forty-seven (30%) of isolates and fourteen (9.3%) had the grade (–) or clear zone around the colony as shown in Figure 4.

Commonly, the hemolytic index is 0.69–0.89 in optimal aerobic environments. In this study, all experimental *Candida spp* isolations from blood samples had hemolysis activity when cultivated on SDA involving 7% human blood and 3% glucose.

Out of all *Candida spp.* isolates, the biofilms were 81(54.4%) negative, 28(18.6%) strong positive, and 41(27%) weak positive resulted from patients with leukemia and dialysis who are lying in hospital. The number of recovered *Candida spp.* from experimental samples was *C. glabrata* 34(49.2%), followed by *C albicans* (15.8%), *C. krusei* (12.5%), *C.tropicalis* (11.6%), and *C. parapsilosis* (10.9%). Among all *Candida isolates*, 46% were positive biofilm, and 54% cases were harmful biofilm. However, within the positive *Candida* biofilm, 21.73 % were strong biofilm producers, and 78.27% were weak; Figure 5 shows the biofilms.

Determination of MICs for Candida glabrata

The results of the susceptibility analysis of *Candida glabrata* isolates are summarized in Table 7. In all examinations, the minimum inhibitory concentrations (MICs) of the control *C. glabrata* isolated yeast were within the recognized limits (data are not shown). However, the CLSI's wild-brand MIC distributions were tested against six commercial antifungal drugs, namely: Fluconazole, Voriconazole, Caspofungin, Micafungin, Amphotericin B, and Flucytosine. The results showed that Fluconazole was the most active antifungal against *Candida glabrata* with different concentrations, whereas micafungin was the lowest antifungal resistance against *Candida glabrata*.

The MFC ranges for the six drugs though significant change for the different organisms. Fluconazole, Voriconazole, and Flucytosine showed fungicidal activity against *C. glabrata* (MFC50s where appropriate ≥1 g/mL). MFCs of Fluconazole for this yeast was not appropriate (NA) due to the occurrence of small inhibitions tested number for species; table 8 illustrates this.

DISCUSSION

Hospitalization (especially in cancer cases), location of central venous dialysis, and who preceding antimicrobial therapies control the number of candidemia infections. Candidemia in this concept was related to the prolonged stay (more than 15 days) of patients[27]. It is essential to mention that el-

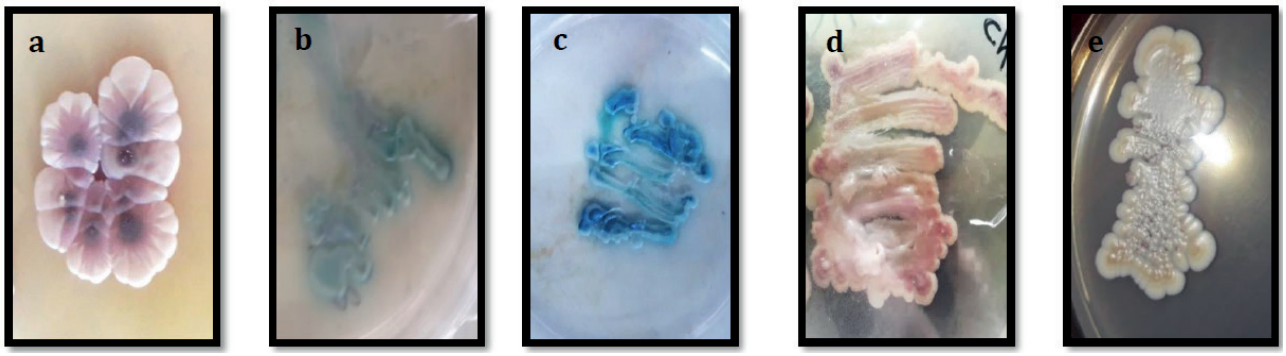

Figure 1. Observation of *Candida spp.* in Chromogenic agar media, (a) *C. krusei*, (b) *C. albicans*, (c) *C. tropicalis*, (d) *C. glabrata*, and (e) *C. parapsilosis*.

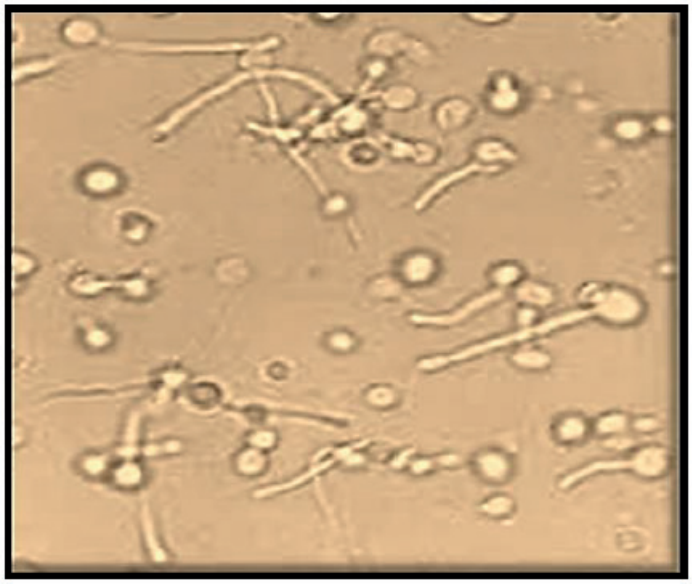

Figure 2. Germ tube of *C. albicans* growth on human serum at 37°C for 2hrs (40 X)..

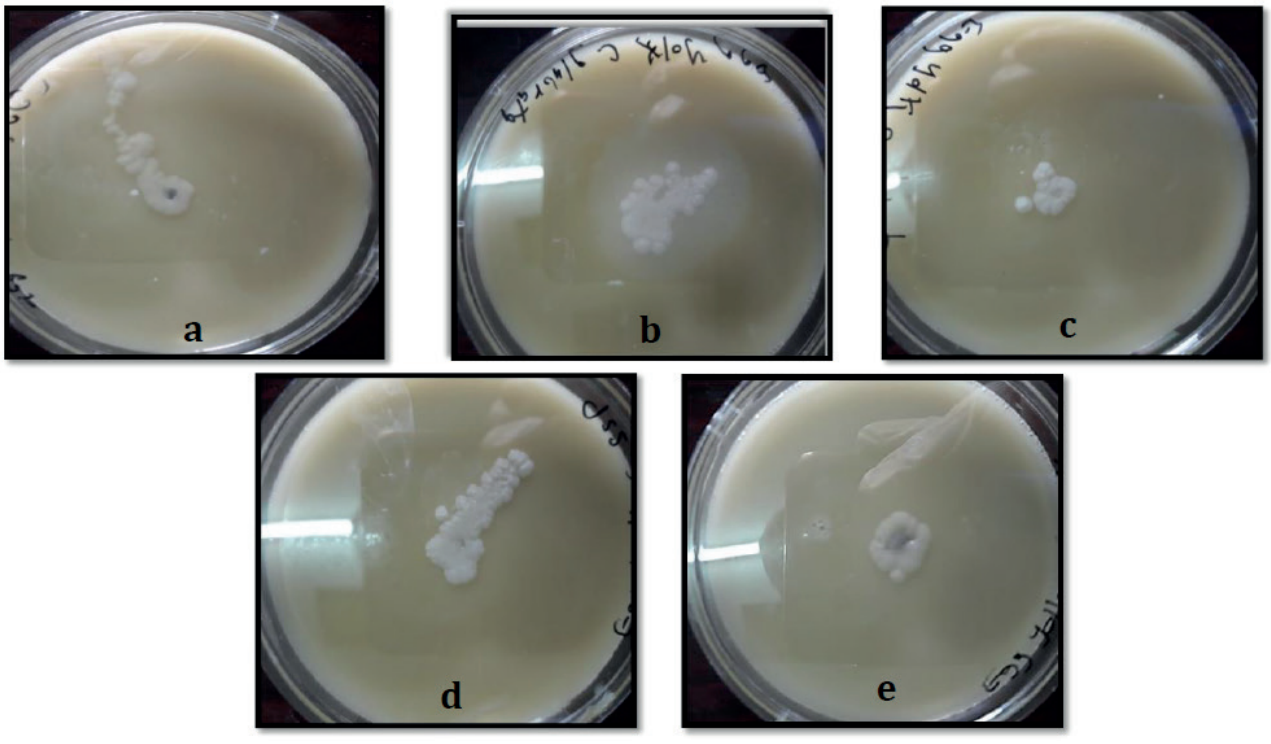

Figure 3. Phospholipase activity of (a) *C. krusei*, (b) *C. albicans*, (c) *C. tropicalis*, (d) *C. glabrata*, and (e) *C. parapsilosis* on egg yolk agar medium at 37 °C for (24-48) hrs.

Candida spp.	Number of Isolates (%)			Total
	Negative	Moderate (++)	Large (+++)	
C glabrata	3	22	42	67
C. albicans	0	11	23	34
C. krusei	1	12	5	18
C. tropicalis	0	6	11	17
C. parapsilosis	2	9	3	14
Total	6(4%)	60(40%)	84(56%)	150

Table 4. Phospholipase activity of *Candida spp* on egg yolk agar medium at 37 °C for (24-48) hrs.

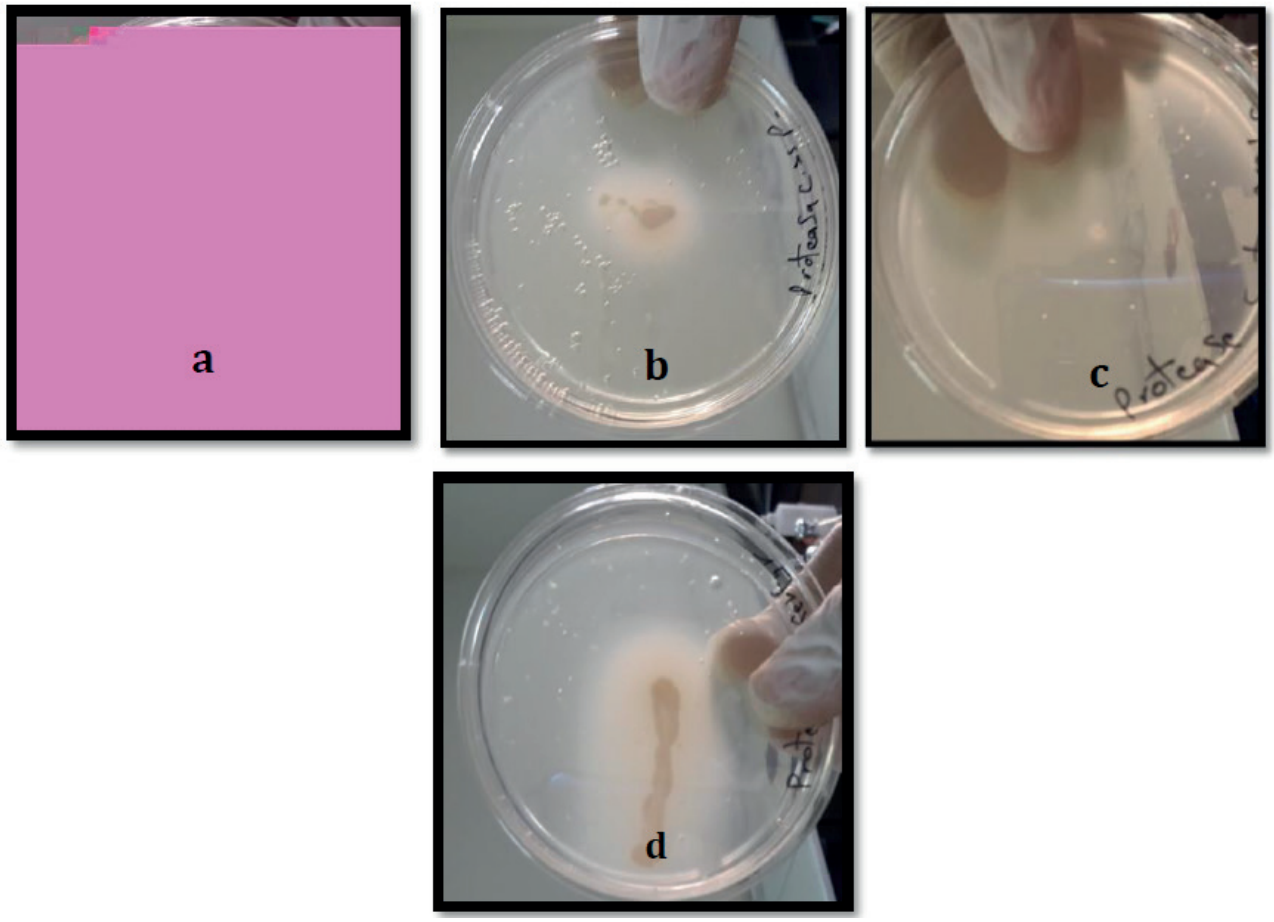

Figure 4. Protease activity from (a) *C. krusei*, (b) *C. albicans*, (c) *C. tropicalis*, (d) *C. glabrata*, and (e) *C.parapsilosis* on bovine-serum albumin agar at 37°C for (24-48) hrs.

Candida ssp.	Number of Isolation (%)			Total
	grade (–)	grade (+)	The double-positive (++)	
C glabrata	4	10	53	67
C. albicans	2	13	19	34
C. krusei	5	9	4	18
C. tropicalis	1	4	12	17
C. parapsilosis	2	11	1	14
Total	9.3%	% 31.4	59.3%	150

Table 5. Protease activity from *Candida spp* on bovine-serum albumin agar at 37°C for (24-48) hrs.

Candida spp.	Biofilm negative no. (%)	Positive biofilm no. (%)		
		Strong	Weak	Total of positive (%)
C. glabrata	33(40.7%)	16(57.1%)	18(43.9%)	34(49.2%)
C.albicans	27(33.3%)	3(10.7%)	4(9.7%)	7(10.1%)
C.krusei	8(9.8%)	4(14.2%)	6(14.6%)	10(14.4%)
C.tropicalis	9(11.1%)	2(7.1%)	6(14.6%)	8(11.5%)
C. parapsilosis	4(4.9%)	3(10.7%)	7(17%)	10(14.4%)
Total	81(54%)	28(18.6%)	41(27.3%)	69(46%)

Table 6. Biofilm formation results of 150 *Candida* isolated by Congo red agar.

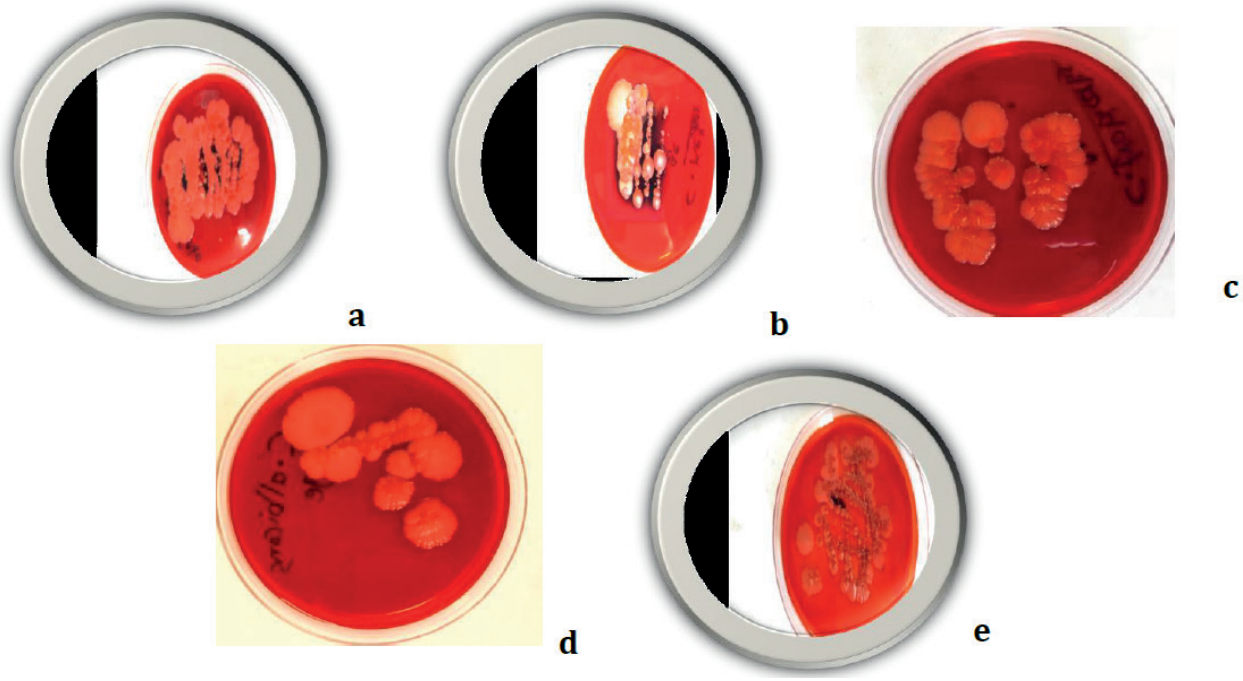

Figure 5. Biofilm formation resulted by (a) *C. krusei*, (b) *C. albicans*, (c) *C. tropicalis*, (d) *C. glabrata* and (e) *C. parapsilosis* on Congo red agar

Antifungal for *C.glabrata* (67)	MIC (mg/L)							
	0.03	0.06	0.125	0.25	0.5	1	S%	R%
	S							
Fluconazole	4	3	6	9	19	23	95.5%	4.5%
Voriconazole	1	2	6	15	17	9	74.6%	25.4%
Caspofungin	-	-	2	7	12	9	44.7%	55.3%
Micafungin	-	2	-	6	8	3	28.3%	71.7%
Amphotericin B	-	4	10	11	14	20	88%	12%
Flucytosine	2	4	8	11	18	19	92.5%	7.5%

S: sensitive; R: resistant

Table 7. Minimum Inhibitory Concentration (MIC) for *C. glabrata* isolates.

Candida spp.	Antifungal Agent	MFC (mg/mL)	
		MFC50	Range
C. glabrata	Fluconazole	0.03 -1	NA
	Voriconazole	0.03 -1	NA
	Caspofungin	0.03 - 0.125	0.125
	Micafungin	0.03 - 0.125	0.125
	Amphotericin B	≥ 0.06	0.06
	Flucytosine	0.03-1	NA

Table 8. Minimum Fungicidal Concentrations (MFC) for *C. glabrata*.

NA.: not-appropriate

der patients were more likely to receive insufficient antifungal therapy. Candidemia is connected with high mortality rates, increasing the hospitalization staying period, and raising therapeutic care[28]. The production test of the germ-tube has the benefit of being simple, effective, inexpensive, and fast to identify the *Candida spp*[29]. The chromogenic agar media are different; a selective medium was used to identify Candida spp fast. As a result, the capability to distinguish these species macroscopically was achieved[30].

The impact of the phospholipase enzyme was presented by digesting the cells' membrane of the host, producing cell lysis, and altering the external structures that increase adherence and result in infection. Therefore, phospholipase is possibly used as one of the limits to distinguish virulent hostile strains from non-hostile colonizers[20]. Mohandas and Ballal, (2008)[31] was referred that *Candida spp.* isolated from blood has more phospholipase activity. In two different studies from Turkey *Candida* isolates from blood culture, phospholipase activity resulted was (60.3% - 100%). The differences in results were retained because of distinctions in the sources of yeast isolates and the percentage of occurrence of isolates[32].

The essential protease enzyme of *Candida spp.* evades the attack of host tissues. The majority of tested *Candida spp.* had a positive protease activity. These conclusions propose that generating the protease may play a significant role in the pathogenesis of candidemia produced by *Candida spp.* It has been testified that more than 90% of Candida spp isolates proteinase products[33]. Proteinase production was identified in 74.56% of *Candida spp*. It was also testified that 88% of *Candida spp.* isolated from blood-linked intense proteinase action.

Moreover, *Candida spp.* atmospheric environments did not damage isolates testifying strong proteinase action. The alterations between studies may include product from differences in the sources of incubation, isolates and times[34].

Sachin *et al.* (2012)[35] reported that 94.8% of *C. albicans* samples presented hemolysis activity, whereas hemolysis activity of additional non-*C.albicans* extended from 7% to 60%; similarly, Sathiya *et al.* (2015)[36] described 100% of *Candida spp.* isolates exhibited β-hemolytic.

Form biofilms are associated with pathogenicity and should be considered an important virulence determinant during candidiasis. Biofilms may help fungi maintain the role of commensal and pathogen by evading host immune mechanisms, resisting antifungal treatment, and withstanding the competitive pressure from other organisms[37].

Our results are in good agreement with Khan's *et al.* results (2012)[38], where amphotericin B and Fluconazole showed vigorous antifungal activity against *Candida* due to their MIC standards of under 100 µg/ml. Also, the antifungal proved to be an active inhibitor against *C. glabrata*[39]. According to Hafidh *et al.* (2011)[40], the MFC/MIC relation is used to identify the influence of the antifungal effect against a specific pathogen. When the percentage of MFC:MIC result is between 1:1 and 2:1, the biochemical analysis measured fungicidal. In addition, Mohamadi *et al.* (2014)[41] also testified the fungicidal action of strain, using the destroy time technique in *C. albicans* and expending agar disc diffusion test, for example *C. albicans*, *C. tropicalis*, and *C. glabrata*. Fungicidal action is clinically more significant than fungistatic action. The prophylactic usage of fungistatic treatments has been related to an increased rate of innate or acquired immunity in clinical isolates[24-44].

Conclusions

Candida samples vary in their ability to form virulence factors, such as the production of phospholipase, proteinase, biofilms, and hemolysis when taken from leukemia patients. *Candida spp* samples were more sensitive to Fluconazole followed by Flucytosine, Amphotericin B, Voriconazole, Caspofungin, and Micafungin.

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Conflicts of Interest

The authors declare no conflict of interest.

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