Proteinase, phospholipase, biofilm forming abilities and antifungal susceptibilities of Malaysian Candida isolates from blood cultures

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This study was conducted to determine the proteinase, phospholipase, and biofilm forming abilities of Candida isolates in blood cultures of specimens from patients at the University Malaya Medical Center, Kuala Lumpur, Malaysia. Proteinase and phospholipase activities were detected in 93.7% and 73.3%, respectively, of 15 Candida albicans isolates. Amongst the 26 non-C. albicans Candida isolates, proteinase and phospholipase activities were detected in 88.5% and 7.7% of the isolates, respectively. There was no significant difference in the expression levels of proteinase amongst the Candida isolates studied (P = 0.272), but the phospholipase activity of C. albicans was significantly higher than that of the non-C. albicans Candida isolates (P = 0.003). There was no significant difference in the biofilm forming abilities of C. albicans and non-C. albicans Candida isolates on the polystyrene microtiter wells (P = 0.379). In addition, the findings of this study demonstrate increased resistance of Candida isolates in biofilms to amphotericin and fluconazole, as compared to their planktonic counterparts.

Keywords proteinase, phospholipase, biofilm, antifungal susceptibility, Candida

Introduction

Yeasts of the genus Candida are colonizers of human skin and mucous membranes that have been recognized as opportunistic pathogens among immunocompromised patients. Candidiasis has become a major problem in healthcare settings as it is generally difficult to diagnose and treat [1,2]. The higher incidence of candidiasis amongst hospitalized patients in recent years has been attributed to advances in surgery, cancer treatment, critical care, the increase usage of broad-spectrum antimicrobials and HIV infections [2]. The association of candidiasis with catheter-related infections has been linked to the ability of the yeasts to form biofilms on intravascular devices [2,3]. It has been reported that 40% of patients from whom Candida was isolated from intravenous catheters developed occult fungemia, and the mortality rate for patients with catheter-related candidemia can be as high as 41% [4].

Candida albicans is the most frequently encountered yeast in clinical specimens and the most pathogenic species. However, recent reports indicate a trend towards an increasing prevalence of infections caused by non-C. albicans Candida species. Candida parapsilosis, C. tropicalis and C. glabrata are now approaching C. albicans as the most frequent cause of candidemia in some institutions [2,5]. Hydrolytic enzymes such as secretory aspartyl proteinases, phospholipases and adherence of Candida species to host tissues have been regarded as the major determinants of the pathogenicity of C. albicans [6–9]. However, little is known about the pathogenicity of non-C. albicans Candida species.

This study was conducted to determine some of the virulence traits of C. albicans and non-C. albicans Candida species isolated from the blood cultures of patients at the
University Malaya Medical Center, in Kuala Lumpur, Malaysia. In addition the susceptibilities of the yeast cells (in biofilm and planktonic stage) against amphotericin and fluconazole were determined.

**Material and methods**

**Clinical isolates**

*Candida* isolates recovered from blood cultures of 41 Malaysian patients (University Malaya Medical Center, Kuala Lumpur, Malaysia) were included in the study. The isolates were identified to their respective species (15 *C. glabrata*, nine *C. parapsilosis*, eight *C. tropicalis*, five *C. krusei*, two *C. krusei*, two *C. rugosa*) using the API 20C AUX system (bioMerieux, Marcy, l’Etoile, France).

**Determination of proteinase and phospholipase activity of Candida isolates**

The proteinase agar clearance assay was performed according to Ruma-Hyanes et al. [10]. Briefly, a chemically defined medium (20 ml) containing 1.5% agar, 13 mM glycine, 29.4 mM KH₂PO₄, 10 mM MgSO₄, and 3 μM thiamine was prepared as the basal layer in a Petri dish. Five milliliters of this medium to which 1.0% (w/v) bovine serum albumin and 1.0% (w/v) galactose was added was then poured over the basal layer and allowed to set overnight. A loopful of an overnight yeast culture was then aseptically inoculated onto the medium and after 6 days of incubation at 30°C, the proteinase and phospholipase activities of the yeast cells were measured. To assess the proteinase activity, the egg-yolk agar was prepared by incorporating a 50% (v/v) egg yolk agar was prepared by incorporating a 50% (v/v) egg yolk into the basal layer and allowed to set overnight. A loopful of an overnight yeast culture was then aseptically inoculated onto the agar. After 6 days of incubation at 30°C, residual protein in the agar was fixed by flooding the agar with 10% (w/v) tannic acid for 5 min. The diameters of the colonies (a) and the diameters of the clearing zone around the colonies (b) were measured.

To assess the phospholipase activity, the egg-yolk agar was prepared by Melo et al. [11]. Briefly, an overnight yeast culture was suspended in RPMI 1640 broth medium supplemented with L-glutamine (Sigma, USA) to a final concentration of 10⁷ cells/ml (OD₅₃₀nm = 0.38). One hundred microliters of the yeast suspension was then transferred to each well of a flat-bottomed, 96-well polystyrene microtiter plate (Nunclon, USA). After incubation under shaking at 75 rpm at 37°C for 1.5 h, unattached cells were removed by washing twice with 150 μl phosphate-buffered saline (PBS). Fresh RPMI 1640 medium (100 μl) was then added to the microtiter wells to allow proliferation of the biofilm. The metabolic activity of the biofilm was measured by the XTT reduction assay after 48 h of incubation. The biofilm in each well was washed twice with 200 μl PBS before adding a solution containing 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) with menadione. The absorbance of the resultant solution (100 μl) after incubation for 1 h in the dark was measured with a spectrophotometer at 490 nm. Examination of biofilm morphology was carried out with an inverted light microscope (Olympus, USA) at 200× magnification. The experiments were performed in triplicate for each isolate.

**Determination of planktonic and biofilm MICs**

The in vitro minimum inhibition concentration (MIC) of planktonic cells of each isolate to amphotericin and fluconazole was determined using the microbroth dilution method as described in the CLSI M27-A2 document [13]. Final drug concentrations ranging from 0.0625 to 2 μg/ml were prepared for amphotericin B, whereas for fluconazole, drug concentrations of 0.125–64 μg/ml were used. The MIC for each isolate was determined after incubation at 37°C for 48 h. The MIC of amphotericin B was defined as the lowest concentration at which there was an absence of growth. The MIC of fluconazole was defined as the lowest drug concentration which achieved 80% growth inhibition as compared to the growth of the drug-free control. The biofilm MIC for each yeast isolate was determined as described by Melo et al. [14]. Final drug concentrations ranging from 0.015–8 μg/ml were prepared for amphotericin B, whereas for fluconazole, drug concentrations of 0.25–128 μg/ml were used. The biofilm MIC of each isolate was determined on the basis of a 50% reduction in metabolic activity compared with the metabolic activity of the biofilm in microtiter wells without the drug. The experiments were performed in duplicate for each isolate.

**Results**

**Proteinase and phospholipase activities of Candida isolates**

A total of 14 (93.7%) of the 15 *C. albicans* isolates and 23 (88.5%) of the 26 non-*C. albicans* Candida isolates
exhibited proteinase activity (Table 1). Phospholipase activity was detected in 11 (73.3%) of the *C. albicans* isolates but only two (7.7%) of the non-*C. albicans* *Candida* isolates, i.e., one isolate each of *C. parapsilosis* and *C. rugosa*.

**Biofilm formation of Candida isolates**

Table 1 shows the quantitation of biofilm metabolic activity of *Candida* isolates using the XTT-reduction assays. A total of 14 yeast isolates, including six *C. albicans*, four *C. parapsilosis* and four *C. tropicalis* were considered as high biofilm producers, as indicated by the high XTT OD readings (≥0.200). Strain to strain variability was observed in the biofilm metabolic activity among the isolates of the same species, as reflected by the standard deviation in the OD readings of each species. Biofilms of *C. albicans*, *C. tropicalis* and *C. parapsilosis* demonstrated a dense network of yeast cells with extensive filamentation after 48 h of incubation. The filamentation was not obvious for the *C. rugosa*, *C. krusei* and *C. glabrata* isolates.

**Antifungal susceptibilities of biofilm and planktonic cells**

The results of the antifungal susceptibility testing of the biofilm and planktonic cells of the yeast isolates are summarized in Table 2. While none of the *C. albicans* isolates were resistant to amphotericin, two *C. tropicalis* isolates were considered as resistant to this antifungal (MIC ≥ 2 μg/ml). The concentrations of amphotericin necessary to inhibit 50% of the isolates (MIC$_{50}$) were 0.25 μg/ml for both *C. albicans* and non-*C. albicans* *Candida* isolates. In vitro fluconazole resistance (≥64 μg/ml) was detected in *C. albicans* (four isolates), *C. tropicalis* (one isolate), *C. parapsilosis* (three isolates), *C. glabrata* (one isolate), and *C. krusei* (one isolate), respectively. The concentration of fluconazole necessary to inhibit 50% of the isolates (MIC$_{50}$) was 2 μg/ml for both *C. albicans* and non-*C. albicans* *Candida* species.

There was an increase (more than 32 folds) in the biofilm amphotericin MICs for all *Candida* isolates as compared to the planktonic cells, except for *C. glabrata* (Table 2). *C. glabrata* demonstrated similar MIC$_{50}$ (0.25 μg/ml) against amphotericin for both biofilm and planktonic cells. Decreased antifungal activity of fluconazole against *Candida* biofilms was reflected by the higher MIC$_{50}$ (≥128 μg/ml) of all the isolates investigated in the current study.

**Discussion**

Proteinase and phospholipase are hydrolytic enzymes that have been identified as playing essential roles in the pathogenicity of *C. albicans* [7–9]. The production of these enzymes has also been demonstrated in non-*C. albicans Candida* species in this and other studies [9,15,16]. There was no significant difference in the proteinase activity between *C. albicans* and the non-*C. albicans Candida* isolates in the current study (P = 0.272), suggesting the importance of this enzyme to both groups of the yeasts. Collectively, the detection rates for proteinase activity of *C. albicans* isolates and non-*C. albicans Candida* isolates from blood cultures in this study were regarded high (93.7% and 88.5% respectively) as compared to a previous study (56.7% and 43.9%, reported by Oksuz *et al.* [15]), whereby isolates from anatomically distinct sites of healthy adults were used.

Our findings concur with the results of previous studies, in which phospholipase activity was detected in higher percentages (53.8–73%) in *C. albicans* isolates as compared to those of non-*C. albicans Candida* isolates (2–17%) [15–18]. The phospholipase activity of *C. albicans* was also significantly higher as compared to the non-*C. albicans Candida* isolates in the current study (P = 0.003). Unlike phospholipase of *C. albicans* which has been implicated in the early steps of host invasion [7], the low and undetectable phospholipase activity in the majority of the non-*C. albicans Candida* species suggests that the enzyme is probably not a significant virulence factor for these species.

<table>
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<tr>
<th>Table 1</th>
<th>Extracellular enzymes and biofilm forming abilities of <em>Candida</em> isolates studied.</th>
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<tbody>
<tr>
<td><strong>Candida isolates</strong></td>
<td><strong>Proteinase assay</strong></td>
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<td><strong>Pz values (No./% positive isolate)</strong></td>
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<tr>
<td><em>C. albicans</em> (n = 15)</td>
<td>0.476 ± 0.206 (14/93.7)</td>
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<tr>
<td>Non-albicans <em>Candida</em> species (n = 26)</td>
<td>0.549 ± 0.214 (23/88.5)</td>
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<tr>
<td><em>C. parapsilosis</em> (n = 9)</td>
<td>0.478 ± 0.23 (8/88.9)</td>
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<tr>
<td><em>C. tropicalis</em> (n = 8)</td>
<td>0.541 ± 0.231 (7/87.5)</td>
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<tr>
<td><em>C. glabrata</em> (n = 5)</td>
<td>0.637 ± 0.231 (4/80.0)</td>
</tr>
<tr>
<td><em>C. krusei</em> (n = 2)</td>
<td>0.671 ± 0.040 (2/100.0)</td>
</tr>
<tr>
<td><em>C. rugosa</em> (n = 2)</td>
<td>0.561 ± 0.150 (2/100.0)</td>
</tr>
<tr>
<td>All (N = 41)</td>
<td>0.523 ± 0.212 (37/90.2)</td>
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*Note*. The production of the proteinase and phospholipase of the yeasts was designated as Pz = a/b, as described by Price *et al.* [11].

Consistent with other studies, the findings in the present investigation demonstrated the variable ability of Candida isolates to form biofilms in vitro [19–21]. There was no significant difference in the biofilm forming abilities between C. albicans and non-C. albicans Candida isolates ($P = 0.379$), although biofilm production was more commonly observed for isolates of C. tropicalis, followed by C. albicans on the surface of catheter materials than noted in a previous study [21]. In addition, there was no obvious association of biofilm production with proteinase and phospholipase activity of the isolates presently investigated, although recent findings indicated that aspartyl proteinase production might be enhanced during the biofilm formation phase of C. albicans [22].

The decreased in vitro antifungal activities of amphotericin and fluconazole against biofilms of Candida isolates noted in the current study, is in agreement with previous studies that Candida biofilms are more resistant to antifungal agents than their planktonic counterparts [19–21]. According to Ramage et al. [23], drug resistance in biofilms is attributed to the high density of cells within the biofilms, expression of resistance genes and the presence of the biofilm matrix and ‘persister’ cells. A recent study reported that although ‘persister’ populations were present in biofilms of C. albicans, C. krusei and C. parapsilosis after exposure to amphotericin, they were absent from those of C. glabrata [24]. This may be a possible explanation for the lower resistance of C. glabrata biofilms against amphotericin in the current study, other than the low biofilm formation ability of the isolates [21].

In conclusion, the herein reported findings demonstrate that proteinase and phospholipase are stable characteristics of C. albicans. While proteinase activity was detected in most non-C. albicans Candida isolates, the phospholipase activity of these isolates was considered as weak and insignificant. Several amphotericin and fluconazole resistant Candida isolates were reported in the current study. However as the number of Candida isolates was small, we are not able to comment on the overall incidence of antifungal resistance of Candida isolates in Malaysia until more extensive work is carried out. Antifungal therapy by amphotericin and fluconazole seems to be insufficient for treatment of Candida biofilms as complete killing of biofilm cells at the highest concentration (8 μg/ml for amphotericin and 128 μg/ml for fluconazole) was not possible for the majority of the herein investigated isolates.

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References


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