Candida albicans isolates from a Malaysian hospital exhibit more potent phospholipase and haemolysin activities than non-albicans Candida isolates

Chin, V.K.1, Foong, K.J.1, Maha, A.2, Rusliza, B.3, Norhafizah, M.2, Ng, K.P.4 and Chong, P.P.1,5
1Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia
2Department of Pathology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia
3Department of Human Anatomy, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia
4Department of Microbiology, Faculty of Medicine, University of Malaya, Malaysia
5Department of Microbiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

*Corresponding author email: cpp@upm.edu.my

Received 4 April 2013; received in revised form 21 May 2013; accepted 28 May 2013

Abstract. This study was aimed at determining the phospholipase and haemolysin activity of Candida isolates in Malaysia. A total of 37 Candida clinical isolates representing seven species, Candida albicans (12), Candida tropicalis (8), Candida glabrata (4), Candida parapsilosis (1), Candida krusei (4), Candida orthopsilosis (1) and Candida rugosa (7) were tested. In vitro phospholipase activity was determined by using egg yolk plate assay whereas in vitro haemolysin activity was tested by using blood plate assay on sheep blood Sabouraud’s dextrose agar (SDA) enriched with glucose. Phospholipase activity was detected in 75% (9 out of 12) of the C. albicans isolates. Among the 25 non-C. albicans Candida isolates, phospholipase activity was detected in only 24% of these isolates. The phospholipase activity of C. albicans was significantly higher than that of the non-C. albicans Candida isolates (P=0.002). Haemolysin activity was detected in 100% of the C. albicans, C. tropicalis, C. glabrata, C. krusei, C. parapsilosis, and C. orthopsilosis isolates while 75% of the C. krusei isolates and 12.3% of the C. rugosa isolates showed haemolysin activity. The haemolytic activity of C. albicans was significantly higher than that of the non-C. albicans Candida isolates (P=0.0001). The findings in this study indicate that C. albicans isolates in Malaysia may possess greater virulence potential than the non-albicans species.

INTRODUCTION

The yeasts of the genus Candida which can exist as either commensals or pathogens had drawn the attention from microbiologists and infectious disease specialists for many decades (Hall et al., 2009; Ishida et al., 2009). With the advances in medical and surgical intervention, abuse of broad-spectrum antibiotics and corticosteroids, as well as an increasing population of immunocompromised patients including cancer patients undergoing chemotherapy and HIV-positive patients, the incidence of candidiasis has increased enormously (Leroy et al., 2009). Candida albicans is the most frequently encountered yeast recovered from human infections. However, there is an increasing prevalence of infections caused by non-C. albicans Candida (NAC) species and this increment may be due to widespread use of antifungal drugs (Dan et al., 2002). Some studies have shown that the incidence of infections caused by NACs such as Candida tropicalis, Candida glabrata and Candida parapsilosis have shown an increasing trend gradually surpassing C. albicans as the most frequent cause of candidemia in some regions (Pfaller & Diekema, 2007).

Many factors like germ tube and hyphae formation, phenotypic switching, surface
recognition molecules, and production of hydrolytic enzymes such as phospholipase, secreted aspartyl proteinase, have been proposed as factors contributing to disseminated infection in susceptible hosts. Extracelluar hydrolytic enzymes, such as phospholipase play an important role in disseminated candidiasis by breaking down the phospholipids in host cell membranes, which facilitate adherence and penetration and hence invasion of host (Schaller et al., 2005).

Haemolysin is another putative virulence factor which contributes to the pathogenesis of Candida in disseminated infection. Production and secretion of haemolysin by Candida, followed by lysis of red blood cells, allow Candida to acquire iron from host, facilitate hyphae invasion and establish disseminated infection in host (Luo et al., 2001; Tsang et al., 2007).

Most of the studies on haemolysin and phospholipase enzymatic activity are focused on C. albicans and information on other medically important non-C. albicans Candida species is limited (Cutler et al., 1991; Luo et al., 2001; Yigit et al., 2009). Hence, the present study was conducted with an aim to determine the in vitro phospholipase and haemolysin activities of Candida isolates in Malaysia as there is limited information on the phospholipase and haemolysin production by Candida isolates in Malaysia.

MATERIALS AND METHODS

Candida species identification
Candida clinical isolates were identified through CHROMagar based on colour and morphology changes, and also through molecular approach by using universal primers which target the internal transcribed spacer region (ITS) of Candida species followed by sequencing of the PCR products for confirmation of the Candida species.

Determination of Phospholipase activity
Phospholipase activity of Candida species were detected by egg yolk agar plate method (Price et al., 1982). The egg yolk medium consisted of 13.0 g SDA, 11.7 g NaCl, 0.11 g CaCl₂ and 10% sterile egg yolk. A loopful of an overnight yeast culture (approximately 10⁸ cells/ml determined through cell counting using a haemocytometer) was aseptically inoculated onto the medium and incubated at 37°C for 2-4 days. The diameter of the precipitation zone (a) and the diameter of the precipitation zone plus the diameter of the colony (b) were measured.

Phospholipase index was designated as \( P_z = \frac{a}{b} \), as described by Price et al. (1982), where \( a \) is the diameter of the precipitation zone produced by the Candida species, and \( b \) is the total diameter of the colony and the precipitation zone.

Determination of Haemolysin Activity
Haemolysin activity was evaluated with a blood plate assay as described previously (Manns et al., 1994; Luo et al., 2001). Media was prepared by adding 7 ml of sheep blood to 100 ml of SDA supplemented with glucose at a final concentration of 3% (w/v). The final pH of the medium was 5.6±0.2. A loopful of an overnight yeast culture (approximately 10⁸ cells/ml determined through cell counting using a haemocytometer) was aseptically deposited onto the medium. The plate was then incubated at 37°C in 5% CO₂ for 48 hours. Candida albicans ATCC140154 strain was used as positive control in this study.

After incubation, the diameter of the colony and the diameter of the translucent zone of haemolysis were measured. The ratio of \( x/y \), which represent haemolysis index (HI value) was measured, where \( x \) is the total diameter of the translucent zone plus the colonies, and \( y \) is the diameter of the colonies. The assay was conducted in duplicate for each yeast isolate tested.

Statistical analysis
Statistical analysis was performed to compare the phospholipase and haemolysin activity profiles among the C. albicans and non-C. albicans Candida isolates using Mann-Whitney test and independent t-test respectively. A p value of <0.05 was considered statistically significant.
RESULTS

Phospholipase activity of Candida albicans versus non-albicans Candida species

The extracellular phospholipase activities of the C. albicans and non-albicans isolates are summarized in Table 1. As many as 9 out of 12 or 75% of the C. albicans isolates investigated in this study demonstrated phospholipase activity whereas 24% (6 out of 25) of the non-albicans isolates expressed detectable phospholipase activity. Three of the phospholipase expressors from the non-albicans group were from C. tropicalis species and the remaining expressors were from the Candida krusei, Candida orthopsilosis and Candida rugosa species. None of the C. glabrata or C. parapsilosis isolates had exhibited any phospholipase activity. Figure 1 depicts the comparison between the C. albicans and non-albicans isolates in terms of the phospholipase index. Figure 2 depicts the phospholipase production by C. albicans and selected non-albicans Candida species on egg yolk agar. The phospholipase activity of C. albicans was statistically significantly higher than that of the non-albicans Candida isolates (P=0.002).

Table 1. Extracellular phospholipase production by Candida isolates

<table>
<thead>
<tr>
<th>Candida isolates</th>
<th>Phospholipase index, mean±S.D. (No. of isolates/ % of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida albicans (n=12)</td>
<td>0.825±0.024 (9/75.0)</td>
</tr>
<tr>
<td>Non-albicans Candida species (n=25)</td>
<td>0.906±0.018 (6/24.0)</td>
</tr>
<tr>
<td>Candida tropicalis (n=8)</td>
<td>0.977±0.003 (3/37.5)</td>
</tr>
<tr>
<td>Candida parapsilosis (n=1)</td>
<td>1.000±0.000 (0/0.0)</td>
</tr>
<tr>
<td>Candida glabrata (n=4)</td>
<td>1.000±0.000 (0/0.0)</td>
</tr>
<tr>
<td>Candida krusei (n=4)</td>
<td>0.920±0.000 (1/25.0)</td>
</tr>
<tr>
<td>Candida orthopsilosis (n=1)</td>
<td>0.923±0.000 (1/100.0)</td>
</tr>
<tr>
<td>Candida rugosa (n=7)</td>
<td>0.871±0.000 (1/12.3)</td>
</tr>
<tr>
<td>Total (N=37)</td>
<td>0.857±0.046 (15/40.5)</td>
</tr>
</tbody>
</table>

Note: The production of phospholipase of the yeasts was designated as described by Price et al. (1982)

Haemolysin Activity of Candida albicans versus non-albicans Candida species

The extracellular haemolysin activities of the C. albicans and non-albicans isolates are summarized in Table 2. All of the C. albicans, Candida tropicalis, C. glabrata, C. parapsilosis and C. orthopsilosis isolates investigated in this study demonstrated haemolysin activity whereas 75% (3 out of 4) of C. krusei isolates and 12.3% (1 out of 7) of Candida rugosa isolates expressed detectable haemolysin activity. Figure 3 depicts the comparison between the C. albicans and non-albicans isolates in terms of the haemolysis index. Figure 4 illustrates the haemolysin production by C. albicans and selected non-albicans Candida species on sheep blood agar. The haemolysin activity of C. albicans was statistically significantly higher than that of the non-albicans Candida isolates (P=0.0001).

DISCUSSION

Phospholipase enzyme is an important pathogenic factor as it is involved in the invasion and destruction of host tissue during disseminated candidiasis. Phospholipase
Figure 1. Histogram depicting the comparison of phospholipase activities between Candida albicans and non-Candida albicans species. Candida albicans exhibited significantly higher phospholipase activity than non-C. albicans species (P<0.05). Bar represents ± 1 standard deviation. The number of Candida species in each group is indicated in parenthesis. Candida albicans possesses lower phospholipase index than the non-Candida albicans species which indicates that Candida albicans has stronger phospholipase activity than non-C. albicans species.

Figure 2. Phospholipase production of representative Candida isolates on egg yolk agar by (a) Candida albicans, (e) Candida krusei, and (h) Candida tropicalis. The other isolates of selected Candida species (b,c,d,g,f) showed no phospholipase production. A distinct, well-defined, dense white zone of precipitation was visible around the colony when there is presence of phospholipase activity.
Table 2. Haemolytic activity of *Candida* species on sheep blood Sabouraud-dextrose agar (SDA)

<table>
<thead>
<tr>
<th>Candida isolates</th>
<th>Alpha</th>
<th>Beta</th>
<th>None (gamma)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>haemolysis index</td>
<td>mean±S.D.</td>
<td>(No. of isolates/ % of isolates)</td>
</tr>
<tr>
<td><em>Candida albicans</em> (n=12)</td>
<td>–</td>
<td>2.492±0.120</td>
<td>(12/100.0)</td>
</tr>
<tr>
<td>Non-albicans <em>Candida</em> species (n=25)</td>
<td>–</td>
<td>1.939±0.199</td>
<td>(18/72.0)</td>
</tr>
<tr>
<td><em>Candida tropicalis</em> (n=8)</td>
<td>–</td>
<td>2.064±0.087</td>
<td>(8/100.0)</td>
</tr>
<tr>
<td><em>Candida parapsilosis</em> (n=1)</td>
<td>–</td>
<td>1.738±0.000</td>
<td>(1/100.0)</td>
</tr>
<tr>
<td><em>Candida glabrata</em> (n=4)</td>
<td>–</td>
<td>2.066±0.107</td>
<td>(4/100.0)</td>
</tr>
<tr>
<td><em>Candida krusei</em> (n=4)</td>
<td>–</td>
<td>1.707±0.025</td>
<td>(3/75.0)</td>
</tr>
<tr>
<td><em>Candida orthopsilosis</em> (n=1)</td>
<td>–</td>
<td>1.667±0.000</td>
<td>(1/100.0)</td>
</tr>
<tr>
<td><em>Candida rugosa</em> (n=7)</td>
<td>–</td>
<td>1.600±0.000</td>
<td>(1/12.3)</td>
</tr>
<tr>
<td>Total (N=37)</td>
<td>–</td>
<td>2.160±0.323</td>
<td>(30/81.1)</td>
</tr>
</tbody>
</table>

( – ) indicates no activity

Note: The production of haemolysin of the yeasts was designated as described by Manns *et al.* (1994) and Luo *et al.* (2001)

![Figure 3. Histogram depicting the comparison of beta-haemolytic activities between *Candida albicans* and non-*Candida albicans* species. *Candida albicans* exhibited significantly higher beta-haemolytic activity than non-*Candida albicans* species (P<0.05). Bar represent±1 standard deviation. The number of *Candida* species in each group is indicated in parenthesis. High haemolysis index indicates stronger haemolysin activity](image)
enzyme breaks down the host phospholipid constituents in host membrane, leading to cell lysis and hence facilitates adherence and establishes infection in host cell. Therefore, phospholipase production can become a parameter to distinguish virulent invasive strains from non-invasive colonizers (Sachin et al., 2012). Price et al. (1982) had described a plate method to detect phospholipase activity in C. albicans by incorporating egg yolk into Sabouraud dextrose agar-based medium. Candida isolates which possess phospholipase activity will produce a distinct, well-defined, dense white zone of precipitation around the colony when grown on this medium.

Our study aimed to determine the in vitro phospholipase activity of medically important Candida isolates in Malaysia. The results showed that extracellular phospholipase activity was detected in higher percentages (75%) in C. albicans isolates as compared to the non-albicans Candida isolates (24%). This finding concurs with previous studies. Samaranayake et al. (1984) screened the phospholipase activity of 41 Candida isolates and found that 79% of the C. albicans strains produced extracellular phospholipases. However, none of the C. tropicalis, C. glabrata, and C. parapsilosis strains produced the enzymes.

Oksuz et al. (2007) reported in a similar study conducted on isolates in Turkey that 53% of the C. albicans and 14% of the non-C. albicans Candida isolates were phospholipase-positive. In a study on isolates from Portugal, Pinto and co-workers reported that 99.4% of the C. albicans isolates displayed phospholipase activity (Pinto et al., 2008). Collectively, these findings together with our latest finding show that phospholipase production is remarkably higher in C. albicans isolates than in non-C. albicans Candida isolates and subsequently implies that phospholipase enzyme is a major virulence factor for C. albicans whereas the low and undetectable phospholipase activity in the majority of the non-C. albicans Candida species suggests that the enzyme probably does not contribute significantly towards the virulence of these species.

Another interesting issue is that reports from different countries show that phospholipase production varies among different regions and Borst & Fluit (2003) surmised that variation in virulence factors might be associated with geographical region and infection type. Hence, it is imperative to profile the phospholipase production of different regions to investigate the causal relationship between the severity of

Figure 4. Haemolysin production of Candida isolates on sheep blood agar by (a) Candida tropicalis, (b) Candida glabrata, (c) Candida krusei, (e) Candida parapsilosis and (f) Candida albicans. In contrast, (d) Candida rugosa showed no haemolysin production on sheep blood agar. A translucent, well-defined, ring shaped formation was visible around the colony when there was presence of haemolysin activity.
candidiasis in that particular region and phospholipase production.

Iron acquisition by pathogenic organisms is important for their survival and for establishing infection within the mammalian host. Human diseases of iron overload often correlate with increased bacteria overload. Most pathogens acquire iron source indirectly from iron-containing compounds such as haemoglobin as there is no free iron circulating in human host. Pathogens, equipped with specialised mechanisms, will destroy and extract the iron from haemoglobin for its survival. Enzymes involved in this destruction activity are termed as haemolysins (Manns et al., 1994; Luo et al., 2001; Tsang et al., 2007).

Studies on haemolysin activity in Candida species are limited. Manns et al. (1994) first reported that a complement mediated haemolysis was induced by C. albicans. However, there were no reports on the haemolytic activity of non-albicans Candida species. Meanwhile, Luo et al. (2001) demonstrated the variable expression of haemolysin by different Candida species, both qualitatively and quantitatively by using a modified plate assay introduced by Manns and co-workers (1994). The report by Luo et al. (2001) demonstrated that aside from C. albicans, some of the other Candida species may also possess haemolytic activities, although the mechanisms involved have to be further explored. Moreover, Luo et al. (2001) suggested that haemolysis can be categorized into complete (beta), incomplete (alpha) or no haemolysis (gamma or no).

In our present study, in vitro haemolytic activity of the Candida isolates on sheep blood SDA medium was tested. The modified plate assay with sheep blood SDA medium as described by Luo et al. (2001) is a simple, reproducible, sensitive and fast screening method for accessing the haemolytic activity of Candida species. Yigit et al. (2009) reported that sheep blood SDA is most sensitive and appeared to give the best indication of haemolytic activity of Candida species compared to rabbit blood SDA and human blood SDA.

Our present results showed that extracellular haemolysin activity was detected in higher percentages (100%) in C. albicans isolates as compared to the non-albicans Candida isolates (72%). All C. albicans, C. tropicalis, C. glabrata, C. krusei, C. parapsilosis, and C. orthopsilosis strains in comparison to 75% of C. krusei and 12.3% of C. rugosa strains showed beta haemolysis on sheep blood SDA medium after 48 hours post inoculation. The haemolysin activity was compared between the C. albicans and non-albicans Candida species (independent t-test) and the result showed that C. albicans possesses stronger haemolysin activity compared to non-albicans Candida species (p=0.002) as indicated in Figure 3. Besides that, we have calculated the haemolytic index among Candida species and the result showed that Candida isolates display different haemolytic index with C. albicans having the highest haemolytic index, followed by C. glabrata, C. tropicalis, C. parapsilosis, C. orthopsilosis, C. krusei in descending order and C. rugosa being the lowest (Table 2, Figure 2). One possible explanation for the difference in haemolytic indices of different Candida species is the existence of species-specific hemolysins whereby these hemolysins may vary in molecular size and have different diffusion rates (Luo et al., 2001).

Haemolysin production might be affected by the species of Candida and the amount of oxygen present in the media. Yenisehirli et al. (2010) reported that all C. albicans strains isolated from various clinical samples showed beta hemolysis in aerobic conditions while 98.5% of all C. albicans isolates expressed hemolytic activity including alpha, beta, and gamma hemolysis in both anaerobic conditions and in conditions with reduced oxygen. In another study, İcl et al. (2012) reported that 91.1% of the C. albicans and 88% of the non-albicans Candida species showed haemolysin activity under aerobic conditions while 40% of the C. albicans and 68% of the non-albicans Candida species showed haemolysin activity under anaerobic conditions.
Tsang et al. (2007) reported that haemolysin production by C. albicans is higher in diabetic patients than in non-diabetic individuals, and suggested that increased blood glucose concentration may directly or indirectly influence the haemolysin production by C. albicans. Yigit et al. (2011) also reported that there is production of haemolysin by both C. albicans and non-albicans Candida species. Sachin et al. (2012) reported that 94.8% of C. albicans isolates showed haemolytic activity while haemolytic activity of other non-albicans Candida species ranged from 7% to 60%.

The findings in the present study concurs with previous study in impyling that haemolysin could be an essential putative virulence factor of Candida species whose actual role and contribution towards the pathogenesis mechanism has yet to be fully explored. Furthermore, haemolysin production was found in most of the Candida species, and unlike phospholipase, which was secreted mostly by C. albicans, haemolysin seems to be a more prominent and significant virulence factor for both C. albicans and non-albicans Candida species.

In conclusion, this study not only reiterates the importance of phospholipase activity of Candida isolates but also provides new information on haemolysin activity produced by Candida isolates in Malaysia. We showed that both phospholipase and haemolysin activities are remarkably high in C. albicans and the overall significantly higher production of both enzymes in C. albicans than in non-albicans species could be one underlying reason of the greater success of C. albicans species in causing infections in human hosts. While haemolysin activity was detected in most of the non-albicans Candida isolates, the phospholipase activity was only exhibited in a minority of these isolates, reflecting the non-essential characteristic of phospholipase as a virulence factor among the non-albicans group. More extensive large-scale studies to profile the phospholipase and haemolysin activities as well as other virulence factors of Candida species are crucial so that universal virulence patterns of this important fungal genus in different geographical regions could be identified, and subsequently the evaluation of the documented virulence features could be adopted in routine clinical microbiology practice.

Acknowledgements. We are grateful to Universiti Putra Malaysia for the financial support through RUGS Grants (Project number: 04-01-12-1607RU and 04-02-12-1761RU).

REFERENCES


