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

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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 immuno-compromised 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. Extracellular 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 $CaCl_2$ and 10% sterile egg yolk. A loopful of an overnight yeast culture (approximately 10^8 cells/ml determined through cell counting using a haemocytometer) was aseptically inoculated onto the medium and incubated at 37ºC 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 Pz = 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 10^8 cells/ml determined through cell counting using a haemocytometer) was aseptically deposited onto the medium. The plate was then incubated at 37ºC in 5% CO_2 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 phosphoplipase 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-*C. albicans Candida* isolates (P=0.002).

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. parapsilopsis* 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

Table 1. Extracellular phospholipase production by *Candida* isolates

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

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

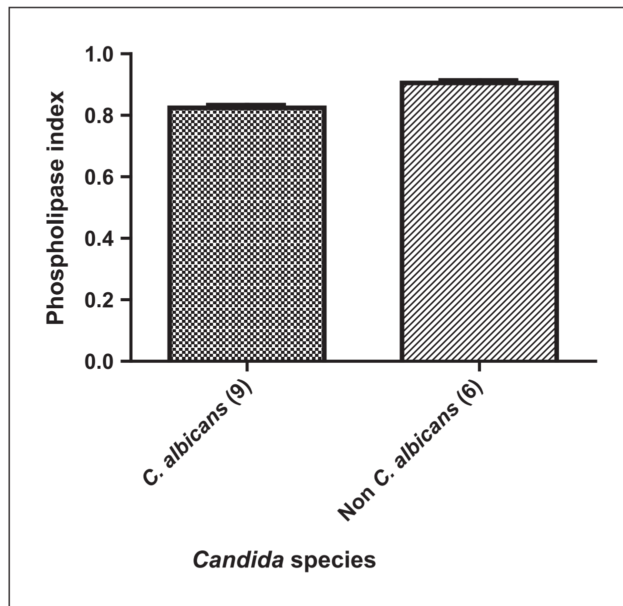

Figure 1. Histogram depiciting the comparison of phospholipase activities between *Candida albicans* and non-*Candida albicans* species. *Candida albicans* exhibited significantly higher phospholipase activity than non-*C. albicans* species (P<0.05). Bar represents ± 1 standard deviation. The number 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

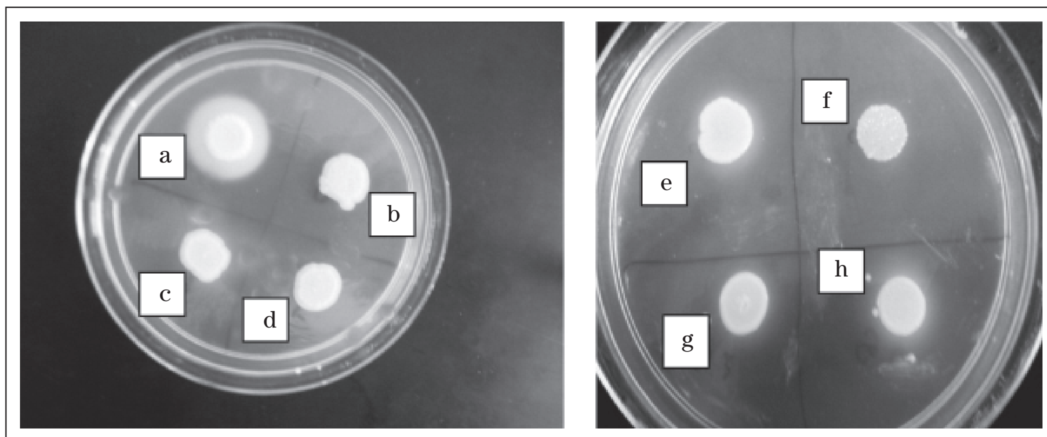

Figure 2. Phospholipase production of representative *Candida* isolates on egg yolk agar by (a) *Candida albicans*, (e) *Candida krusei*, and (h) *Candida tropicalis*. The other isolates of selected *Candida* species (b,c,d,g,f) showed no phospholipase production. A distinct, well-defined, dense white 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)

Candida isolates	haemolysis index, mean±S.D. (No. of isolates/ % of isolates)		
	Alpha	Beta	None (gamma)
Candida albicans (n=12)	_	2.492±0.120 (12/100.0)	_
Non-*albicans Candida* species (n=25)		1.939±0.199 (18/72.0)	
Candida tropicalis (n=8)	_	2.064±0.087 (8/100.0)	_
Candida parapsilosis (n=1)	_	1.738±0.000 (1/100.0)	_
Candida glabrata (n=4)	_	2.066±0.107 (4/100.0)	_
Candida krusei (n=4)	_	1.707±0.025 (3/75.0)	1
Candida orthopsilosis (n=1)	_	1.667±0.000 (1/100.0)	_
Candida rugosa (n=7)	_	1.600±0.000 (1/12.3)	6
Total (N=37)		2.160±0.323 (30/81.1)	

(–) 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)

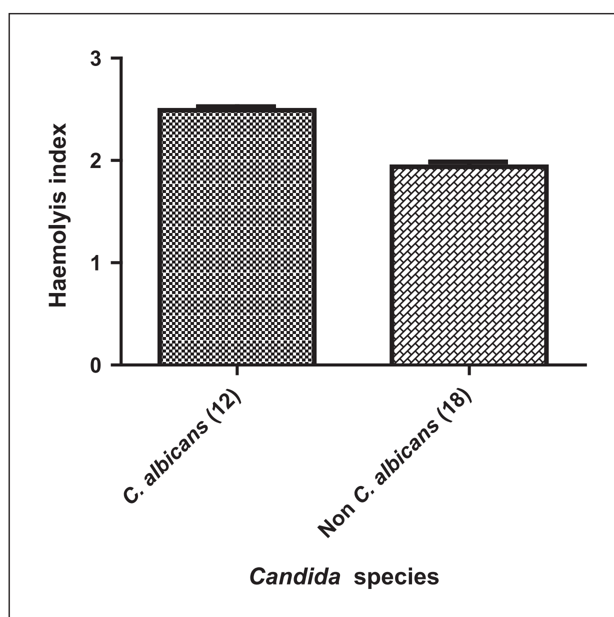

Figure 3. Histogram depiciting the comparison of beta-haemolytic activities between *Candida albicans* and non-*Candida albicans* species. *Candida albicans* exhibited significantly higher beta-haemolytic activity than non-*Candida albicans* species (P<0.05). Bar represent±1 standard deviation. The number of *Candida* species in each group is indicated in parenthesis. High haemolysis index indicates stronger haemolysin activity

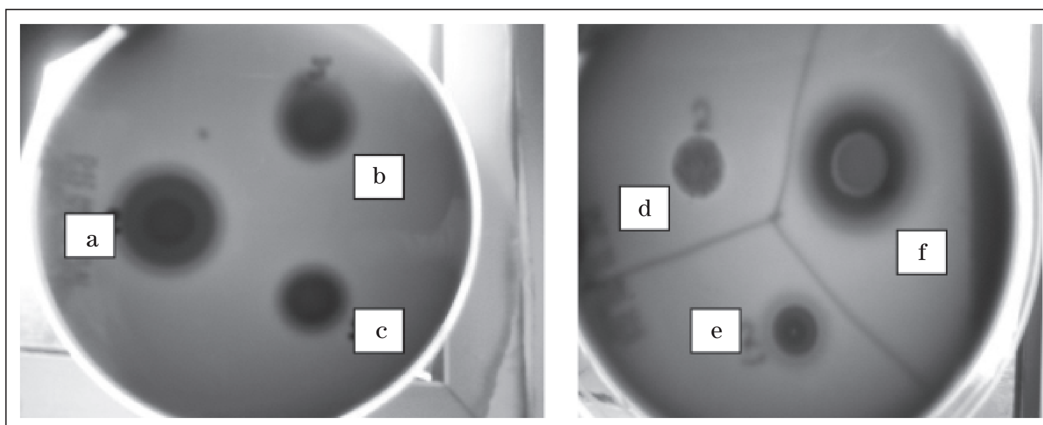

Figure 4. Haemolysin production of *Candida* isolates on sheep blood agar by (a) *Candida tropicalis*, (b) *Candida glabrata*, (c) *Candida krusei*, (e) *Candida parapsilosis* and (f) *Candida albicans*. In contrast, (d) *Candida rugosa* showed no haemolysin production on sheep blood agar. A translucent,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

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

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. orthosilopsis*, *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. Yenişehirli *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, İncİ *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 phosholipase 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.

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