


estimation of the number of cells in the human body. *Annals of Human Biology, 40*(6), 471.


Wachtler, B., Wilson, D., Haedicke, K., Dalle, F., & Hube, B. (2011). From attachment to damage: defined genes of *Candida albicans* mediate adhesion, invasion and damage


Full Length Research Paper

Chrome agar Candida for species level identification of isolates of Candida sp. from oral cavity

Deepa, K.¹*, T. Jeevitha² and A. Michael¹

¹Department of Microbiology, PSG College of Arts and Science, Coimbatore, India.
²Department of Microbiology, Karlapam University, Coimbatore, India.

Received 17 October, 2014; Accepted 1 December, 2014

Recently, Candida has become an important nosocomial pathogen. They are normal flora of skin, mouth, gut and vagina of healthy humans. They become opportunistic with immunocompromised and immunosuppressed individual. Since it is not possible to identify the species directly on Sabouraud’s dextrose agar (SDA), CHROM agar for candida is used for easy recognition of species by colour of the colonies. This study was conducted in 38 patients with symptoms of oral candidiasis aged 25 to 75 years. Oral swabs were taken from oral cavity and were cultured on Sabouraud's dextrose agar and CHROM agar for candida. Gram staining and germ tube test were done with the samples. Four different species of Candida were isolated from the samples using CHROM agar, that is, Candida albicans, Candida tropicalis, Candida glabrata and Candida krusei. It was observed that, of the 38 isolates, C. albicans was obtained in higher rate (58%) followed by C. tropicalis (24%), C. krusei (16%) and C. glabrata (2%). C. albicans produced light green colonies, C. tropicalis produced dark blue with purple diffusion colonies, C. glabrata showed pink with a darker mauve center colonies, C. krusei produced pink with pale borders colonies. Thus, CHROM agar candida medium was found to be helpful in direct and easy identification of multiple yeast species simultaneously.

Key words: Candida albicans, oral swabs, CHROM agar, differential medium.

INTRODUCTION

Yeasts, especially Candida albicans is a member of the native born microbial flora of the skin, mucus membranes of the gut, mouth and vagina in healthy human. Although, C. albicans rarely causes infections in healthy human without predisposing factors, immune-suppressed patients can suffer from mucosal, cutaneous or systemic candidiasis. Oropharyngeal candidiasis is the most common opportunistic infection. Oral thrush is a common form of the oropharyngeal candidiasis and its clinical features include white patches appearing as discrete lesions on the buccal mucosa, throat, tongue and gum linings that develop into confluent pseudo-membranes resembling milk curds (Marsh and Martin, 2009). The incidence of candidiasis has increased markedly with the advent of diseases like AIDS and the development of immune suppressive therapy (Smitha and Shashanka, 2011). Among the various species of Candida, C. albicans was the most frequently isolated species (72.7%)
(Back-Brito et al., 2009). Although C. albicans remains the major species isolated, non-albicans such as C. glabrata, C. krusei and C. tropicalis also involved in the incidence of candidiasis. In a study conducted by Vijaya et al. (2011), non C. albicans was isolated at a higher rate (55.8%) than C. albicans. Isolation and prompt identification of infecting microorganism from the mixed yeast population are required for early antifungal therapy. 

Traditional method of identification of Candida species is germ tube formation by the fungi in serum (Mackenzie, 1962). In most clinical investigations, fungal pathogens are routinely cultured on Sabouraud’s Dextrose Agar (SDA) (Baveja, 2010). The drawback with these media is that, the colonies on these media are very similar in appearance and their subsequent identification requires considerable investigative time (Zarei Mahmoudabadie et al., 2000; Beighton et al., 1995).

CHROM agar for Candida is a differential culture medium which facilitates the species level identification of Candida isolates of various clinical specimens. These chromogenic media provide different colours of colonies secondary to chromogenic substances that react with enzymes secreted by the organisms (Murray et al., 2005; Yucesoy et al., 2001). A major advantage of these media is that, identifications of species can be done in shorter duration within 48 h with great accuracy (Pfaller et al., 1995). In addition, mixed yeast infections are seen in the oral cavity frequently in immunocompromised patients, CHROM agar is useful because differences in the colour of the colony make the identification simple and selective (Odds and Bernaerts, 1994; Pfaller et al., 1996). Therefore, the present study was conducted to evaluate the performance of CHROM agar for the isolation, direct presumptive identification and species differentiation of Candida from oral specimens.

MATERIALS AND METHODS

Preparation of CHROM agar Candida

CHROM agar Candida (Himedia India) was prepared according to the manufacturer’s instructions. CHROM agar Candida is composed of (per litre): peptone (10 g), glucose (20 g), agar (15 g), chloramphenicol (0.5 g) and Chromogenic mix (2 g). Twelve grams of CHROM agar Candida powder (one vial) was added to 250 ml of sterile distilled water in a sterile Erlenmeyer. The suspension was completely dissolved by boiling (<100°C) and mixing. The medium does not require sterilization by autoclave, therefore after cooling in a water bath at 45°C, the agar was poured into sterile Petri dishes (Odds and Bernaerts, 1994). After being allowed to cool, the plates were stored at 4°C prior to use. CHROM agar was prepared as per the instruction manual. Candida species isolate were inoculated on CHROM agar and incubated at 37°C for 48 h.

Collection of samples

A total of 38 clinical samples were obtained from patients attending tertiary care Hospital, Coimbatore, TamilNadu, India, with symptoms of oral candidiasis. Oral swabs were collected with all aseptic precautions using sterile swabs from tongue and buccal mucosa by gently rubbing a sterile cotton swab over the lesional tissue (18) (Axe’ll et al., 1985). The swabs were then dispensed in a test tube containing sterile SDA broth.

Processing of samples

The samples were inoculated on HiCHROM Candida differential agar and Sabouraud’s Dextrose agar and incubated at 37°C for 48 h. From that Gram staining was done. Germ tube test was done which is the standard laboratory method to differentiate the C. albicans from other Candida species. The test involved the induction of hyphal outgrowths (germ tubes) when sub cultured in serum at 37°C for 2 - 4 h (Williams and Lewis, 2000). Growth on the CHROM agar was observed with in 24 h in most of the cases. For few isolates, the plate had to be incubated for up 48 h to appreciate the growth. Colour of the colonies was noted and the species was identified.

RESULTS AND DISCUSSION

A total of 38 species isolated from oral specimen were studied for morphological characteristics by Gram staining and cultural characteristics by growth on SDA. Gram positive budding yeast cells were observed in Gram staining. On SDA (Figure 1a) creamy white colored, smooth, pasty convex colonies were observed. After 48 h incubation at 37°C, positive cultures produced colonies of 1 to 5 mm in diameter. On CHROM agar appearance of Candida species were as follows: C. albicans - Green (Figure 1b), C. tropicalis - metallic blue (Figure 1c), C. krusei - pink (Figure 1d) and C. glabrata - Mauve (Table 2).

The germ tube test (Figure 1e) was used for the confirmation of C. albicans. C. albicans alone gave positive result for germ tube test (Table 2). A distribution of Candida species isolated is shown in Table 1. Of the 38 isolates obtained, predominantly isolated Candida species was C. albicans (58%) and then C. tropicalis (24%), C. krusei (16%) and C. glabrata (2%). Distribution of specimen between different age group is shown in Table 3.

Among the 38 Candida species isolated from the oral cavity, C. albicans was found to be predominant with 58%. This observation correlated with the previous studies. Manjunath et al. (2012) found that, C. albicans was the most common isolate from both HIV and non-HIV infected patients. This observation was also reported (Odds and Bernaerts, 1994; Al-Dwairi et al., 2014). According to Back-Brito et al. (2009) and Williams and Lewis (2000), the majority of yeast isolates from oral swabs were C. albicans, but it was often recovered in association with other yeasts. This was followed by C. tropicalis 24%, C. krusei 16% and C. glabrata 2% (Table 1). In our study, the isolation rates of Candida species is high in ages ranging from 36-70 years old. This observation is more or less similar with the results shown by Zaremba et al. (2006) and Pinho Resende et al. (2002).
Figure 1. Growth of Candida sp. on SDA (a), Candida albicans on CHROM agar (b), Candida tropicalis on CHROM agar (c), Candida krusei on CHROM agar (d) and Germ tube formation by Candida albicans (e).

Table 1. Distribution of different species of Candida isolated from oral cavity

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates</th>
<th>Percentage of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>22</td>
<td>58%</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>9</td>
<td>24%</td>
</tr>
<tr>
<td>C. krusei</td>
<td>6</td>
<td>16%</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>1</td>
<td>2%</td>
</tr>
</tbody>
</table>

Table 2. Growth characteristics of Candida species isolated from oral cavity.

<table>
<thead>
<tr>
<th>Species</th>
<th>Growth on CHROM agar</th>
<th>Germ tube test</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>Green</td>
<td>Positive</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>Metallic blue</td>
<td>Negative</td>
</tr>
<tr>
<td>C. krusei</td>
<td>Pink</td>
<td>Negative</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>Mauve</td>
<td>Negative</td>
</tr>
</tbody>
</table>
It has generally been assumed that old age represent a predisposing condition for increased candidal colonization. Lockhart et al. (1999) found that frequency and intensity of carriage of candidal colonization increased as a function of age. According to Sumitra and Megha (2014), sensitivity and specificity of CHROM agar for C. albicans were 100 and 96%, C. tropicalis were 100% and 100%, C. krusei were 100% and 100% and C. glabrata 75% and 100%, respectively. Germ tube test has been the gold stranded method for species differentiation of Candida yeast. But it may lead to false positive and false negative results. Though SDA has been used for routine culturing of yeast cultures, precise identification by colony appearance and colour is not possible with mixed cultures (Jean-Philippe et al., 1996). In our study, with in 48 h, candida species were differentiated based on colony colour and morphology.

Hence, the identification of Candida species is technically simple, rapid and cost effective as compared to technically demanding time consuming and expensive conventional method. In recent years, other differential media have been developed that allow identification of certain Candida species based on colony appearance and colour following primary culture (Houang et al., 1997). The advantage of such media is that the presence of multiple Candida species in a single infection can be determined which can be important in selecting subsequent treatment options (Odds and Bernaerts, 1994). CHROM agar Candida is a new Chromogenic differential culture medium that is used for the isolation and identification of some of the most clinically important yeast pathogens on the basis of colony colour. CHROM agar Candida has previously been shown to be an effective and selective medium for the direct identification of Candida species from clinical materials (Odds and Bernaerts, 1994; Pfaller et al., 1996). This medium has previously also been used for the isolation and identification of yeasts from dental samples (Beighton et al., 1995) and from swabs of soft tissues in oral cavity (Odds and Bernaerts, 1994). A major advantage of CHROM agar is the ability to detect mixed cultures of yeasts in clinical specimens.

### Table 3. Age distribution between the collected isolates.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>No. of isolates</th>
<th>Percentage of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>21-35</td>
<td>9</td>
<td>24%</td>
</tr>
<tr>
<td>36-50</td>
<td>17</td>
<td>45%</td>
</tr>
<tr>
<td>51-70</td>
<td>12</td>
<td>31%</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>100%</td>
</tr>
</tbody>
</table>

Conclusion

CHROM agar Candida medium was found to be helpful, allowing direct and presumptive identification of C. albicans and the easy recognition of association of multiple yeast species. Thus, CHROM agar for Candida was proved to be easy to use, time saving and appears to be well suited for routine use in the clinical mycology laboratories.

### Conflict of Interests

The author(s) have not declared any conflict of interests.

### ACKNOWLEDGEMENTS

The authors thank the Principal and the Management of PSG College of Arts and Science, Coimbatore, India for providing facilities to carry out the work. They also thank the Principal and the Management of Karpagam University, Coimbatore, India for their consistent support and help during this study.

### REFERENCES


In vitro evaluation of virulence factors of Candida species isolated from oral cavity

K. Deepa¹*, T. Jeevitha² and A. Michael¹

¹Department of Microbiology, PSG College of Arts and Science, Coimbatore, India.
²Department of Microbiology, Karpagam University, Coimbatore, India.

Received 21 January, 2015; Accepted 10 March, 2015

The yeast Candida is a normal flora of the skin and the mucous membrane and it then becomes pathogen in immunocompromised people. Various virulence factors are contributing to establishment of the infection in the host. Adherence of the pathogen to host tissues, yeast-hyphal transition and extracellular hydrolytic enzymes secretion are important virulence factors of Candida species. These hydrolytic enzymes play important roles in pathogenicity of Candida infection. The present study was conducted with an aim to determine in vitro phospholipase, protease, haemolysin, esterase activities and biofilm formation in oral Candida isolates. A total of 38 Candida species were isolated from oral cavity of patients with symptoms of oral candidiasis. The specimens were identified by standard mycological techniques up to species level and were investigated for production of hydrolytic enzymes and biofilm formation. Phospholipase activity was in 52.6% of isolates, 86.8% produced protease and haemolysin activity was seen in 63.1%, esterase activity was demonstrated in 50% of isolates, 78.9% of Candida isolates showed biofilm formation. Candida albicans showed more extracellular hydrolytic enzyme activity, whereas, Candida tropicalis showed more biofilm formation. Both the C. albicans and Non-albicans Candida (NAC) species are capable of producing extracellular hydrolytic enzymes and biofilm formation.

Key words: Candida species, virulence factors, extracellular hydrolytic enzymes, biofilm formation.

INTRODUCTION

The dimorphic fungus Candida sp. can respond rapidly to environmental changes, and this flexibility could allow this organism to take advantage of impaired immunity and facilitate establishment of disease. Although Candida is normal flora of skin and mucous membranes of healthy people, they cause infections that range from superficial infections to life-threatening systemic infections in immuno-compromised and immunosuppressed people. Various virulence factors are contributing to the colonization and pathogenicity of Candida infection, including the expression of adhesins and invasins on the cell surface, yeast-hyphal morphogenetic transformation, the formation of biofilms, phenotypic switching and the secretion of hydrolytic enzymes (François et al., 2013).
Among the various factors, extracellular hydrolytic enzymes of which SAPs (secreted aspartyl proteinases) are considered to be one of the major virulence factors play a major role in overgrowth of the Candida, since these enzymes pave way to adhere, penetrate and for tissue invasion (Schaller et al., 2005).

Aspartyl proteinases are secreted by pathogenic species of Candida in vivo during infection. The enzymes are secreted in vitro when the organism is cultured in the presence of exogenous protein (usually bovine serum albumin) as the nitrogen source. Phospholipase enzymes, another important virulence factor, are associated with the function related to host cell damage, adherence and penetration (Kabir et al., 2012). They destroy phospholipids in the host cell, hence results in the damage to the cell membrane, cell lysis and facilitating tissue invasion (Bhat et al., 2011). There are four secreted phospholipase, A to D (PLA, PLB, PLC and PLD). Their activity is very high during tissue damage because these enzymes carry out hydrolysis of one or more ester linkages of glycerophospholipids on the host cell membrane. Furthermore, Candida albicans is able to acquire elemental iron from host tissues through haemolysin production, iron chelators (siderophores) and iron-transport proteins, which then is used by the fungus for metabolism, growth and establishment of infection in humans (Weinberg, 1978; Almeida et al., 2009). The ability of C. albicans to utilize hemoglobin as an iron source was first described by Moors et al. (1992). In humans, iron is found in some proteins, including hemoglobin (a component of erythrocytes). The first step of C. albicans infection in vivo involves binding to erythrocytes through receptors of the complement system. Next, C. albicans produces a hemolysis factor that induces lysis of the erythrocyte. This factor most likely corresponds to a mannoprotein bound to the cell surface of the fungus (Almeida et al., 2009; Watanabe et al., 1999). Almeida et al. (2008) observed that C. albicans caused greater damage to oral epithelial cells containing elevated concentrations of ferritin as compared to cells with lower iron levels.

Traditionally, antifungal drugs were developed either to inhibit or to kill the pathogenic organism. Because of the development of anti-fungal resistance to various antifungal drugs by the pathogen, there is a need to develop new antifungal strategy which specifically targets the virulence factors. The study of virulence factors provides a way to specifically target virulence of Candida sp. Therefore, the present study was conducted with an aim to determine in vitro phospholipase, proteinase, haemolysin, esterase activities and biofilm formation in oral Candida isolates.

MATERIALS AND METHODS

Collection of samples

A total of 38 clinical samples were obtained from patients attending Tertiary Care Hospitals, Coimbatore, TamilNadu, India, with symptoms of oral candidiasis. Oral swabs were collected with all aseptic precautions using sterile swabs from tongue and buccal mucosa by gently rubbing over the lesional tissue. The swabs were then dispensed in a test tube containing sterile SDA broth. Then, they were identified by Gram staining, lactophenol cotton blue test, germ tube test, carbohydrate fermentation test; urease test, morphology on HiCHROM agar and corn-meal agar with Tween-80. Culture on Candida HiCHROM agar was for the species identification whereas corn-meal agar was for demonstration of chlamydospores.

Preparation of the yeast suspension

Yeast suspension was prepared from the isolates. A small amount of stock culture was inoculated on Sabouraud dextrose agar (SDA) containing chloramphenicol by using a sterile loop and incubated at 37°C for 24-48 h. Then, the yeasts were harvested and suspended in sterile phosphate buffered solution (PBS) at turbidity equal to optical density (OD) of 0.5 McFarland. The final suspension was adjusted to contain 1 x 10^7 yeast cells/ml.

Determination of phospholipase activity

The extracellular phospholipase activity of Candida sp. was determined by growing them on egg yolk agar and measuring the size of zone of precipitation by the method prescribed by Samaranayake et al. (1984). The egg yolk medium was prepared according to Tsang et al. (2007) and Mohandas (2011). A 10 ml suspension of yeast cells per ml saline was placed on the egg yolk medium and left to dry at room temperature. The culture was then incubated at 37°C for 48 h, after which the diameter of the precipitation zone around the colony was determined. Phospholipase activity was measured by dividing colony diameter by the diameter of the precipitation zone (pz) around the colony formed on the plate. The pz was scored as follows: pz = 1, negative phospholipase activity; pz = 0.64-0.99, positive phospholipase activity; and pz <= 0.63, very strong phospholipase activity (Price et al., 1982). The lower the pz value, the higher the enzymatic activity.

Determination of protease activity

Extracellular protease activity of Candida sp. was analyzed in terms of bovine serum albumin (BSA) degradation by the technique described by Staib et al. (1965). To determine protease activity, bovine-serum albumin agar (0.1% KHPO4, 0.05% MgSO4, 4% agar and 1% bovine serum albumin) was employed (Tsang et al., 2007). The final pH was adjusted to 4.5. Ten microliters of previously prepared yeast suspension was inoculated into the wells punched onto the surface of the medium onto the plates; these were then incubated at 37°C for 10 days in both aerobic and anaerobic conditions. After incubation, the plates were fixed with 20% trichloracetic acid and stained with 1.25% amidoblack. Decolourization was performed with 15% acetic acid. Opaqueness of the agar, corresponding to a zone of proteolysis around the wells that could not be stained with amidoblack, indicated degradation of the protein. The presence of protease activity was determined by the formation of a transparent halo around the yeast colonies. The diameter of stained zones around the well was considered as a measure of protease production. The protease activity (Prz) was determined in terms of the ratio of the diameter of the well to the diameter of the proteolytic unstained zone. Proteinase activity (Prz) was determined by the method described by Price et al. (1982). Prz was scored as follows: Prz = 1, negative protease activity, Prz =
Table 1. Various virulence factors exhibited by Candida sp.

<table>
<thead>
<tr>
<th>Organism</th>
<th>No. of isolates</th>
<th>Phospholipase (P₂) activity No. (%)</th>
<th>Proteinase (Pr₂) activity No. (%)</th>
<th>Haemolytic (H₂) activity No. (%)</th>
<th>Esterase activity No. (%)</th>
<th>Biofilm formation No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans</td>
<td>22</td>
<td>22 (100)</td>
<td>20 (90.9)</td>
<td>14 (63.6)</td>
<td>13 (59)</td>
<td>19 (86.3)</td>
</tr>
<tr>
<td>C. tropicalis</td>
<td>9</td>
<td>4 (44.4)</td>
<td>9 (100)</td>
<td>7 (77.7)</td>
<td>3 (33.3)</td>
<td>8 (88.8)</td>
</tr>
<tr>
<td>C. krusei</td>
<td>6</td>
<td>2 (33.3)</td>
<td>3 (50)</td>
<td>2 (33.3)</td>
<td>2 (33.3)</td>
<td>2 (33.3)</td>
</tr>
<tr>
<td>C. glabrata</td>
<td>1</td>
<td>1 (100)</td>
<td>1 (100)</td>
<td>1 (100)</td>
<td>1 (100)</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>20 (52.6)</td>
<td>33 (86.8)</td>
<td>24 (63.1)</td>
<td>19 (50)</td>
<td>30 (78.9)</td>
</tr>
</tbody>
</table>

0.64-0.99, positive proteinase activity; and Prz ≤ 0.63, very strong proteinase activity. Thus, a low Prz indicated high production of the enzyme.

Determinantion of haemolysin activity

To determine hemolytic activity, SDA (Oxoid) containing 7% sheep blood and 3% glucose with a final pH adjusted to 5.6 ± 0.2 was employed. Ten microliters of yeast suspension was inoculated onto plates; these were then incubated at 37°C for 48 h in aerobic condition. After incubation, a transparent/semitransparent zone around the inoculation site was considered as positive hemolytic activity (Manns et al., 1994). The ratio of the diameter of the colony to that of the translucent zone of haemolysis (mm) was used as the haemolytic index (Hz value).

Determinantion of esterase activity

To determine esterase activity, Tween-80 opacity test medium was used. The test medium with a pH adjusted to 6.8 consisted of 1% peptone, 0.5% NaCl, 0.01% CaCl₂ and 1.5% agar. After cooling the medium (50°C), 0.5% of Tween-80 was added. Ten microliters of previously prepared suspension was carefully deposited on the Tween-80 opacity test medium. This was then incubated at 30°C for 10 days in aerobic conditions. Esterase activity was considered as positive in the presence of a halo pervious to light around the inoculation site (Slifkin, 2000).

Determinantion of biofilm formation

Candida sp. was evaluated for biofilm formation using the method described by Melek et al. (2012). Sterile 96-well microplates were used to evaluate biofilm formation. Yeast culture was inoculated using a loop into a tube containing 2 ml of brain heart infusion broth (BHIB) medium with glucose (0.25%) and incubated at 37°C for 24 h. Then, all tubes were diluted at a ratio of 1:20 by using freshly prepared BHIB. From this final solution, 200 μL was placed into the microplate, which was then incubated at 37°C for 24 h. After incubation, the microplate was rinsed with PBS 3 times and then inverted to blot. Then 200 μL of 1% crystal violet was added to each well, followed by incubation for 15 min. After incubation, the microplate was again rinsed with PBS 3 times. Then 200 μL of ethanol : acetone mixture (80:20 w/v) was added to each well. They were read at 450 nm using an enzyme-linked immunosorbet assay (ELISA) reader and the OD was recorded for each well. Three wells were used for biofilm formation and the arithmetical mean of 3 readings was used in analysis. Enterococcus faecalis ATCC 29212 was employed as the control strain. Sterile BHIB without microorganism was employed as the negative control. Samples with an OD higher than the cutoff value were considered positive, whereas those with lower value than cutoff were considered negative.

RESULTS AND DISCUSSION

Of the 38 isolates, 22 isolates (58%) were identified as C. albicans, while 9 (24%) were identified as Candida tropicalis, 6 (16%) as Candida krusei, 1 (2%) as Candida glabrata (Table 1). These isolates were studied for the production of hydrolytic enzymes such as phospholipase, proteinase, esterase, haemolytic activity and for the biofilm formation (Figure 1).

Phospholipase activity was found in 20 (52.6%) isolates and positivity for proteinase activity was found in 33 (86.8%) Candida isolates. Hemolysin activity was seen in 24 (63.1%) isolates and esterase activity was found in 19 (50%). About 30 (78.9%) isolates gave positive result for biofilm formation. Maximum phospholipase (100%) activity and esterase activity (59%) was seen in C. albicans whereas maximum proteinase (100%) activity, haemolysin (77.7%) production and biofilm formation (88.8%) was seen in C. tropicalis. C. krusei and C. glabrata also showed positive results for all the activities (Table 1).

C. albicans is an opportunistic pathogenic microorganism that has developed several virulence factors facilitating the invasion of host tissues (Schaller et al., 2005). The ability of Candida species to persist on mucosal surfaces of healthy individuals is an important factor contributing to its virulence. This is particularly important in the oral cavity, where the organism has to resist the mechanical washing action of a relatively constant flow of saliva toward the esophagus (Sitheeque and Samaranayake, 2003). Various virulence factors contribute to the colonization and pathogenicity of C. albicans infection, including the expression of adhesins and invasins on the cell surface (Cannon and Chaffin, 1999), yeast-hyphal morphogenetic transformation, phenotypic switching (Francois et al., 2013), the secretion of hydrolytic enzymes (Schaller et al., 2005), iron acquisition from the environment (Manns et al., 1994), the ability to form biofilm on various surfaces (Williams and Lewis, 2011).

Many different hydrolytic enzymes are identified in
**Virulence factors**

<table>
<thead>
<tr>
<th>No. of isolates</th>
<th>Biofilm formation</th>
<th>Esterase activity</th>
<th>Haemolytic (Hz) activity</th>
<th>Proteinase (Prz) activity</th>
<th>Phospholipase (Pz) activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>C.glabrata</td>
<td>C.krusei</td>
<td>C.tropicalis</td>
<td>C.albicans</td>
</tr>
</tbody>
</table>

Figure 1. Virulence factors produced by different *Candida* sp. isolated from oral cavity.

*Candida* sp. including secreted aspartyl proteinase, phospholipase, lipase and esterase. The production of hydrolytic enzymes helps in colonization of host surfaces, increase adhesion by degrading host surface molecules, allow penetration into host tissues by digesting host cell membranes or evasion of host defense mechanism by digesting cells and molecules of the host immune system hence modulate host immune responses (Calderone et al., 2002).

It has been reported that the enzymatic activity of *Candida* sp. may vary depending on the species and source of isolates (Mohandas and Ballal, 2002). In this study, out of 38 isolates, phospholipase activity was detected in 100% of the *C. albicans*. Tsang et al. (2007) also reported the same positivity rate of phospholipase activity, in samples from patients with oral *Candida* infection. Previous studies have reported phospholipase activity in 30 to 100% of Candidal isolates from various groups of patients and from various sites (Price et al., 1982; Wu et al., 1996). As shown in Table 1, 100% of *C. albicans* produced phospholipase, among the NAC species, *C. tropicalis* followed by *C. krusei* showed maximum phospholipase production. Phospholipase enzyme digests the host cell membrane phospholipid causing cell lysis and changes in the surface features that enhance adherence and consequent infection and hence phospholipase production may be used as one of the parameters to distinguish virulent invasive strains from non-invasive colonisers. About 90.9% of *C. albicans* showed proteolytic activity in the present study. *C. tropicalis* showed 100% proteolytic activity followed by *C. krusei*. This observation was similar to the reports given by previous workers (Marcos-arias et al., 2011).

It was noted that haemolysin activity was higher in *C. tropicalis* (77.7%) followed by *C. albicans* (63.63%). Manns et al. (1994) defined the condition under which *C. albicans* can display haemolytic activity and found that haemolysis is non-existent when no glucose is available in the culture medium. Rossoni et al. (2013) found that non-*Candida* species also produced same haemolytic activity as *C. albicans*. *C. albicans* secretes a haemolytic factor that causes the release of haemoglobin, which is then used as an iron source by the organisms. Watanabe et al. (1999) demonstrated that mannoprotein released from *C. albicans* bound to the band 3 protein on RBCs, thereby promoting their disruption. They detected the haemolytic activity in the culture supernatant of *C. albicans in vitro*. In the oral cavity, extracellular iron is bound mainly to lactoferrin, a protein present in saliva, while intracellular iron is stored as ferritin. Although, this element is bound to proteins and/or is present in the cytoplasm of cells, oral infections with *C. albicans* are frequent, suggesting that this yeast is able to take up different forms of iron from the oral cavity (Almeida et al., 2008).

About 59% of *C. albicans* expressed esterase activity (Pakshir et al., 2013; Aktas et al., 2002). It has been reported that both *C. albicans* and non-*albicans Candida* sp. express esterase activity. Rudek et al. (1978) demonstrated that esterase activity would appear to be a common feature of *Candida* species that are frequently isolated from clinical specimens. Kumar et al. (2006) reported that Tween 80 opacity test cannot be used as the sole phenotypic trait in the differentiation of *C. albicans* and *C. dubliancis* though it appears to be simple, economical and easy method to perform for use in small clinical laboratories. Melak et al. (2012) detected that *C. albicans* showed esterase activity in aerobic conditions but not in anaerobic conditions.

Biofilm formation is one of the most important virulence factors of *Candida* sp. (Figure 1). *Candida* biofilms occur on tissue surfaces as well as the biomaterials of medical devices. As reported by Gultekin et al. (2011), no biofilm formation was detected in any *C. albicans* strains by...
microplate method, while it was found in 50% of non-
albicans Candida sp. Demirbilek et al. (2007) also
detected that the biofilm formation rate was higher in non-
albicans Candida sp. than in C. albicans strains by the
microplate method. In our study, the biofilm formation
rates were found to be higher in C. tropicalis (88.8%) than C. albicans (86.3%). According to the above
mentioned studies, the biofilm formation rate was higher
in non-albicans Candida species as compared to C. albicans isolates.

Conclusion

It is necessary to understand the pathogenicity mechanisms of the Candida sp. for the development of new antifungal strategy. Developing anti-fungal therapies against selective target virulence factor is very crucial nowadays because of the multi-drug resistance developed by Candida sp. Hence, our study on virulence factors of Candida sp. pave way for the better understanding of the various virulence factors exhibited by Candida sp.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

The authors thank the Principal and the Management of PSG College of Arts and Science, Coimbatore, India for providing facilities to carry out the work. They also thank the Principal and the Management of Karpagam University, Coimbatore, India for their consistent support and help during this study.

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Acceptance Certificate

Date: 03-Feb-2016

Manuscript Number  AJB/07.03.15/14552

Manuscript Title: Phenotyping identification of Candida albicans from oral cavity of immunosuppressed patients

Corresponding Author: Deepa Kirishnaswamy

Corresponding Author Email  deepa.shappire@gmail.com

Author(s): DEEPA KIRISHNASWAMY JEEVITHA T MICHAEL A Appalaraju B

Date Accepted: 02-Nov-2015

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