EVALUATION OF PHENOTYPIC METHODS FOR SPECIATION OF CANDIDA AND IN VITRO PRODUCTION OF VIRULENCE FACTORS FROM VULVOVAGINAL CANDIDIASIS

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ABSTRACT

Background: Vulvovaginal Candidiasis (VVC) is an extremely common infection in women of all strata of society. In order to colonize, infect and evade host defense mechanisms, Candida possesses a repertoire of virulence attributes which includes adhesion factors, phenotypic switching and extra cellular lipolytic and proteolytic activity. VVC can be caused by both Candida albicans and nonalbicans Candida (NAC). However identification is laborious and intricate by traditional methods in rural laboratories.

Aim: Study was performed to evaluate the performance of a chromogenic medium for identification of Candida and also to study their virulence properties like phospholipase, proteinase, haemolysin and biofilm production.

Methods: A total of 40 Candida isolates from VVC was processed by both conventional and CHROM agar. These isolates were further tested for virulence factors such as phospholipase, proteinase, haemolysin and biofilm.

Results: There was 100% agreement in identification of isolates by conventional and chromogenic medium. The isolates demonstrated phospholipase activity in 52.5%, caseinase in 50%, haemolysin in 25% and biofilm in 100%.

Conclusion: Data suggested CHROM agar could be used in rural settings. Our study showed that capacity of all Candida spp to fabricate biofilm reveals the pathogenic potential of the isolates.

INTRODUCTION

Vulvovaginal candidiasis (VVC) is not a reportable disease and is often diagnosed without confirmatory tests and treated with over-the-counter (OTC) medications, and thus the exact incidence is unknown. It is estimated that around 75% of all women experience at least one episode of VVC during their childbearing years, of whom about half have at least one recurrence (Sobel et al., 2007). Candida spp., mostly C. albicans, can be isolated in the vaginal tracts of 20 to 30% of healthy asymptomatic nonpregnant women at any single point in time and in up to 70% if followed longitudinally over a 1-year period (Bauters et al., 2002 and Beigi et al., 2004). If the balance between colonization and the host is temporarily disturbed, Candida can cause disease such as VVC, which is associated with clinical signs of inflammation. Such episodes can happen sporadically or often can be attributed to the presence of a known risk factor, e.g., the disturbance of local microbiologic flora by antibiotic use (Achkar and Fries, 2010). Considering the ever changing antifungal spectrums, identification of yeasts to the species level has now become essential, for efficient diagnosis and treatment. Identification of yeasts requires evaluation of microscopic morphologies and a whole range of biochemical studies (Murray et al., 2005). Routine identification of Candida species in the clinical microbiology laboratory is based upon the morphological characteristics such as the formation of pseudohyphae and terminal chlamydospores, clusters of blastoconidia at septa when grown on Corn meal agar at room temperature and the formation of germ tube in serum at 37°C. In addition, carbon source assimilation and fermentation tests or commercially available kits are also used as additional diagnostic tests (Fotedar and Al-Hedaiithy, 2003). In order to facilitate rapid identification, several chromogenic substrate containing culture media have been developed.
These media yield microbial colonies with varying colors secondary to chromogenic substrates that react with enzymes secreted by microorganisms (Murray et al., 2005 and Peng et al., 2007). HiCrome Candida Differential Agar (HiMedia, Mumbai, India) employs this methodology to differentiate several Candida yeasts by color and morphology (Baradkar et al., 2010). It is a yeast differential and selective medium that allows the presumptive identification of C. albicans from other Candida spp. Yeast populations are differentiated by colony morphologies and colours which are generated by a chromophore in the agar (Odds and Bernaerts, 1994). Extracellular hydrolytic enzymes seem to play an important role in candidal overgrowth, as these enzymes facilitate adherence and tissue penetration, and hence invasion of the host. Among the most important hydrolytic enzymes produced by C. albicans are phospholipases and secreted aspartyl proteinases. Furthermore, the ability of C. albicans to acquire elemental iron through haemolysin production is pivotal in its survival and ability to establish infections within humans (Tsang et al., 2007).

Biofilms are a collection of microorganisms surrounded by the slime they secrete. The ability to form biofilms is associated with the pathogenicity and as such should be considered as an important virulence determinant during candidiasis. Biofilms may help maintain the role of fungi as commensal and pathogen, by evading host immune mechanisms, resisting antifungal treatment, and withstanding the competitive pressure from other organisms. Consequently, biofilm related infections are difficult to treat (Bailie and Douglas, 1999). Thus the purpose of our study was determining the utility of HiChrom agar in identification of Candida and also to study the in vitro phospholipase, proteinase, haemolysin and biofilm activities in Candida species isolated from vulvovagina.

MATERIALS AND METHODS

This study was carried out in the department of Aarupadai Veedu Medical College and Hospital, Puducherry, India during the period of August 2010 to September 2012. The study was started after getting the ethical clearance from the scientific research committee of the institution. An informed written consent was obtained from all the subjects. Women with clinically diagnosed vulvovaginal candidiasis were enrolled in the study. Inclusion criteria for the study group were women of all age groups, attending gynaecology clinic with complaints of itching white discharge per vaginum and also clinically on per speculum examination presence of curdy white discharge. Women with clinically diagnosed vulvovaginal candidiasis on antifungal treatment were excluded.

Isolation and identification of Candida

Two high vaginal swabs were collected from each patient. One vaginal swab was subjected to KOH wet mount microscopy and Gram's stain for presence of budding yeast and pseudohyphae. Subsequently, second swab was inoculated on SDA for yeast isolation. Traditional methods as per standard procedure for identification were used such as germ tube formation test, chlamydospore production test, carbohydrate assimilation and fermentation test. Also Candida growth and differentiation of species were also determined by CHROME agar (Hi- media Mumbai).

Virulence factors

The virulence factors studied were enzymatic activity (phospholipase, and caseinase), haemolysin production and biofilm formation.

Phospholipase production

The extracellular phospholipase activity of Candida spp was determined by the egg yolk agar plate method as described by Samaranayake et al. (1984). Briefly 5 μL of inoculum containing 10^8 Candida cells /ml was aseptically inoculated onto egg yolk agar. The plates were incubated at 37°C for 3 days and were examined for the presence of precipitation zone around the colony. The presence of precipitation zone indicated expression of phospholipase enzyme. The phospholipase index (Pz) was calculated by dividing the diameter of the colony by the precipitation zone. A Pz value of 1 indicated negative phospholipase activity; Pz < 1 indicated phospholipase production by the isolate. The lower the Pz value, the higher the phospholipase activity (Deorukhkar and Saini, 2014).

Caseinase production

Caseinase activity was measured by single diffusion technique in SDA agar plates provided with 1% casein. Plates were inoculated with yeast colonies and incubated at 37°C for 48 hrs. zone of clearance was observed by addition of 30% trichloro acetic acid (Dorothi et al., 2002).

Haemolysin activity

Haemolytic activity was measured on sheep blood Sabouraud dextrose agar plate by the method described by Manns et al (1994). Briefly 10 μL of standard inoculum containing 10^8 Candida cells/mL was aseptically inoculated onto the plate. Zone of hemolysis around the colony was considered positive and the test strain produced hemolysin. Hemolytic activity (Hz) was calculated by dividing the diameter of the colony to the translucent zone of hemolysis.

Biofilm formation

Biofilm formation of Candida spp was determined by the tube method (Yigit et al., 2011). Colonies from the surface of SDA plate were inoculated into a polystyrene tube containing 10 ml of Sabouraud-dextrose broth (SDB) supplemented with glucose (final concentration 8%). After incubation at 35°C for 48 h, the broth in the tubes was gently aspirated. The tubes were washed with distilled water twice and then stained with 2% safranin for 10 min. They were then examined for the presence of an adherent layer. Biofilm production was scored as negative (−), weak (+), moderate (+++) or strong (+++).

RESULTS

A total of 40 Candida spp were isolated from VVC. Candida albicans was the most frequently isolated species accounting...
for 65% of the total isolates followed by C. glabrata 22.5%, C. tropicalis 7.5%, C. parapsilosis 2.5%, C. krusei 2.5% (Table 1). Candida albicans constituted 65% which was more than nonalbicans 35%. These 40 isolates were subjected to identification using CHROM agar. There was 100% agreement in the identification of the isolates by CHROM agar method as shown in Table 2. Thus the sensitivity and specificity of CHROM agar was 100% for all the strains. All the isolates were tested for virulence factors like phospholipase, caseinase, haemolysin production and biofilm formation. Our present study aimed at determining the in vitro phospholipase activity in all the strains of C. albicans and non albicans isolated from VVC. As shown in Table 3 positivity of phospholipase activity was detected in 21 (52.5%) isolates and among them maximum activity was seen in non albicans 64.3% and 46.2% C.albicans produced phospholipase. Caseinase production was produced by 50% isolates with maximum among C.albicans 53.8% whereas non albicans produced 42.9% (Table 3). Table 4 shows the hemolysin production of the isolates. Hemolysin activity was seen in 10(25%) isolates. Hemolysin activity was more in C.krusei 75%, followed by C.tropicalis 50%, C.glabrata 50%, C.parapsilosis 25% and least in C.albicans 18%. In the present study all Candida isolates 100% had the ability to produce biofilm invito. Furthermore 50% of the isolates had the maximum ability to form biofilm, 30% were moderate producers, while 20% were weak producers (Table 5).

<table>
<thead>
<tr>
<th>S. No</th>
<th>Species</th>
<th>No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>C. albicans</td>
<td>26 (65)</td>
</tr>
<tr>
<td>2.</td>
<td>C. glabrata</td>
<td>09(22.5)</td>
</tr>
<tr>
<td>3.</td>
<td>C. tropicalis</td>
<td>03(7.5)</td>
</tr>
<tr>
<td>4.</td>
<td>C. parapsilosis</td>
<td>01(2.5)</td>
</tr>
<tr>
<td>5.</td>
<td>C. krusei</td>
<td>01(2.5)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>40(100)</td>
</tr>
</tbody>
</table>

### Table 2. Speciation of Candida by CHROM Agar

<table>
<thead>
<tr>
<th>S. No</th>
<th>Species</th>
<th>Colour on HICHROM Agar</th>
<th>No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>C. albicans</td>
<td>Light green</td>
<td>26(65)</td>
</tr>
<tr>
<td>2.</td>
<td>C. glabrata</td>
<td>Purple</td>
<td>09(22.5)</td>
</tr>
<tr>
<td>3.</td>
<td>C. tropicalis</td>
<td>Dark Blue</td>
<td>03(7.5)</td>
</tr>
<tr>
<td>4.</td>
<td>C. parapsilosis</td>
<td>Cream</td>
<td>01(2.5)</td>
</tr>
<tr>
<td>5.</td>
<td>C. krusei</td>
<td>Pink</td>
<td>01(2.5)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Organisms</th>
<th>No of isolates</th>
<th>Phospholipase No (%)</th>
<th>Caseinase No (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.albicans</td>
<td>26</td>
<td>12 (46.2)</td>
<td>14 (53.8)</td>
</tr>
<tr>
<td>C.nonalbicans</td>
<td>14</td>
<td>9 (64.3)</td>
<td>6 (42.9)</td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>21 (52.5)</td>
<td>20 (50)</td>
</tr>
</tbody>
</table>

### DISCUSSION

VVC is an infection caused by abnormal growth of yeasts in the mucosa of the female genital tract (Consolaro et al., 2004). It is a frequent diagnosis in the daily practice of gynaecology and accounts for large numbers of visits to general practices in Puducherry. Around 75% of adult women will experience at least one episode of VVC during their lifetime, of which 5% will develop recurrent vulvovaginal candidiasis, with at least four symptomatic episodes of vaginitis in one year (Sobel et al., 2007). Although clinical occurrence of various Candida spp is reported: yet, the most commonly implicated species is still C. albicans. This is responsible for 80% of symptomatic episodes of VVC, but still its incidence is declining and non albicans species rapidly supervening (Wei et al., 2010). This declining trend was also observed in our study though C. albicans were isolated at a higher frequency. Out of 40 Candida isolates in the present study, species identification revealed that 26 (65%) were C.albicans, whereas 14(35%) isolates belonged to non albicans Candida. Relatively recent studies showed different C. albicans colonization rate; 90% in China, Tibet (Wei et al., 2010), 94% in Iran, Ahvas (Mahmoudabadi et al., 2010), 46.9% in India (Ahmad and Khan, 2009). In many part of the world, NCA isolates notably C. glabrata effect 10 to 20% of women (Corsello et al., 2003; Buscemi et al., 2004).

In Turkey, India, and Nigeria, cases due to C. glabrata range between 30 to 37 (Achkar and Fries, 2010). In our study C. glabrata was the second commonest isolated (22.5%). Vaginal culture is the most accurate method for the diagnosis of VVC. Among the various culture methods, there appears to be no difference between Sabouraud agar, Nickerson's medium, or Microstix-candida medium. CHROM agar Candida is a selective fungal medium that includes chromogenic substances allowing for quick identification of several different Candida spp, based on their color, which also facilitates the detection of mixed infections with more than one species of Candida. Antigen detection or serological tests as well PCR-based diagnosis are either not yet reliable or not clinically useful because they are too sensitive (Achkar and Fries, 2010). For differentiation between different species of Candida conventionally Germ tube test, chlamydospore formation, sugar fermentation and assimilation tests are being used which are laborious and time consuming. CHROM AGAR is a rapid method to differentiate between different Candida species. It facilitates the detection and identification of Candida species from mixed culture and provides result in 24-48 hours (Devi and Maheshwari, 2014). In our study, sensitivity and specificity of CHROM agar for Candida spp were 100%. However a study by Shymala et al though showed 100% sensitivity and specificity for C. albicans yet sensitivity for C.tropicalis was only 68%, C parapsilosis 23.08%, C.krusei 44% and C.glabrata 66.67%.
Our results were however consistent with study by Sumithra Devi (2014) where sensitivity was 100% for C. albicans, C. tropicalis, C. Krusei where as 75% for C.glabrata. All the isolates identified by the conventional methods in our study were identified by the CHROM agar without difficulty. Since the traditional methods are laborious and time consuming it can be replaced by HICHROM agar in rural laboratories. Virulence attributes have been investigated in other mucosal candidiasis models, including VVC. Importantly, recent studies suggest that the presence of vaginal Candida strains with enhanced virulence and tropism for the vagina correlates with the severity of VVC in humans. From these studies we have learned of Candida's exceptional ability to adapt rapidly to environmental stimuli. Many attributes contribute to C. albicans virulence, among them adhesion, hyphal formation, and ability to secrete haemolysin to lyse host erythrocytes and strip iron from hemoglobin molecules, which facilitates hyphal invasion in disseminated candidacies (Odds and Bernaerts, 1994). Our present study showed hemolytic activity was more in C. krusei 66.7%, followed by C. tropicalis and C.glabrata 50% each, C.parapsilosis 20% and C.albicans 15.4%. Studies on the activity of haemolysin in Candida are limited. However many studies showed C. albicans produced maximum haemolysin activity which was in contrast to our study (Sachin et al., 2013; Ruchel et al., 1983). Biofilms are a collection of microorganisms surrounded by the slime they secrete. The ability to form biofilms is associated with the pathogenicity and as such should be considered as an important virulence determinant during candidiasis. Biofilms may help maintain the role of fungi as commensal and pathogen, by evading host immune mechanisms, resisting antifungal treatment, and withstanding the competitive pressure from other organisms. Consequently, biofilm related infections are difficult to treat (Bailie and Douglas 1999). In our study 100% of the candida isolates formed strong biofilm, 30 % formed mild biofilm formation & 20% of the isolates did not form any biofilm.

Conclusion

Non albicans Candida which was in the past considered as nonviral are now implicated as causative agents of VVC. In rural laboratories CHROM agar can be used as a simple diagnostic test for the identification of Candida spp. Detection of virulence factors helps in the establishment of the isolate as pathogen. Also the production of biofilm by all the isolates in our study reveals the pathogenic potential.

REFERENCES


