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RESEARCH ARTICLE

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 repertoir of virulence attributes which includes adhesion factors, phenotypic switching and extra cellular lipolytic and proteolytic activity. VVC can be caused by both *Candidia 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, hemolysin 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.

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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 37^0C. In addition, carbon source assimilation and fermentation tests or commercially available kits are also used as additional diagnostic tests (Fotedar and Al-Hedaithy, 2003). In order to facilitate rapid identification, several chromogenic substrate containing culture media have been developed.

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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 pathogenecity 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 (Baillie 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 37^0 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% where as *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 invitro. Furthermore 50% of the isolates had the maximum ability to form biofilm, 30% were moderate producers, while 20% were weak producers (Table 5).

Table 1. Distribution of *Candida spp* isolated from vaginal discharge in SDA by traditional methods

S. No	Species	No (%)
1.	*C. albicans*	26 (65)
2.	*C. glabrata*	09(22.5)
3.	*C. tropicalis*	03(7.5)
4.	*C. parapsilosis*	01(2.5)
5.	*C. krusei*	01(2.5)
Total		40(100)

Table 2. Speciation of *Candida* by CHROM Agar

S. No	Species	Colour on HICHROM Agar	No (%)
1.	*C. albicans*	Light green	26(65)
2.	*C. glabrata*	Purple	09(22.5)
3.	*C. tropicalis*	Dark Blue	03(7.5)
4.	*C. parapsilosis*	Cream	01(2.5)
5.	*C. krusei*	Pink	01(2.5)

Table 3. Phospholipase and caseinase activity among *Candida spp* obtained from VVC

Organisms	No of isolates	Phospholipase No (%)	Caseinase No (%)
C.albicans	26	12 (46.2)	14 (53.8)
C.nonalbicans	14	9 (64.3)	6 (42.9)
Total	40	21 (52.5)	20 (50)

Table 4. Virulence factor-haemolysin activity

Organisms	No of isolates	Haemolytic No (%)
C.albicans	26	4(15.4)
C.parapsilosis	5	1(20)
C.tropicalis	4	2(50)
C.krusei	3	2(66.7)
C. glabrata	2	1(50%)
TOTAL	40	10(25%)

Table 5. Virulence Factor (Biofilm)

S. No	Biofilm	No(%)
1.	Strongly positive	20 (50)
2.	Moderate	12(30)
3.	Mild	8(20)
Total		40(100)

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, Tibeta (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 serologic 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 adaptability by rapid alterations in gene expression in response to various environmental stimuli. Many attributes contribute to *C. albicans* virulence, among them adhesion, hyphal formation, phenotypic switching (PS), extracellular hydrolytic enzyme production, and biofilm formation (Achkar and Fries, 2010). Phospholipases are a group of enzymes produced by *Candida* species that primarily help in digesting the phospholipids of the host cells leading to cell lysis. *C. albicans* is the major producer of phospholipases, whereas a less proportion of non-*albicans Candida* produce this enzyme.

It is postulated that more sensitive methods are needed to detect the lesser amount of phospholipases produced by non*albicans Candida* (Ghannoum, 2000). In our study 52.5% of the isolates produced phospholipase. Similar finding was observed in a study by Deepa *et al*. (2015) where 52.6% of the isolates produced phospholipase. As phospholipases and aspartyl proteinases of C. albicans are considered important virulence factors , the absence or lowered expression of these enzymes may indicate the less virulent nature of Candida species, when compared with Candida species with higher expression of these enzymes (Mohan das and Ballal 2008). In our study C.albicans accounted only for 46.2% and non albicans 64.3%. However Mahmoudabadi *et al*. (2010), reported that all clinical isolates of *C. albicans* from VVC showed phospholipase activity. On the other hand our *C.albicans* relatively produced less phospholipase yet it was a pathogen suggests that other factors may have contributed to its virulence. An emphasis on hydrolytic enzymes produced by *Candida* spp. can help in understanding the disease process better as these enzymes have activity on a wide array of host substrates. Secreted aspartyl proteinases (SAP) in *Candida* are known to enhance the hypha formation, epithelial cell damage, invasion, and inflammatory responses.

In vivo experimental models also demonstrated an increase in the invasiveness of yeasts with the production of proteinases (Tellagrada *et al.,* 2014). For testing the proteinase activity of the candida isolates caseinase test was performed in our study. Proteinase activity was observed in 50% of the isolates. This result was almost similar to study by Camargo *et al*. (2008) that found 58.3% positive samples for proteinase activity. Haemolysin is another putative virulence factor thought to contribute to candidal pathogenesis. *C. albicans* have the 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 pathogenecity 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 (Baillie 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 nonvirulent 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.

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