Identification and Characterization of *Candida* on CHROMAgar\textsuperscript{TM} in Pregnant Women of Multan, Pakistan

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Abstract

**Introduction:** Infections to vagina with fungi/yeast are ten times more common during pregnancy because of hormonal and biochemical fluctuations. Increased use of antibiotics and presence of high levels of reproductive hormones during pregnancy also stimulate yeast colonization with *Candida* species. These two contributes significantly to be an important risk factor in Vulvovaginal Candidiasis (VVC) and Urinary Tract Infections (UTI), a complication during pregnancies. Overall occurrence of UTI/vaginal yeast infections increases with progression of pregnancy and maximizes by third trimester worldwide.

**Study parameters:** In the current study, pregnant women were look for presence of UTI by *Candida* species in different trimesters within the city of Multan. The total of 70 women with different gestational stages, attending gynecologists, aged 18-39 years were included in the investigation. A clean catch urine samples were cultured on CHROMAgar\textsuperscript{TM} *Candida*, a selective medium, for the isolation and presumptive identification of yeast species. Of all samples, 25 women (36%) were positive for *Candida* yeast infection (symptomatically and asymptotically).

Colonies were identified as *C. albicans* (48%), *C. krusei* (16%), *C. tropicalis* (16%) along with some mixed infections with *C. parapsilosis* and *C. krusei*. Two of the mixed cultures (8%) were isolated with *C. albicans* and *C. tropicalis*, one (4%) with *C. albicans* and *C. krusei*, one (4%) with *C. tropicalis* and *C. krusei*.

**Results:** The highest incidence, 36% (n=9/25), of candiduria was recorded in the age group of 30-35 years. The colony counts in urine were also studied in different gestational stages and age. Total of 23% isolations share high counts (>1000 CFU/mL). While other 33% have intermediate (<600 CFU/mL) and 30% isolates were with low colony counts (<150 CFU/mL). The species distribution in these positive specimens reveals increased percentages of non-albicans species with increasing gestational stage.

**Conclusion:** From these findings it is concluded that incidence of candiduria in pregnant women were higher in 2\textsuperscript{nd} and 3\textsuperscript{rd} trimester of pregnancy with an increase in mixed *Candida* infections in young women. It is recommended to educate the group so that less young individuals are affected.

**Keywords:** Pregnancy; Infection; *Candida albicans*, Non-albicans species

Introduction

Vaginosis in pregnancy is most commonly linked to infection by *Candida* species [1]. In female, *Candida* is also part of normal flora and become opportunistic pathogen. In these women with Vulvovaginal candidiasis (VVC), most common problem and affect women two or more episodes yearly [2-4], can also involve urinary tract [5,6]. The infection accounts for 40-50% of all women cases within their childbearing age [7]. Accurate prevalence of these infections are quite difficult to assess as one third to three quarter of affected women 'feel' asymptomatic [7,8]. Recent randomized screening in pregnant women indicates complications like candidiasis, bacterial vaginitis, and/or trichomoniasis, are on the increase that could result in spontaneous preterm birth in 46% of pregnancies by 15-19 weeks of gestation [9]. It has been reported that about 75% females develop it at least once in their lifetime [10]. *Candida albicans* is most frequently (90%) isolated form compared to other non albicans species [11]. Recent reports on *Candida* infections suggests that there is a shift in colonization pattern by *C. glabrata* [12] and other non-albicans species within developed countries [13]. From data, it is estimated that *Candida glabrata* account for 4-5% in these country individuals. While other non albicans species appear to be still less common that includes *Candida tropicalis* and *Candida parapsilosis* [14].

It is very difficult to ascertain the involvement of *Candida* species in vaginosis and later with candiduria. During vaginosis more than 50% of these individuals are asymptomatic and harbor endogenous vaginal microbiota [15]. This asymptomatic vaginosis very efficiently gets into urinary tract that could result without tissue invasion [16,17]. Milder signs and symptoms in urinary *Candida* infection don't permit proper diagnosis [18]. Factors like socio-economic variables, maternal smoking, other genital infections and short cervix contributes to increase colonization by *Candida* species [19]. These UTIs are also associated to number of gynecological and obstetric complications,
such as preterm birth [20], premature rupture of the membranes, chorioamnionitis [18], postpartum endometritis, inflammatory pelvic disease, intrauterine growth retardation [21], and low birth weight of the new born [1]. There are reports of transfer of *Candida* species from vaginal infection to new born also [5,22]. Increased UTI colonization and candiduria are believed to be due to higher incidences of vaginosis resulting from high estrogen levels, high glycogen content, and use of antifungal agents in treatment of VVC [21,23]. Identification of shared similar drug resistance markers in *Candida* and its species, from urine and vaginal, demonstrates their common ancestral origin [19].

Pregnancy is typically broken into three periods, or trimesters [20]. It has been elucidated with good conviction that *Candida* species can be cultured from the urine or vagina in more than >25% of pregnant women who are approaching term [24]. Prevalence survey in 228 hospitals from 29 European countries determined that just 9.4% of nosocomial UTIs were caused by *Candida* species [11]. During this period considerable high rates are observed with non-albicans species. This isolation frequency of non-albicans species especially in some countries, are unclear and dubious of its selection [20]. This scenario is further compounded with presence of antifungal resistance in non-albicans *Candida* species that is a worry for future [25].

**Material and Methods**

**Sample area and collection**

Total of 70 randomly collected urine samples from pregnant women in different pregnancy trimesters was included in the study. The subjects were between 18 to 39 years. Sampling was done in local winter-spring season (more marriages in winter months). Urine was collected from antenatal patients’ visiting medical centers, maternity homes of Multan, Pakistan. Patients were inquired to complete a detailed questionnaire. The questionnaire includes demographic data (age, age at marriage, cousin marriage), number of pregnancies/deliveries, trimester, signs and symptoms related to *Candida* infection. The midstream urine was collected in sterile urine containers (China) and transferred to Micro/Molecular Biology Laboratory (MMBL) at IPAB, Bahauddin Zakariya University, Multan 60800 in a cooler packed with ice packs for further processing.

**Media preparation and sample processing**

CHROMAgar™ *Candida* was provided by CHROMAgar™, Paris, France. Media plates with antibiotics were made according to the manufacturer instructions. Prepared plates were stored in dark at 4°C till further use. Individual sample (1 mL) was centrifuged in sterile 1.7 mL microfuge tube (China) at 1400 g for 1 minute (Heareus, Germany). Samples having more than 10 WBC/HPF were selected for inoculation on to CHROMAgar™ *Candida* plates (0.1 mL of neat urine) with a pasture glass spreader in a clean bench surface (Dalton, UK). Plates were incubated (Memmert, Germany) at 36 ± 0.5°C for 48 hours. Colonies were identified according to manufacturer’s instruction or described by Odds and Bernaerts.

**Statistical analysis**

Data was formulated from the questionnaire for all parameters and were analyzed for ANOVA and correlation of significance by SPSS 17 (SPSS Inc., USA).

**Results**

**Isolation and identification**

Direct presumptive identification of yeast species is a challenging task. Several brands of chromogenic media are available for rapid identification of yeast. This also includes CHROMAgar™ *Candida* [26,27]. This media provides straight forward advantage in time over conventional identification methods in mixed yeast infections. This is also important in significant clinical bearing [28]. In a total of 70 samples, only 25 samples (36%) turned positive for *Candida* species (Table 1).

The chromogenic substances in media help to identify *C. albicans*, *C. krusei*, *C. tropicalis*, and *C. parapsilosis*. The *C. albicans* colonies were identified as green, *C. tropicalis* as steel blue, and *C. krusei* as fuzzy rose colored colonies. *C. parapsilosis* appears with pink to pale yellow colonies [29]. Other groups have also reported further success in differentiation of *C. dubliniensis* from *C. albicans* on CHROMAgar™ *Candida* medium which our group did not performed. From the total positive cultures, *C. albicans* (48%) share the maximum load (Table 1).

Non albicans *Candida* share just 32% in pure culture category. Mixed culture infections of *C. albicans* with either *C. krusei* or *C. tropicalis* (12% and 8% respectively) as non albicans were also recorded in the study.

### Table 1: Relationship between age and *Candida* species infection in pregnant women.

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Pure culture</th>
<th>Mixed culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>C. albicans</em></td>
<td><em>C. krusei</em></td>
</tr>
<tr>
<td>18-23 Y (n=21)</td>
<td>2 (10%)</td>
<td>2 (10%)</td>
</tr>
<tr>
<td>24-29 Y (n=29)</td>
<td>4 (13%)</td>
<td>1 (3%)</td>
</tr>
<tr>
<td>30-35 Y (n=19)</td>
<td>6 (32%)</td>
<td>1 (5%)</td>
</tr>
<tr>
<td>36-41 Y (n=1)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>n=20</td>
<td>-</td>
<td>-</td>
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<tr>
<td>n=5</td>
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<td>-</td>
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</tbody>
</table>

Pregnancy trimester and Candida species

Breakup of pregnancy into trimester showed that most positive yeast infection occurred in the 3rd trimester (36%) (Table 1). Out of 15 positive cultures only 2 had mixed culture outcome (Table 2). In the 2nd trimester, highest number of specimens collected turned positive (47%). The species isolated and identified in all trimester specimens showed that 80% have single organism (Table 1).

Table 2: Relationship between age and vaginal candida burden in pregnant women.

Interestingly, 60% of positive colorizations were within 3rd trimester (Table 2). Presence of C. krusei C. albicans or C. tropicalis or C. parapsilosis were identified in 4 cultures (20%) and 4 mixed cultures respectively (Table 2).

Age, Candida species and yeast burden

There were 15 Candida albicans cultures (60%) isolated that were either pure or mix with C. krusei or C. tropicalis. All mixed infections (20%) were, interestingly, present in all young age groups (18-23 years) and (24-29 years). In the middle age group, only single organism infection (predominantly C. albicans- 67%) occurred (Tables 2 and 3).

Talking about the yeast burden, most infections (>600 CFU/mL) were identified in middle age groups (24-29 years and 30-35 years) (Table 3). This group also is dominated by pure cultures by 2/3 positivity with C. albicans. Low yeast counts were predominantly noticed in only younger age groups (Table 3).

Table 3: Distribution of positive culture with trimesters of pregnancies.

Statistical analysis

ANOVA and correlation analysis on various factors indicated that yeast infections were highly significant (p=0.000 at 95% CI) to the pregnancy in this study. Analysis also correlates to Candida cell counts which is highly significant to pregnancy month (Pearson’s Correlation 0.872 and significance of 0.000 at 0.01% CL) and trimester (Pearson’s Correlation 0.447 and significance of 0.000 at 0.01% CI).

Data also specify that numbers of pregnancies are also significantly placed to the age and symptoms (Tables 4 and 5).

Table 3: Distribution of positive culture with trimesters of pregnancies.
Interestingly, as the age of patient increases, chance of symptomatic rather asymptomatic infection also increases. This criterion of positively relate to number of pregnancies and deliveries (Table 5). Correlation dissection showed that in 1st and 2nd trimesters yeast burden is highly significant to pregnancy month and infecting *Candida* species (Tables 4 and 5). Comparison of same parameters in 2nd and 3rd trimester (most positive cultures) indicates that pregnancies correlates highly to symptoms and the month of pregnancy. *Candida* counts, however, were highly negatively rerated to *Candida* species and deliveries (Tables 5 and 6).

### Pearson’s Correlations (2 tailed)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Age</th>
<th>Symptoms</th>
<th>Trimester</th>
<th>Month</th>
<th>Hospital</th>
<th>Pregnancies</th>
<th>Deliveries</th>
<th>Candida Sp.</th>
<th>CFU/mL</th>
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<tr>
<td>Age</td>
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<td>Symptoms</td>
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<tr>
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<tr>
<td>Pregnancy month</td>
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<td>0.811**</td>
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<td></td>
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<tr>
<td>Hospital</td>
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<td></td>
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<tr>
<td>Pregnancies</td>
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<td>0.466**</td>
<td>0.307**</td>
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<tr>
<td>Deliveries</td>
<td>0.372**</td>
<td>0.340**</td>
<td>0.823**</td>
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<td>0</td>
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<tr>
<td><em>Candida</em> Species</td>
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<td>-0.333**</td>
<td>-0.300**</td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>0.002</td>
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<tr>
<td>CFU/mL</td>
<td>0.398**</td>
<td>0.301**</td>
<td>-0.230*</td>
<td>-0.245*</td>
<td>1</td>
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<tr>
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</table>

**Table 4**: Pearson’s correlation of *Candida* species.

**Table 5**: Pearson’s correlations of trimester 2 and 3 (n=59).
Use of such culture medium also addresses the important issue of grave concern around the world [3,12,22]. In our hands, results did not follow because of antibiotic resistance [19,38].

However, lower incidences of Candida albicans are reported from Tanzanian pregnant women [36-38] where hygienic and socio-economic conditions may have played the differences. In North America, Europe and Latin American studies with CHROMAgar™ show that Candida albicans share the burden 51.5%, 47.8%, and 36.5%, respectively [18,23]. Taken together, in our study in this part of the world, we observed similar isolation rate as indicated in above mentioned studies. In Mexican study, on the contrary, other isolated species includes were Candida parapsilosis, Candida tropicalis, Candida krusei, and Saccharomyces cerevisiae [18,19,23]. In many other studies on specifically candiduria, Candida albicans is shown to share 50-70% burden followed by C. glabrata, and C. tropicalis, which is the third most common species [39,40]. Similar findings were recorded in this study also except presence of C. glabrata which we did not follow because of antibiotic resistance [19,38].

In present study, we also looked at the nature of asymptomatic and symptomatic yeast infection as per reported worldwide for Candida species. We find little lower symptomatic reporting of candiduria that is reported in other parts of the world. This may be linked to less education in the females. Significance of trimesters of pregnancy were also ascertained in the investigation. Our data show that 36% of the collected samples were positive with non albicans species (Table 2). Importantly of these, most common non albicans species, was Candida tropicalis in our case study (n=9/25) (Table 1). Similar patterns were seen in other studies [3,5,39,40]. Portuguese data from Negri et al. [39] provide good support to the notion that non albicans strains are becoming more important day by day.

The age distribution data in the study show that Candida infection is predominantly present in middle age (30-35 years) group. Majority of these isolates were pure infection of Candida albicans (55.6%) (Table 1). This is in agreement with the results of American and Brazilian studies [13,40]. On the contrary, Venezuelan study provided similar good support to age groups for other Candida infections [41]. Our data also signifies to the fact that more non albicans species and mixed infections were isolated in 18-23-year group (Table 1). We also incline to infer from our data that non albicans species are on rise [2,10,17]. In this study, C. tropicalis is next most prevalent species after C. albicans. Repetitiony and co-workers [42] suggested that higher cases of this species tend to be a result of patients being neutropenic which isn't been convincible address in literature. This high level of colonization of non albicans were observed in older subjects which is not seen in our study (Table 1). It is also documented that occurrence of nonalbicans species (up to 20-30% in some cases) are associated to recurrent candidiasis [12]. Quantification of viable yeast cell indicates that 23% of the isolates have higher than >1000 CFU/mL of urine. Other studies

### Table 6: Analysis of variance with age.

<table>
<thead>
<tr>
<th></th>
<th>Within groups</th>
<th>Between groups</th>
<th>0.004*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deliveries</td>
<td></td>
<td></td>
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<tr>
<td>Pregnancies</td>
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</table>

**Discussion**

During pregnancy, vagina is more susceptible to many infections because of its proximity, results in higher incidences of lower urinary tract colonization [16,17]. In a literature citation, candiduria indicates an asymptomatic presentation in patients, yet presence of yeast is noted in the urine as a serendipitous in routine urinalysis or urine culture [16]. These infections have lower cure rates [25] with more resistant species [20]. It has been demonstrated further that micro biota of vagina is of similar patterns as observed in urine [30]. Recent literature also highlights yeast drug resistance in non albicans yeast strains than in any other [4,22]. Thus, rapid identification of these yeast strains provides valuable information concerning treatment regimens in clinical settings [27,31]. Rein and colleagues [32] provided an outlined for infections in 60-90% women. In our study, total of 70 patients, 17 women were placed in 2nd trimester of pregnancy out of which 8(47%) were positive for Candida species (Table 2).

In 3rd trimester, one third (36%) specimens were positive. Jointly together, they share two third of women get candiduria during their antenatal days which is statistically important (Table 5). This is contradictory to recent finding where trimester doesn’t have any adage [33]. In number of other studies, however, demonstrated that from the conception day till it reaches 2nd and 3rd trimester, Candida infections were recorded highest [5,25,34]. Similar conclusion was drawn from study of Rein et al. [32] where 10% of infections were in 1st trimester as compared to 36-55% in the 3rd trimester. Some other studies provide some controversies on rates of candiduria. These studies show that 3rd trimester has similar rates to that of 23-26 (2nd trimester) weeks of gestation [35]. This provides a positive backing to our study (Table 3).

CHROMAgar™ Candida has previously been reported to provide valuable results in identifying Candida species [2,26,27]. The accurate identification of Candida species on colony color and morphology provide fast and decisive offering for clinical treatment regimens [2,28]. Use of such culture medium also addresses the important issue of non albicans species which are emerging all around and is a matter of grave concern around the world [3,12,22]. In our hands, results conclude that all major pathogenic Candida species were conveniently identified on CHROMAgar™ Candida. This advantage of early identification is an essential step for clinician to use specific treatment protocols in lowering yeast burden in pregnant women. This provides lowering of complication during pregnancies [19,25]. In our study, Candida albicans was isolated in all cultures (either pure or mixed). The non albicans group shares, Candida krusei (28%), Candida tropicalis (20%), and Candida parapsilosis (4%) (Table 2). In a large multicenter study from Spain, Candida albicans were recovered as high as 68%, followed by Candida glabrata (8%) and Candida tropicalis (4%) [18]. The occurrence of such numbers has also been reported by da Silva et al. [17] from Brazil. Another large sample study from Iran, provided similar results as of our study results [16].

The advantage of early identification is an essential step for clinician to use specific treatment protocols in lowering yeast burden in pregnant women. This provides lowering of complication during pregnancies [19,25]. In our study, Candida albicans was isolated in all cultures (either pure or mixed). The non albicans group shares, Candida krusei (28%), Candida tropicalis (20%), and Candida parapsilosis (4%) (Table 2). In a large multicenter study from Spain, Candida albicans were recovered as high as 68%, followed by Candida glabrata (8%) and Candida tropicalis (4%) [18]. The occurrence of such numbers has also been reported by da Silva et al. [17] from Brazil. Another large sample study from Iran, provided similar results as of our study results [16].
have also provided similar results [43-45]. Of these 1/3 of positive specimens had intermediate numbers and 30% had low counts (<150 CFU/mL) (Table 3). Interestingly low yeast counts were detected in younger subjects with more non-albicans species.

Over and above, worldwide and our data indicates that infections with C. krusei, C. tropicalis and other non-albicans species have gone up [18,44]. Occurrence of this high rate is quite disquieting. Recent literature highlights the notion that C. krusei along with C. tropicalis are involved in invasive fungal infections [18,19]. Other studies demonstrate that these organisms also show resistance to teriazoles with high mortality [4]. Presence of these species in relatively high number of young pregnant women from Punjab, Pakistan is alarming. Possible explanation to the scenario may be due to malnutrition, lower immunity levels and use of antifungal agents that needs to be further investigated like other workers have done in other parts of the world (Tables 1 and 2) [19,45].

**Conclusion**

This study, first of its kind in south Punjab, concludes finally that candidiasis in Pakistani women is at the same rate, same species distribution as per seen in other parts of the developing world [43]. A larger future project would provide more insight into Candida species infections in non-pregnant and pregnant (different trimesters) women would provide discernment in resident microbiota that could contribute to this pathological conditions.

**Acknowledgement**

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**References**