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Research Article

## Molecular and phenotypic identification of *Candida* isolates from pregnant women in Ogbomoso, Southwestern Nigeria

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### ABSTRACT

**Background:** Vulvovaginal candidiasis (VVC), often referred to as a yeast infection is a common gynaecologic disease, affecting 3 out of 4 women in their lifetimes. More than 40% of affected women will have 2 or more VVC episodes, and infection occurs more frequently in pregnant women. This study was carried out to provide information on the appropriate diagnostic method required to differentiate the causative agents of VVC among pregnant women.

**Methods:** In this study, vaginal specimens were collected from one hundred (100) pregnant women aged between 17-44 years and of gestation age of 14-36 weeks who were attending antenatal clinic at LAUTECH Teaching Hospital, Ogbomoso. The species identification was performed using chromogenic medium, induction of fungal germ tube formation, and PCR using universal primers of internal transcriber spacer (ITS1 and ITS4); (ITS1 [5'-TCCGTAGGTGAACCTGCGG-3'] and ITS4 [5'-TCCTCCGCTTATTGATATGC-3']) and *Candida albicans*-specific primers [5'-GGTTTGCTTGAAAGACGGTAG-3'] and [5'-AGTTTGAAGATATACGTGGTAG-3'] that target sequences site of the intergenic spacer region (ITS) of the fungal rRNA genes (18S and 28S) were used for this assay.

**Results:** Forty (40) *Candida* species from 100 specimens were isolated in Sabouraud dextrose agar (SDA) medium. Of 19 strains of *C. albicans* that were identified by chromogenic agar (CHROMagar), 17 were confirmed as true positive by PCR while 2 were false positive. The CHROMagar had 89.4% sensitivity and 90.4% specificity. In comparison, GTT was better in correctly identifying those strains that were confirmed as *C. albicans* by PCR (Sensitivity=94%) while CHROMagar was better in identifying the strains that were not confirmed as *C. albicans* by PCR (Specificity=90.4%).

**Conclusions:** The combine uses of chromogenic agar and PCR have the advantage of efficient differentiation and identification of *Candida* species.

**Keywords:** Candida isolates, Pregnant women, Molecular identification

### INTRODUCTION

*Candida* is a part of human flora. It becomes pathogenic when certain conditions are present and becomes opportunistic infection.<sup>1</sup> The major etiological agent is

*Candida albicans*, whereas different *Candida* species can cause a variety of infections, including *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. guilliermondii*, *C. glabrata*, and *C. kefyer* which represent many clinical forms of candidiasis.<sup>2</sup>

Candidiasis is a primary or secondary mycotic infection caused by members of the genus *Candida*.<sup>3</sup> The clinical manifestations may be acute, subacute or chronic. The infection may be localized in mouth, throat, skin, scalp, vagina, nails, bronchi, lungs or in the gastrointestinal tract or become systemic as in septicemia, endocarditis and meningitis.<sup>4</sup> *Candida* infections have become a problem of growing significance.<sup>5</sup> Candidiasis is the most common opportunistic fungal infection.<sup>6</sup>

Vulvovaginal candidiasis (VVC), often referred to as a yeast infection, is a common gynaecologic disease, affecting 3 out of 4 women in their lifetimes.<sup>7</sup> More than 40% of affected women will have two or more VVC episodes, and infection occurs more frequently in pregnant women.<sup>7,8</sup> It is believed that higher estrogen levels and higher glycogen content in vaginal secretions during pregnancy increase a woman's risk of developing VVC.<sup>9</sup> Also women in childbearing years, have episodes of VVC. Vulvovaginal candidiasis is caused by abundant growth of yeast, belonging to the *Candida* species in the vaginal mucosa.<sup>10</sup>

Generally, women are said to present with clinical symptoms of vulvovaginal candidiasis when the high vaginal yeast count is greater or equal to 10<sup>5</sup> CFU/ml of vaginal fluid.<sup>11</sup> There are however, several reports implicating symptomatic vulvovaginal candidiasis and increase in rate of transmission and spread of HIV infection in several parts of sub-saharan Africa and beyond.<sup>12</sup> Recurrent vaginal candidiasis is also common in female patients with acquired immune deficiency syndrome (AIDS), suggesting a role for depressed cell-mediated immunity in candidiasis.<sup>12</sup>

Appropriate and rapid laboratory assays are important to identify *Candida* species in clinical samples to diagnose Candidal vulvovaginitis of women. This type of candidiasis can be very important and significant among HIV positive, pregnant, and immunodeficiency women due to the development of systemic candidiasis in this group of patients.<sup>13</sup>

The currently used laboratory assays such as direct microscopy, culture and biochemical methods to detect and identify *Candida* species accurately, are time-consuming, and lack the necessary validity. Also, these assays fail to precisely differentiate between the pathogenic *Candida* species with none or less pathogenic species of *Candida*. Since vaginal candidiasis is particularly a health concern in pregnant women, a prompt and reliable diagnostic process is vital in order to choose appropriate therapeutic protocol. This study was therefore conducted to provide information on the appropriate diagnostic method required to differentiate the causative agents of candidiasis in pregnant women.

## METHODS

### Study area

This study was carried out at the Department of Microbiology of Obafemi Awolowo University. The participants were recruited at the Ladoko Akintola University of Technology (LAUTECH) Teaching Hospital, Ogbomoso, Oyo State. The hospital is located in Ogbomosho North Local Government Area, Ogbomoso.

### Study design

The study was a cross sectional survey of one hundred (100) pregnant women aged between 17-44 years and of gestation age of 14-36 weeks who were attending routine antenatal clinic for the first time between July 2014 - Jan 2015 at LAUTECH Teaching Hospital in Ogbomoso. Socio-demographic information and obstetric history was obtained from participants by the use of structured questionnaire. Pre-test counseling for sexually transmitted infections (STIs) was given to each subject by a trained counselor before specimens were collected from them.

### Inclusion criteria

Pregnant women on booking at the hospital were included while members of staff of the hospital, patients with previous history of preterm labor or spontaneous abortion and those that did not give their consent were excluded from the study.

### Sample collection

The vaginal smears were obtained from the posterior fornix of the vagina using sterile swabs. The swab sticks were well labeled accordingly. Samples collected were analyzed within one hour of collection. In case of delay, a drop of phosphate buffer saline was added to the swabs stick container and stored in the refrigerator at 4 to -8 °C and assayed within 1-3 hours. Positive patients were given post-test counseling and those infected were treated and it was well documented in their case note.

### Germ tube test (GTT) to identify *C. albicans*

Yeast isolates from Saboraud dextrose agar were screened for germ tube production in serum broth using 0.5 ml of human serum. The broth was dispensed into small test tubes, and each tube was inoculated with yeast colonies from the culture plate. The tube was incubated at 35–37 °C for 3 hours. A drop of the serum containing the yeast culture was placed on a clean microscope glass slide, and covered with a cover glass. The preparation was examined using the X10 and X 40 objective lenses. Sprouting yeast cells (tube-like outgrowths) also known as germ tubes were observed in positive samples. Germ tube negative species were regarded simply as *Candida*

species. When sprouting yeast cells were seen they were reported as *C. albicans*. *Candida glabrata* ATCC 22018 was used as negative control.<sup>14</sup>

#### Cultivation on the selective medium (CHROMagar)

Isolates from Sabouraud dextrose agar (SDA) were inoculated on CHROMagar (Oxoid, Basingstoke, UK) using an inoculating loop and incubated at 37°C for 48 hours. The method is based on the differential release of chromogenic breakdown products from various substrates by *Candida* species following differential exoenzyme activity.<sup>15</sup> Identification of yeast was done based on the colour of each colony. Using this method, the following *Candida* species were identified; *C. glabrata* (wet dark pink colonies), *C. tropicalis* and *C. dublinensis* (wet blue colonies) and *C. albicans* (wet, green colonies).<sup>16</sup>

#### DNA extraction and amplification

The DNA of all the 40 isolates was extracted using zymo DNA extraction kit for bacteria and fungi (Inqaba Biotec, South Africa). DNA extraction was done by picking three colonies from the culture plate using sterile loop and suspended into 500 µl of distilled water in a micro-centrifuge tube. The suspension was centrifuged at 10,000 rpm for 1 min and the supernatant was discarded. To the sediment, 200 µl of distilled water was added after which the cells were dislodged and 750 µl of lysis solution was added to the tube. This was thoroughly mixed for about 15 min and vortexed for another 10 min. The lysis tube was spun for 10,000 rpm for 1 min after which 400 µl of the supernatant was transferred into the provided zymo-spin filter and spun at 7,000 rpm for 1 min. Elution buffer was added and centrifuged at 10,000 rpm for 30 seconds to elute DNA. The eluted DNA was stored at 2°C until use. The DNA was quantified using nano drop spectrophotometer (Inquaba Biotec, South Africa) and quantified and gel electrophoresis was done to confirm the presence of DNA.

The universal primers of internal transcriber spacer (ITS1 and ITS4); ITS1 [5'-TCCGTAGGTGAACCTGCGG-3'] and ITS4 [5'-TCCTCCGCTTATTGATATGC-3'], and *C. albicans*-specific primers [5'-GGTTTGCTTGAAAGACGGTAG-3'] and [5'-AGTTTGAAGATATACGTGGTAG-3'] that target sequences site of the intergenic spacer region (ITS) of the fungal rRNA genes (18S and 28S) were used for this assay. The PCR mixture consisted of 5 µl of master mix, 1 µl of each primer and 1 µl of template DNA make up to 25 µl with nuclease free water. All were mixed and vortexed for 1 second and slightly spun at 500 rpm for 1 min.<sup>17</sup> It was loaded in a thermo cycler alongside with controls and set at an initial denaturation temperature of 95°C, denaturation at 95°C, Annealing temperature of 55°C, and final extension of 72°C for 35 cycles. The PCR products were electrophoresed on a 1.5% (w/v) agarose gel. Electrophoresis was performed at 100 V in 1X TAE buffer gels were stained after electrophoresis in ethidium

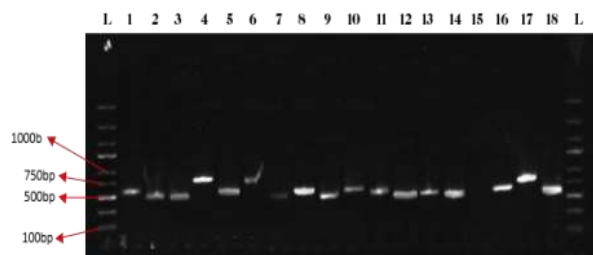
bromide for 15 minutes and de-stained in distilled water for 30 minutes. Gels were visualized under ultraviolet (UV) light using UVitec trans illuminator (Avebury, Cambridge). *C. albicans* ATCC 90028, *Candida tropicalis* ATCC 1909, *Candida dublinensis* ATCC 10232 and *Candida glabrata* ATCC 22018 were used as positive control organisms while *Escherichia coli* ATCC 25922 was used a negative control organism.<sup>17</sup>

#### Data analysis

Frequencies were obtained and percentages were calculated for study variables. Statistical Package for Social Sciences (SPSS) version 18.0 (SPSS, Inc., Chicago, Ill) was used for the analysis. Sensitivity and specificity values were determined using graph pad prism 6 (GraphPad Software, Inc. USA).

## RESULTS

Of the 100 pregnant women investigated for candidiasis, four (4) *Candida* species were identified by PCR. These were *C. albicans* 18(18%), *C. dublinensis* 8(8%), *C. glabrata* 13(13%) and *C. tropicalis* 1(1%). *Candida albicans* yielded an amplicon size of 535 bp, *Candida glabrata* yielded 871 bp, *Candida tropicalis* 524 bp and *Candida dublinensis* 530 bp (Figure 1).



Lane L:1kb ladder, Lane 1: *C. albicans* control, Lane 2: *C. dublinensis* control, Lane 3: *C. tropicalis* control, Lane 4: *C. labrata* control, lane 5: *C. albicans*, lane 6: *C. glabrata*, lane 7: *C. dublinensis*, Lane 8: *C. albicans*, Lane 9: *C. tropicalis*, Lane 10 and 11: *C. albicans*, Lane 12: *C. dublinensis*, Lane 13: *C. albicans*, Lane 14: *C. dublinensis*, Lane 15: Negative control, Lane 16: *C. glabrata*

**Figure 1: Agarose gel electrophoresis of *Candida* species.**

The result of phenotypic identification of *Candida spp.* was compared with genotypic identification as shown in Table 1. Of all the *Candida spp.* isolated (n=40), germ tube test identified 21 (52.5%) as *C. albicans* while 19 (47.5%) could not be identified. CHROMagar identified 19 (47.5%) as *C. albicans*, 11 (27.5%) as *C. glabrata*, 2 (5.0%) as *C. tropicalis* and 8 (20%) were identified as *C. dublinensis*. All the two isolates that were identified as *C. tropicalis* by CHROMagar were also confirmed by PCR. Of all the 19 strains of *C. albicans* identified by

CHROMagar, 2 (10.5%) were re-identified by PCR as *C. glabrata* and *C. dublinensis* (Table 1).

Sensitivity and specificity values of CHROMagar and germ tube test in comparison with the Multiplex PCR for identification of *C. albicans*, *C. glabrata* and *C. dublinensis* are reported in Table 2. Eighteen (18) strains of *C. albicans* were confirmed as true positive by PCR out of 21 that were identified by GTT as *C. albicans*. The GTT had 94% sensitivity and 81% specificity. Of 19 strains of *C. albicans* that were identified by CHROMagar, 17 were confirmed as true positive by PCR. The CHROMagar had 89.4% sensitivity and 90.4% specificity. In comparison, GTT was better in correctly identifying those strains that were confirmed as *C. albicans* by PCR (Sensitivity=94%) while CHROMagar was better in identifying the strains that

were not confirmed as *C. albicans* by PCR (Specificity=90.4%).

**Table 1: Comparison of phenotypic identification with genotypic identification of *Candida spp.***

Species	Phenotypic method		PCR Method
	Germ tube	CHROMagar	
<i>C. albicans</i>	21	19	17
<b>Other <i>Candida spp.</i></b>			
<i>C. glabrata</i>	0	11	12
<i>C. tropicalis</i>	0	2	2
<i>C. dublinensis</i>	0	8	9
Unidentifiable spp.	19	0	0
Total	40	40	40

**Table 2: Comparing sensitivity and specificity of germ tube test and CHROMagar.**

	Polymerase chain reaction		Sensitivity	Specificity
	+ve <i>C. albicans</i>	-ve <i>C. albicans</i>		
<b><i>C. albicans</i></b>				
GTT positive=21	17	4	94.4%	81.8%
GTT negative=19	1	18		
CHROMagar positive=19	17	2	94.0%	81.0%
CHROMagar negative=21	2	19		
<b><i>C. dublinensis</i></b>				
CHROMagar positive=9	8	1	88.9%	96.8%
CHROMagar negative=31	1	30		
<b><i>C. glabrata</i></b>				
CHROMagar positive=12	11	1	91.6%	96.2%
CHROMagar negative=28	1	27		
<b><i>C. tropicalis</i></b>				
CHROMagar positive=2	2	0	100%	100%
CHROMagar negative=38	0	38		

Key: +ve: Positive; -ve: Negative

Out of 9 strains of *C. dublinensis* that were identified by CHROMagar, 8 were re-identified as true positive by PCR. One (1) from the 31 strains that were identified as negative for *C. dublinensis* was re-identified as positive by PCR method. CHROMagar sensitivity and specificity for *C. dublinensis* were 88.8% and 96.8% respectively.

Out of 12 strains of *C. glabrata* that were identified by CHROMagar, 11 were re-identified as true positive by PCR. One (1) from the 28 strains that were identified as negative for *C. glabrata* was re-identified as *C. glabrata* by PCR. CHROMagar sensitivity and specificity for *C. glabrata* were 91.6% and 96.2% respectively. Two (2) strains of *C. tropicalis* identified by CHROMagar were confirmed by PCR as true positive.

## DISCUSSION

A previous study revealed a high incidence of asymptomatic vulvovaginal candidiasis among pregnant

women in Ogbomoso with infection rate of 25%.<sup>19</sup> In a study conducted by Merlino et al. all *C. albicans* were reported to be germ tube positive but two *C. tropicalis* gave false positive results.<sup>20</sup> In this present study, 52.5% of the *Candida spp.* isolated were identified as *C. albicans* using GTT, 47% were identified by CHROMagar, while 42.5% were identified by PCR as *C. albicans*. Kazemi et al. reported that 65.4% of *C. albicans* was detected by germ tube formation, 53% by Chlamydoconium formation and 62.4% by molecular studies.<sup>21</sup>

While germ tube test was not able to identify other species of *Candida* (47.5%), CHROMagar identified 27.5%, 5.0% and 20% as *C. glabrata*, *C. tropicalis* and *C. dublinensis* respectively; PCR was able to identify 30.0% *C. glabrata*, 5.0% *C. tropicalis* and 22.5% *C. dublinensis*. Although germ tube test is performed quickly, false positive results by *C. tropicalis* and *C. dublinensis* generated by the test and the use of

potentially dangerous sera are the major drawbacks of the test.<sup>22</sup>

The identification of other species of *C. albicans* was bettered with the use of CHROMagar and PCR. Mahens et al. used PCR to differentiate *C. albicans* and *C. dubliniensis*. They concluded that PCR has the potential of detection in low amounts of DNA and helps the rapid diagnosis of pathogenic fungi that is critical in treatment of candidiasis.<sup>23</sup>

The sensitivity and specificity of germ tube test for *C. albicans* were 94% and 81% respectively. Some authors evaluated and reported sensitivity and specificity of germ tube test as 84.6% and 100% respectively.<sup>24</sup> Hoppe et al. estimated both specificity and sensitivity as 98%.<sup>25</sup>

Of 19 strains of *C. albicans* that were identified by CHROMagar, 17 were confirmed as true positive by PCR while 2 were false positive. The CHROMagar had 89.4% sensitivity and 90.4% specificity. In comparison, GTT was better in correctly identifying those strains that were confirmed as *C. albicans* by PCR (Sensitivity=94%) while CHROMagar was better in identifying the strains that were not confirmed as *C. albicans* by PCR (Specificity=90.4%).

In this study, the identification of *Candida* isolates was performed successfully using germ-tube test, CHROMagar test and PCR. The isolated *Candida* species were *C. albicans*, *C. tropicalis*, *C. glabrata* and *C. dubliniensis*. In a comparison among PCR, germ-tube, and CHROMagar test results, all three assays confirmed the presence of *C. albicans*, but germ-tube test did not confirm the identity of 19 other *Candida* isolates. Therefore, this study shows the best specificity of CHROMagar over the GTT Method, when compared with the PCR Method. The combine use of CHROMagar and PCR has the advantage of efficient detection of various *Candida* isolates thus; the use of PCR is technically demanding.

## CONCLUSIONS

Even though CHROMagar method had so long been established, however, the use of this method for diagnostic purposes is not common in Nigeria due to the cost implication. Most diagnostic centers in Nigeria report the presence of *Candida* in a given sample has *C. albicans* while others will be categorized as *Candida spp.* We therefore advise that efforts should be intensified to further detect other *Candida spp.* by using both the CHROMagar and PCR methods in order to improve both diagnostic and treatment outcome of candidiasis among pregnant women. Further studies are however required to appropriately classify the *Candida* isolates by sequencing the PCR products.

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