

RESEARCH ARTICLE

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## Study of Virulence Factor Production in *Candida* Species Colonizer Isolated from Diabetic Patients

Karishma Wasnik<sup>1</sup>, Archana Wankhade<sup>1\*</sup>  and Pritam Wasnik<sup>2</sup>

<sup>1</sup>Department of Microbiology, All India Institute of Medical Sciences, Raipur, Chhattisgarh, India.

<sup>2</sup>Department of General Medicine, All India Institute of Medical Sciences, Raipur, Chhattisgarh, India.

### Abstract

*Candida* is a yeast like fungus. It causes candidiasis which is a spectrum of disease from cutaneous, mucosal, systemic & multisystem dissemination. It is a coloniser of mucocutaneous surfaces of body but it is an opportunistic fungus causing severe type of infection. Objective of the study was to detect colonization of *Candida* species in diabetic patient & to detect virulence factors i.e., phospholipase, proteinase, esterase activity & homolysis activity in isolated strains of *Candida* from colonised diabetic patients. Throat swab and urine sample were collected from diabetic patients visiting the General Medicine OPD/Ward diagnosed with diabetes mellitus before six months and was submitted in microbiology department. *Candida* species were identified tested for production of virulence factors. Total 30 diabetes mellitus patients diagnosed 6 months before. In 41-65 years age groups, *Candida* colonization was found predominantly, 63% patients. Amongst these 78% had fasting sugar levels greater than the normal limits. The study revealed a strong correlation between higher fasting blood sugar levels and *Candida* colonization. The predominant isolate was *Candida albicans* in throat followed by *Candida glabrata*. Out of 27 *Candida* species isolates, 16 strains showed production of virulence factors. Amongst these 44 % (7/16) strains were positive for proteinase production, 38 % (6/16) were positive for esterase production, 13% (2/16) strains were positive for hemolysin and single strain (6%, 10/16) showed the production of phospholipase. The study concludes that *Candida albicans* was the predominant colonising species found followed by *Candida glabrata* in diabetic patients. Non-*Candida albicans* species can be seen as an emerging colonizing species in the diabetic patients and thereby are increasingly gaining clinical importance. Colonized or commensal *Candida* species showed in vitro production of virulence factor.

**Keywords:** Diabetes, *Candida*, Immunocompromised, Colonization, Proteinase, Esterase, Hemolysin

\*Correspondence: archukeche@gmail.com

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## INTRODUCTION

*Candida* is yeast like fungus. It causes candidiasis which is a spectrum of disease from cutaneous, mucosal, systemic & multisystem dissemination. *Candida albicans* and other species of *Candida* are member of the normal human commensal,<sup>1</sup> but in diabetic patients it forms severe colonization. This type of colonization makes them more prone to develop deeper mucosal colonization with further dissemination via blood.<sup>2</sup> It may be due to immune suppression, cancer chemotherapy, intravascular catheters, cardiothoracic or gastrointestinal surgery, and endocrine disorder like diabetes mellitus or HIV infections.<sup>3</sup>

Rodrigues et in his study analysed the development of candidiasis in diabetic patients by outlining various characteristics of *Candida* sp. Including hydrolytic enzymes & biofilm & correlated with the development of candidiasis.<sup>4</sup> Life threatening infections like candidemia usually acquired in hospital when colonised patients are introduced with IV or urinary catheters.

*Candida* is having some virulence factors which is having role in proliferation. So, the study was planned to detect the colonization of *Candida* species in diabetic patients and detection of virulence factors like phospholipase, proteinase, esterase & hemolysin in the isolated strains of *Candida* species.

### Objectives

#### Primary Objectives

1. To detect colonization of *Candida* species in diabetic patient
2. To detect virulence factor phospholipase, proteinase, esterase activity & hemolysin activity in isolated strains of *Candida* from colonised diabetic patients.

#### Secondary Objectives

1. To identify *Candida* species isolated from colonized diabetic patients

## MATERIALS AND METHODS

The Cross-sectional Study was conducted at tertiary care hospital after approval Institutional

Ethics Committee. The study duration was 15<sup>th</sup> July to 1<sup>st</sup> September 2019. It involved human patients diagnosed with diabetes mellitus of age >18 years before 6 months. Patients were asked for the written informed consent to participate in the study. Two samples (throat swab/oropharyngeal swab, urine sample) were collected at the time of enrolment. Samples were submitted in the department of Microbiology. It was processed for isolation, identification & detection of virulence factor by following the standard protocol. The sample was processed for microscopy with Gram stain & KOH mount.

Culture of samples on two sets of SDA & SDA with Chloramphenicol (incorporated to inhibit bacterial growth) were inoculated. One set will be incubated at 37°C & another at room temperature i.e., 25°C to 29°C in BOD incubators. Identification of colony was done with Gram stain, Germ tube test, chlamydospore formation, sugar assimilation, sugar fermentation method & by inoculating it on HiCrome agar.

If growth of yeasts from at least two different sites were considered as coloniser. virulence factors were detected in the isolates by detecting enzymatic activity.<sup>5-8</sup>

The phospholipase activity was carried out in egg yolk agar medium. The proteinase was detected in Bovine serum albumin medium. Esterase activity was detected by using Tween 80 medium. Hemolysin will be detected by SDA with Sheep blood agar. Yeast suspension was inoculated onto plates & incubated at 37 °C for 48 h. Hemolysis was considered as positive hemolytic activity. ATCC *C. albicans* 10231 strain will be used as Control strain in the study.

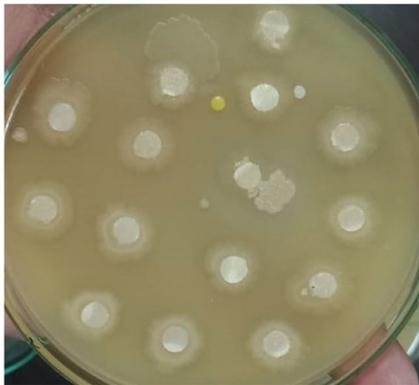
## RESULTS

This study was carried out in the Department of Microbiology. The clinical samples were collected from diabetic patients who visited the Medicine department in the study duration. Total 30 diabetes mellitus patients diagnosed 6 months before, were included in the study. The predominant age group observed was 41-65 years (80%) and amongst these 63% (15/24) were females. Fasting sugar levels  $\geq 126$  was seen in 77% (24/30) & postprandial sugar levels  $\geq 200$  was seen

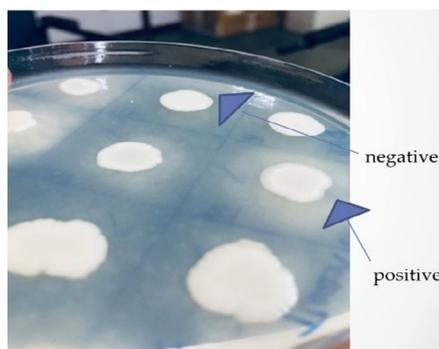
in 53% (16/30) of patients. Amongst these 47% (14/30) were having both fasting & postprandial sugar levels above the normal limits.

Following the standard protocol, samples were processed for isolation *Candida* species were isolated and identified. Total 19 patients had colonization of *Candida* species. Total 27 *Candida* strains were isolated from clinical samples of 19 patients (Figure 4). These were processed for identification of species & production of virulence factors.

The predominant isolate was *Candida albicans* in throat followed by *Candida glabrata* in urine as shown in (Figure 4). Amongst the 19 patients with colonization, 79% (15/19) patients had fasting sugar level more than the normal limits. When fasting blood sugar is more than normal limit there is strong correlation for *candida* colonization was observed (Pearson's test) shown in Table 1.



**Figure 1.** Proteinase activity detection on Bovine serum albumin agar medium



**Figure 2.** Esterase activity detection on Tween 80 medium

*Candida* isolates were processed for detection of virulence factors-Phospholipase, Proteinase, Esterase and Hemolysin by following the mentioned methodology. (Figure 1 to 3).

Out of 27 *Candida* species isolated from throat swabs and urine samples, 16 strains showed production of virulence factors. Amongst these 44 % (7/16) strains were positive for Proteinase production, 38 % (6/16) were positive for Esterase production, 13% (2/16) strains were positive for Hemolysin and single strain showed the production of Phospholipase. (Table 1)

Esterase production was predominantly seen in *Candida glabrata* 50% (3/6) followed by *Candida albicans* 33% (2/6), *Candida krusei* 17% (1/6).

Hemolysin was produced by one strain of *Candida glabrata* and one strain of *Candida parapsilosis*.

Phospholipase production was observed only by a single strain of *Candida glabrata*.

Virulence factors production in *candida* coloniser was seen comparatively more in patients with fasting sugar level more than normal limit. (Figure 5).

Similar findings were observed for Proteinase production in case of postprandial sugar levels, but Esterase production was observed to be equal in patients having postprandial sugar levels within normal limits and above normal limits. (Figure 6).

There is strong correlation of production of virulence factor for *candida* isolates from patients having postprandial blood sugar more than >200 mg/dl. (Figure 6).



**Figure 3.** Hemolysin activity detection in 5% sheep blood agar

All the four virulence factors were not produced by any isolate but multiple virulence factors production was seen in the various *Candida* isolates (Table 2).

## DISCUSSION

Colonization means that a person has the organism somewhere on their body but does not have an infection or symptoms of infection.<sup>2</sup> Diabetes mellitus (DM) is a metabolic disorder that predisposes individuals to fungal infections, due to an immunosuppressive effect on the patient, also making them susceptible to infections those related to *Candida* species. The study revealed a strong correlation between higher fasting blood sugar levels and *Candida* colonization. (Pearson test). Similar findings were revealed by studies carried by Kumar et al.,<sup>9</sup> Khazal et al.<sup>10</sup> and Lotfi-Kamran et al.<sup>11</sup> This is due to the higher levels of blood glucose which lead to increased adherence of *Candida* species to buccal and mucosal surfaces due to the presence of various adhesive molecules in *Candida*. Pallavan B et al. found that increase in the oral candidal colonization in diabetic patients as compared to non-diabetic individuals.<sup>12</sup> Rizzi et al observed that there is a high risk of Urinary tract infections and of Genital infections in diabetic

patients. Only genital infections were found to be associated with poor glycemic control.<sup>13</sup>

Kumar et al found *C. albicans* as the predominant species followed by *C. tropicalis* and *C. krusei*.<sup>9</sup> This difference obtained in the isolation percentage of different *Candida* species may be due to the difference in distribution of *Candida* in various geographical areas, the time of sampling, or the use of different methods for yeast recovery.<sup>9,14-16</sup> The predominant isolate was *Candida albicans* followed by *Candida glabrata*. Study conducted at All India Institute of Medical Sciences, New Delhi, India, in diabetic patients with vulvovaginal candidiasis showed *C. glabrata* as the primary species responsible for vulvovaginal candidiasis in the patients.<sup>15,16</sup>

In a study by Gomes et al *Candida albicans* was the most frequent isolate and had higher virulence in diabetic patient.<sup>17</sup> In a study by Pandey et al.<sup>18</sup> in which Extracellular hydrolytic enzyme activities of the different *Candida* spp. isolated from the blood of the Intensive Care Unit- patients was determined, hemolysin activity was shown by 89.86% of the isolated *Candida* spp., whereas other extracellular enzymes such as proteinase, phospholipase, and esterase were produced only by 84.72%, 55.69%, and 37.97% of the strains, respectively was produced.<sup>18</sup>

**Table 1.** Production of virulence factors by isolated species of *Candida*

Candida species →	<i>Candida albicans</i> (n=10)	<i>Candida glabrata</i> (n=7)	<i>Candida krusei</i> (n=4)	<i>Candida parapsilosis</i> (n=3)	<i>Candida tropicalis</i> (n=1)
Virulence factors ↓					
Phospholipase	0	1	0	0	0
Proteinase	2	1	3	1	0
Esterase	2	3	1	0	0
Hemolysin	0	1	0	1	0
Total	4(18.75%)	6(37.5%)	4(25%)	2(12.5%)	0(0%)

Note: In parentheses depicts percentage out of vertical total

**Table 2.** Distribution of Production of virulence factors by isolated species of *Candida*

Patients with <i>candida</i> species showing all the virulence factors production	0(0%)
Patients with <i>candida</i> species showing 3 virulence factors production but no proteinase	1(3.3%)
Patients with <i>candida</i> species showing 2 virulence factors production (Proteinase & Esterase)	2(6.6%)
Patients with <i>candida</i> species showing any one of the virulence factors production	8(26.6)
Total patients having colonized strains producing virulence factors	11(36.6%)
Total	30(100%)

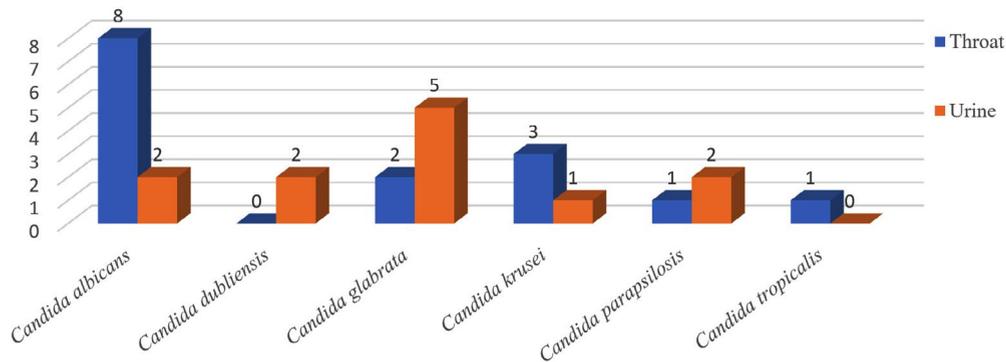
In parentheses depicts percentage out of vertical total

Proteinase production was predominantly seen in *Candida krusei* followed by *Candida albicans*, *Candida glabrata* & *Candida parapsilosis*. Pandey et al.<sup>18</sup> found that strong proteinase activity was shown by the *C. albicans* (93.75%), followed by the *C. tropicalis* (87.5%).<sup>19</sup>

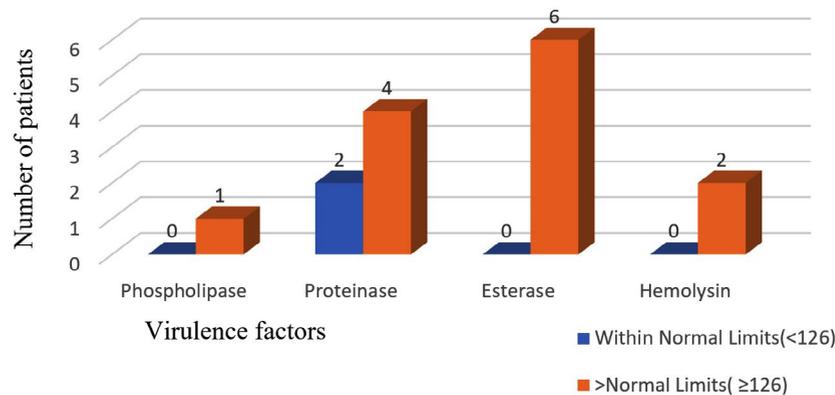
Esterase production was predominantly seen in *Candida glabrata* 50% (3/6) followed by *Candida albicans* 33% (2/6), *Candida krusei* 17% (1/6). Pandey et al found that 56% *C. albicans* showed esterase activity and 54% *C. tropicalis* showed the esterase activity.<sup>18</sup> Similarly, Kumar et al. reported *C. albicans* and *C. tropicalis* species with the highest rate of esterase activity.<sup>19</sup> No activity was observed in the isolated strains of the *C. glabrata* and *C. krusei*, which are considered as the intrinsic fluconazole resistant. Fatahinia et al., reported esterase activities of higher level in *C. glabrata* isolated from diabetic patients having oral candidiasis.<sup>20</sup>

Hemolysin was produced by one strain of *Candida glabrata* and one strain of *Candida parapsilosis* Pandey et al.<sup>18</sup> observed that hemolysin activity was shown by 95.8% of *C. tropicalis* and 90.9% strains of the *C. krusei* strains. Only 87.5% strain of *C. albicans* produced hemolysin activity. Hemolysin and proteinase enzymes were produced by >80% of the isolated *Candida* spp. Similar findings of higher hemolysin production have been reported by Riceto et al.<sup>21</sup> Present study demonstrated higher production of proteinase which was similar to the findings by Pandey et al.<sup>18</sup> and Riceto et al.<sup>21</sup> However, hemolysin was produced only by one strain of *Candida glabrata* and one strain of *Candida parapsilosis*.

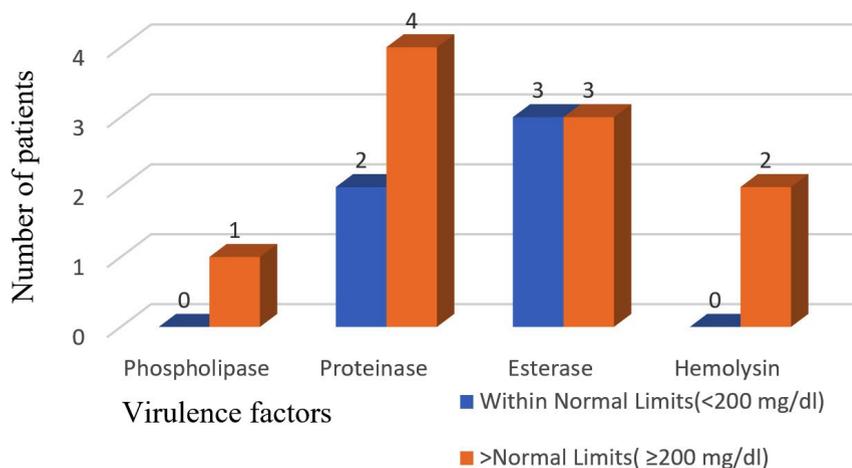
However, phospholipase was produced by only one strain of *Candida glabrata*. Earlier studies also show that *Candida albicans* and *Candida glabrata* represent the two most commonly isolated *Candida* species worldwide.<sup>22,23</sup>



**Figure 4.** Distribution of sample wise isolation of *Candida* species



**Figure 5.** Number of patients showing production of virulence factors with respective fasting sugar level



**Figure 6.** Number of patients showing production of virulence factors with respective post prandial sugar level

The difference in production of virulence factors obtained may be due to intra- and interspecies differences in the virulence of *Candida* species strains.<sup>24,25</sup> Genetic variation in the ribosomal DNA (rDNA) internal transcribed spacer (ITS) region has been studied among fungi.<sup>26</sup> This may also be responsible for the difference obtained in the production of virulence factors within different species and for the difference in present study from other studies.

The isolates of *C. albicans* are the most virulent and produce the most extracellular virulence factors; The expression of proteases, hemolysins, and esterases is correlated with the virulence of *Candida* isolates.<sup>27</sup>

The expression of virulence factors among *Candida* species, may vary depending on the infecting species, geographical origin, type of infection, the site and stage of infection, and host reaction.<sup>28</sup> Studies like this can explore the colonizing newer emerging *Candida* species for example *C. auris* which is resistant to many antifungals can be identified. The study is useful to know the presence or absence of virulence factors in colonized strains of *Candida*. This becomes extremely important when patient condition is deteriorated, & *Candida* is isolated from the colonized site the dissemination of isolated colonized strain may occur which may be fatal. The immunostimulatory factors that the virulence factors offer stimulate dendritic cell activation, T cell infiltration, and activation. The risk of resistance

developing in *Candida* infections can be decreased by focusing on virulence factors.<sup>29</sup> Pawar et al detected PLB gene in 97.3% *C. albicans* isolates.<sup>30</sup>

## CONCLUSION

The study concluded that *Candida albicans* is the predominant species causing colonization followed by *Candida glabrata*. The *candida* isolates showed production of proteinase predominantly followed by esterase. If the patient is colonized with intrinsically drug resistant *Candida* species like *C. auris* or *C. glabrata*, it will help to take preventive measures accordingly, when patients get admitted. Testing of virulence factors may help to know its potential role in invasiveness of *Candida*.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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## DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

## ETHICS STATEMENT

Not applicable.

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