

## Original Research Article

# An *in vitro* study on comparative adherence of oral *Candida albicans* and non-*albicans Candida* to human buccal epithelial cells and its antifungal susceptibility pattern

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## ABSTRACT

**Background:** *Candida spp.* are intra-oral commensals in 40-60% of human subjects and the precise factors involved in the development of Candidiasis are still not clear. The adherence of *Candida* to epithelial cells is one of the main pathogenic characteristics of the genus. Studies have shown that there are variations in the adherence capabilities of different *Candida spp.*, which may explain why some species colonize mucosal surfaces more frequently than others. The aim of this study was to estimate the adherence properties of *Candida spp.* to buccal epithelial cells and to determine the antifungal susceptibility.

**Methods:** 87 isolates of *Candida spp.* from known non-diabetic patients with oral Candidiasis were collected from diagnostic microbiology laboratories in central Kerala. The method of Kimura and Pearsall *et al* was followed with minor modifications to test the adherence.

**Results:** All the three species exhibited adherence with varying capacity. *C. albicans* was significantly more adherent to buccal epithelial cells than *C. tropicalis* and *C. krusei*. Resistance was not observed among the isolates against fluconazole, voriconazole and clotrimazole.

**Conclusions:** In conclusion *Candida spp.* isolated from oral candidiasis patients exhibited a higher adherence capacity to normal human buccal epithelial cells considered an essential virulence property. As anticipated *C. albicans* displayed higher virulence activity than non-*Candida albicans* species.

**Keywords:** *Candida*, Human buccal epithelial cell, Adherence, Antifungal susceptibility

## INTRODUCTION

Yeast cells or blastospores are unicellular, eukaryotic organisms which multiply by a specific process of mitotic cell division known as budding.<sup>1</sup> Yeast and yeast like organisms are the most common fungi isolated in clinical laboratories. *Candida* is one such medically important yeast-like fungi. *Candida spp.* are carried as part of commensal oral flora of 40% of healthy individuals,

normally without disease.<sup>2</sup> There are 20 species of *Candida* among 700 species of microorganisms that are found in the oral cavity of humans.<sup>3,4</sup> However, overgrowth of these organisms (usually as a result of lowered host defence) can cause clinical manifestations.<sup>5</sup> Such pathogenicity can occur when the existing harmony between the host and the organism is altered, hence they are considered to be opportunistic pathogens.<sup>6</sup> There are over 10 species of *Candida* that can cause infection in

humans, 80% of which is *Candida albicans*.<sup>7</sup> Other medically important species include *Candida glabrata* (now renamed as *Nakaseomyces glabrata*), *Candida rugosa* (now renamed as *Diutina rugosa*), *Candida parapsilopsis*, *Candida tropicalis*, *Candida krusei* (now renamed as *Pichia kudriavzevii*), *Candida auris*, *Candida dublinensis*, *Candida kefyr* (now renamed as *Kluyveromyces marxianus*) and *Candida guilliermondii* (now renamed as *Meyerozyma guilliermondii*).

*Candida spp.* can cause infection if there are predisposing conditions related to the host. The infective ability of this yeast-like fungi also depends on several virulence factors such as germ tube production, growth at 37°C, protease and phospholipase production and adherence to epithelial cells.<sup>8</sup> The infection caused by these species are known as Candidiasis. It encompasses infections ranging from superficial infections, such as oral thrush and vaginitis, to systemic and potentially life-threatening diseases such as invasive candidiasis. In the pharmacological treatment of Candidiasis, the two classes of antifungals most commonly used are the polyenes and the azoles.

Adherence of *Candida spp.* to host cells constitute one of the first stages in the development of Candidiasis and is therefore an important factor during the colonization and invasion of tissues.<sup>9-12</sup> Most of the results demonstrate that adhesion seems related to cell surface hydrophobicity.<sup>13,14</sup> *Candida* possesses a set of proteins (adhesins) which mediate their adherence to other microorganisms, abiotic surfaces and to host cells.<sup>15</sup> The aim of this study was to estimate the adherence properties of *Candida spp.* to buccal epithelial cells.

## METHODS

The present cross-sectional study was conducted at School of Medical Education (SME), Kottayam, Kerala between January 2022 and December 2022. 87 isolates of *Candida spp.* from known non-diabetic patients with oral Candidiasis were collected from diagnostic microbiology laboratories in central Kerala. *Candida albicans* MTCC 227, procured from Institute of Microbial Technology (IMTECH), Chandigarh, India, was used as standard control for adherence assay and antifungal susceptibility testing. The isolates were reconfirmed by subculturing on to chromogenic media - HiCrome™, followed by Gram staining and colonies were confirmed to be Gram positive yeast like budding cells. Further identification was done by tests like germ tube and chlamydospore production. All reagents, culture media and antifungal disc were procured from HiMedia Laboratories, Mumbai, India.

Test for adherence of *Candida spp.* to BEC

The method of Kimura and Pearsall et al was followed with minor modifications.<sup>16</sup>

## Collection and washing of BEC

Buccal epithelial cells were collected from healthy individuals (in age range of 20-30 years) with no known coexisting disease or not in receipt of any medication that would affect the results of the study. Individuals with any known coexisting disease and those who have had any antimicrobial in the past 3 months were excluded. BECs, were collected from healthy male and female volunteers by scraping the buccal mucosa with the cotton-tipped sterile swab. Swabs were immersed in sterile PBS (pH 7.2), and vortexed. The cells were suspended in PBS, and washed 3 times in the buffer.

## Harvesting and washing of *Candida spp.*

An overnight culture of *Candida spp.* in Sabouraud dextrose agar was washed 3 times in Phosphate buffered saline by centrifugation at 3000 rpm for 30 minutes.

## Adherence assay

1 ml of washed *Candida* suspension was incubated with 1 ml of BEC suspension and incubated in a water-bath at 37°C for the hour. After this the BEC fungal suspension mixture was added to 5 ml PBS and centrifuged at 3000 rpm for 2 minutes. Supernatant containing the free *Candida* was discarded, the recovered BEC were re-suspended in 5 ml PBS and the process was repeated. Epithelial cells were suspended in 15 ml PBS and filtered allowing passage of any residual free fungi while retaining epithelial cells on the surface. After filtering, a glass microscopic slide was pressed gently against the filter, lifting off the retained epithelial cells. This cell preparation was air dried, alcohol fixed and Gram stained. The number of *Candida* adherent to each of the first 25 epithelial cells was counted. Only fungi that were in contact with the cell surface were counted and epithelial cells that overlapped other cells were excluded from the evaluation. The mean number of fungi per cell was calculated for each preparation.

## Antifungal susceptibility testing

Antifungal disc diffusion susceptibility testing was done as prescribed by CLSI M44-A2.<sup>17</sup> Briefly, Mueller-Hinton agar supplemented with 2% glucose and 0.5µg/ml methylene blue was used for the sensitivity testing. Antifungals used were fluconazole, voriconazole and clotrimazole. Interpretive criteria for fluconazole and voriconazole was as prescribed by CLSI M44-A2<sup>17</sup> and for clotrimazole as per manufacturers' instructions.

This study was approved by the institutional ethical committee (IEC) at the School of Medical Education.

**Statistical analysis**

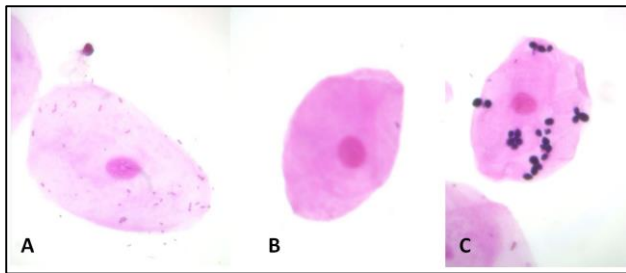
The data was analysed using Microsoft excel 2019 and statistical package for the social sciences (SPSS) 16. Data were expressed as means ± standard deviation (SD).

**RESULTS**

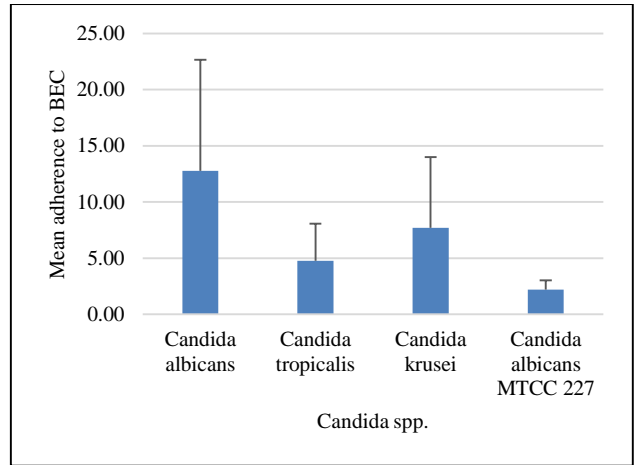
A total of 87 *Candida spp.* was isolated from 65 males (74.7%) and 22 females (25.3%), which belonged to three species viz *Candida albicans* (n= 46), *Candida tropicalis* (n=26) and *Candida krusei* (n=15). *C. albicans* produced light green color, *C. tropicalis* blue color and *C. krusei* purple color as shown in Figure 1.



**Figure 1: candida spp. on micrometm agar. A- candida albicans, B- candida tropicalis, C- candida krusei.**

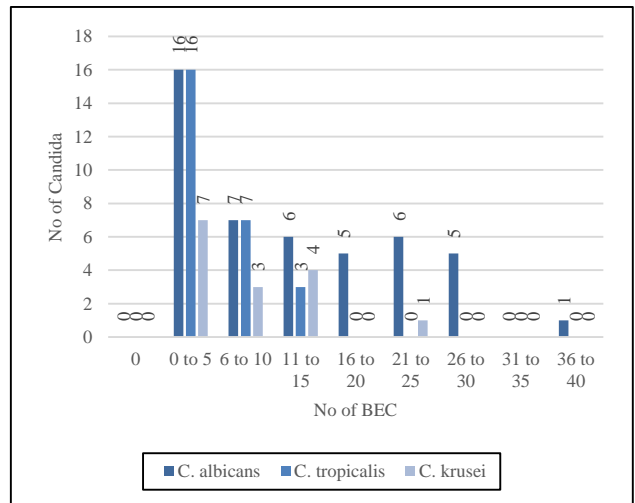


**Figure 2: A- human BEC with adherent bacteria prior to washing. B - washed BEC. C - candida spp. Adhering to BEC post adherence assay.**



**Figure 3: Comparative adherence of candida spp. To BEC.**

All the three species exhibited adherence with varying capacity. Adherence of *Candida spp.* to buccal epithelial cell is shown in Figure 2. *C. albicans* exhibited an average adherence of 12.75 (SD=9.88), *C. tropicalis* 4.78 (SD=3.29) and *C. krusei* 7.71 (SD=6.28). The control strain, *C. albicans* MTCC 227 exhibited an average adherence of 2.2 (SD= 0.82). The comparative adherence to buccal epithelial cells is shown in Figure 3.



**Figure 4: Frequency of adherence of Candida spp. to BEC.**

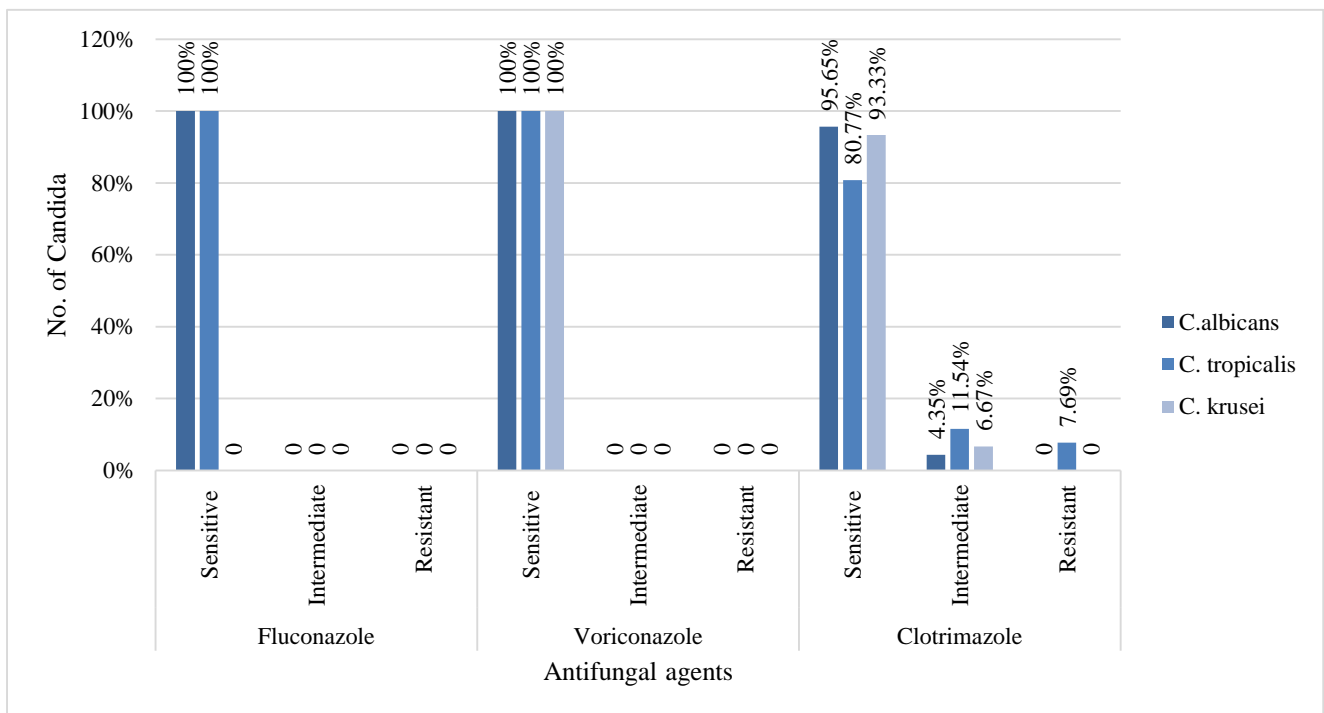
**Table 1: Comparative adherence of Candida spp. to C. albicans MTCC 227 using t-test.**

	t	df	P value	Mean Difference	Lower	Upper
<i>Candida albicans</i>	7.261	45	0.00	10.57913	7.6447	13.5135
<i>Candida tropicalis</i>	3.997	25	0.00	2.57538	1.2482	3.9025
<i>Candida krusei</i>	3.395	14	0.004	5.50667	2.0283	8.9851
95% Confidence interval of the difference						
<i>C. albicans</i> MTCC 227 test value = 2.2						

**Table 2: Comparative adherence between *Candida spp.* using t-test.**

I	J	Mean Difference (I-J)	Std. Error	Sig.	Lower bound	Upper bound
<i>Candida albicans</i>	<i>Candida tropicalis</i>	8.00375*	1.93344	0.00	3.3906	12.6169
	<i>Candida krusei</i>	5.07246	2.343	0.08	-0.5178	10.6628
<i>Candida tropicalis</i>	<i>Candida albicans</i>	-8.00375*	1.93344	0.00	-12.6169	-3.3906
	<i>Candida krusei</i>	-2.93128	2.555	0.49	-9.0274	3.1648
<i>Candida krusei</i>	<i>Candida albicans</i>	-5.07246	2.343	0.08	-10.6628	0.5178
	<i>Candida tropicalis</i>	2.93128	2.555	0.49	-3.1648	9.0274
95% Confidence Interval						

\*The mean difference is significant at the 0.05 level.



**Figure 5: Antifungal susceptibility of *Candida spp.***

Sixteen *C. albicans*, sixteen *C. tropicalis* and seven *C. krusei* adhered in the range of 0 to 5 BEC. Seven *C. albicans*, seven *C. tropicalis* and three *C. krusei* adhered in the range of 6 to 10. Six *C. albicans*, three *C. tropicalis* and four *C. krusei* adhered in the range of 11 to 15. Five *C. albicans* adhered in the range of 16 to 20. Six *C. albicans* and one *C. krusei* adhered in the range of 21 to 25. Five *C. albicans* adhered in the range of 26 to 30. One *C. albicans* adhered in the range of 36 to 40. The frequency of adherence of *Candida spp.* to BEC is shown in Figure 4.

The adherence of clinical isolates of *C. albicans*, *C. tropicalis* and *C. krusei* was compared with *Candida albicans* MTCC 227 (test value = 2.2) using t-test. *C. albicans*(p=0.00) and *C. tropicalis*(p=0.00) adherence was statistically significant when compared to the

standard strain *C. albicans* MTCC 227 while *C. krusei* adherence was insignificant, p=0.004 with a 95% confidence interval of difference as shown in the Table 1.

Adherence of clinical isolates of *Candida spp.* were compared using tukey’s HSD test. *C. albicans* showed a statistically significant level of adherence when compared to *C. tropicalis* (p=0.00) but was insignificant when compared to *C. krusei* (p=0.00). when adherence of *C. tropicalis* was compared to *C. albicans*, it showed a statistical significance, p=0.00 but was insignificant when compared to *C. krusei*. When the adherence of *C. krusei* was compared against *C. albicans* (p=0.08) and *C. tropicalis* (p=0.49) it was found insignificant (Table 2).

Antifungal susceptibility of *Candida spp.* is summarized in Figure 5. The rates of susceptibility to fluconazole

were 100% for *C. albicans* and *C. tropicalis*. *C. krusei* are intrinsically resistant to fluconazole. The rates of susceptibility to voriconazole were 100% for *C. albicans*, *C. tropicalis* and *C. krusei*. The antifungal susceptibility pattern of *C. albicans* against clotrimazole was, 95.65% sensitive and 4.35% intermediate. In case of *C. tropicalis* clotrimazole was sensitive to 80.77%, intermediate to 11.54% and resistant to 7.69%. *C. krusei* exhibited 93.33% and 6.67% susceptibility and intermediate susceptibility respectively.

## DISCUSSION

*Candida spp.* are intra-oral commensals in 40-60% of human subjects and the precise factors involved in the development of Candidiasis are still not clear.<sup>18</sup> The adherence of *Candida* to epithelial cells is one of the main pathogenic characteristics of the genus. Studies have shown that there are variations in the adherence capabilities of different *Candida spp.*, which may explain why some species colonize mucosal surfaces more frequently than others.<sup>19-21</sup> During both mucosal colonization and induction of disease *Candida spp.* interact with epithelial cells. Because the outcomes of these interactions are important in determining whether disease develops, they are subject of intense investigation by multiple laboratories around the world.<sup>22</sup> In this study we evaluated the adherence capabilities of clinical isolates of *Candida spp.* to normal human buccal epithelial cells as it is a widely used model to study the adherence of *Candida spp.* and other microorganisms.<sup>2</sup>

The male to female proportion of distribution was similar to the study of Duggal et al, in non-diabetic individuals.<sup>23</sup> The species wise distribution of *Candida spp.* in our study was similar to that of Duggal et al, A. Kalkanci et al and L. Bulacio et al, i.e, *C. albicans* followed by non-*Candida albicans spp.*<sup>23-25</sup> The species distribution in our study was *C. albicans* 52.9%, *C. tropicalis* 29.9% and *C.krusei* 17.2%.

Adherence is an important pathogenic factor and the relation between yeasts' adherence capacity and their ability to colonize mucous surfaces is an initial necessary requirement in its colonization which may later lead to an infectious process. Many studies have been carried out on the adherence, mainly of *C. albicans* strains, but very few researchers have examined the adherence of *C. albicans* and other non-*Candida albicans spp.* with comparative adherence to standard strain of *Candida albicans*.<sup>26-30</sup> In the present study all the oral *Candida* isolates (100%) showed adherence to buccal epithelial cells, which is in accordance with the study of Arati et al.<sup>31</sup> Adherence of *Candida species* to BEC from cases of oral candidiasis was significant when compared with a standard strain *Candida albicans* MTCC 227.

In the present study, *C. albicans* was significantly more adherent to BECs than *C. tropicalis* and *C. krusei*, which is in accordance with observations of Biasoli et al., Repentigny et al, Samaranayake et al, King et al, and

Critchley et al.<sup>33-36</sup> The number of yeasts attached to buccal epithelial cells was in the range 0 to 36. This is in correlation with study of Sandin et al.<sup>37</sup> We compared the adherence of clinical isolates of *Candida spp.* to *C. albicans* MTCC 227 and found a statistically significant adherence in *C. albicans* and *C. tropicalis* but not *C. krusei*. We further compared the adherence properties between species in which *Candida albicans* showed a statistically significant adherence over *C. tropicalis* but the adherence was not significant when compared to *C. krusei*. The variability in adherence of *Candida spp.* to BEC may be due to different population of cell receptors, environmental and host causes such as dietary and nutritional factors, or hormonal levels.

The most common antifungals used in treatment of candidiasis are azoles, polyenes and echinocandins. In the present study the *Candida* isolates showed high levels of sensitivity to the tested agents viz fluconazole, voriconazole and clotrimazole (Figure 4) which is in accordance with the study of Kuriyama et al.<sup>38</sup> Antifungal susceptibility testing revealed 100% sensitivity to fluconazole by *C. albicans* and *C. tropicalis*. All the three *Candida spp.* showed 100% sensitivity to voriconazole and sensitivity to clotrimazole was 95.67%, 80.77% and 93.33% for *C. albicans*, *C. tropicalis* and *C. krusei* respectively.

Our study has some limitations as the isolates were collected only from a few centres. Our current knowledge of these adhesion events has relied largely on data obtained from in vitro experiments and primarily pertains to yeast interactions with epithelial cells as this ignores the possible role of many important hyphal adhesins. For a broader understanding of virulence factor of *Candida spp.*, other virulence factors like enzymes, phenotypic switching, biofilm formation etc need to be studied.

## CONCLUSION

In conclusion *Candida spp.* isolated from oral candidiasis patients exhibited a higher adherence capacity to normal human buccal epithelial cells considered an essential virulence property. As anticipated *C. albicans* displayed higher virulence activity than non-*Candida albicans* species. As the study revealed significant intra-species variation in *Candida* adhesion it is important to evaluate a larger number of isolates in order to elicit differences in relative adhesion among *Candida* species. Resistance to antifungal agents was not observed among the isolates tested.

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