# Crystal violet staining to quantify *Candida* adhesion to epithelial cells

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**ABSTRACT**

*In vitro* studies of adhesion capability are essential to characterise the virulence of *Candida* species. However, the assessment of adhesion by traditional methods is time-consuming. The aim of the present study is the development of a simple methodology using crystal violet staining to quantify *in vitro* adhesion of different *Candida* species to epithelial cells. The experiments are performed using *Candida albicans* (ATCC 90028), *C. glabrata* (ATCC 2001), *C. parapsilosis* (ATCC 22019) and *C. tropicalis* (ATCC 750). A human urinary bladder epithelial cell line (TCC-SUP) is used. Yeast and epithelial cells were stained with crystal violet, epithelial cells were then destained using intermediate washing, and the dye in the yeast cells was extracted with acetic acid. The method was validated for the different *Candida* reference species by comparison with traditional microscope observation and enumeration. The method was then used to assess *Candida* adhesion to epithelial cells and also to silicone. For all *Candida* spp. high correlation values ($r^2$= 0.9724–0.9997) between the number of adherent yeasts (microscope enumeration) and absorbance values were obtained for an inoculum concentration >$10^6$ cells/mL. The proposed technique was easy to perform and reproducible, enabling the determination of adhesion ability of *Candida* species to an epithelial cell line.

KEY WORDS: Adhesion.
Candida.
Cell line.
Epithelial cells.
Gentian violet.
Silicones.

## Introduction

*Candida* species can adhere to a variety of different surfaces in the human body, thus facilitating the colonisation of many host niches. Remarkably, such niches provide very different environments for growth, and *Candida* has developed specific mechanisms to adapt to the respective conditions. Several studies have shown a correlation between adhesion of organisms and their potential virulence. [1–4]

Adhesion to epithelial cells is well recognised as an essential step in the process of *Candida* colonisation and subsequent infection.[5] *Candida* adhesion to epithelial cells has been investigated to define parameters relevant to the pathogenesis of oral, gastrointestinal, vaginal and urinary candidiasis.[6] Furthermore, *Candida* can also grow on abiotic surfaces (e.g., plastic devices), for example, leading to biofilm formation in catheters, which represents a major problem especially in intensive care units.[2,7]

Over the past decades, a broad range of model systems have been described for the *in vitro* study of *Candida* adhesion to hard surfaces.[8] In most model systems, quantification of yeast cells is obtained by plating, which is labour-intensive and slow.[9] Moreover, yeast adhesion to epithelium can be determined by visual methods (e.g., light, fluorescence, scanning or transmission electron microscopy) or by counting radiolabelled yeast.[6]

The visual method involves incubating standard yeast suspensions with confluent cell monolayers grown on a coverslip. Following removal of unattached yeasts, the number of adherent organisms per unit area of the monolayer is determined by direct microscopy after air drying, Gram staining and mounting on glass slides. Although visualisation allows monitoring of adhesion to individual epithelial cells, it is a very time-consuming technique.[10,11] The radiolabelling method seems to offer an attractive alternative in some situations, although leaching of the isotope can produce misleading results.[6]

Crystal violet (CV) staining, which is commonly used for the indirect quantification of adherent cells and amount of biofilm formed by *Candida* on abiotic surfaces, is a quick and cheap method. It involves a basic dye, which binds to negatively charged surface molecules and polysaccharides in the extracellular matrix,[8,12] and dissolves easily in acetic acid.

The aim of this study is to develop a quick and simple technique to assess the number of *Candida* adherent to epithelial cells, based on the quantification of crystal violet absorbance.

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## Materials and methods

### *Yeasts and growth conditions*

*Candida* species used in this study were *Candida albicans* (ATCC 90028), *C. glabrata* (ATCC 2001), *C. parapsilosis* (ATCC 22019) and *C. tropicalis* (ATCC 750), obtained from the American Type Culture Collection. Strains were kept frozen

at –80°C in Sabouraud dextrose broth (SDB; Liofilchem, Italy) containing 5% (v/v) glycerol. *Candida* spp. were subcultured on Sabouraud dextrose agar (SDA; Liofilchem, Italy) for 24 h and then grown in SDB for 18 h at 37°C at 120 rpm. After incubation, yeasts were harvested at 8000 rpm for 5 min. Cells resuspended in phosphate-buffered saline (PBS, 0.01 mol/L, pH 7.5) were enumerated using a haemocytometer (Boeco, Germany) and the final concentration (specific to each assay) was adjusted with PBS.

### *Epithelial cells*

A human urinary bladder epithelial cell line (TCC-SUP; DSMZ – German Collection of Microorganisms and Cell Cultures) was used. Cells were cultured at 37°C in 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) containing 15% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin/streptomycin (P/S; Gibco, USA) in cell culture flasks. After achieving 80% confluence, cells were detached using a 25% trypsin-EDTA solution (Gibco, USA) and cell concentration was adjusted to $1x10^6$ cells/mL with fresh DMEM without P/S and added to each well of a six-well plate. Wells were washed (x2) with PBS prior to assay.

### *Silicone*

Coupons (2 x 2 cm) were cut from a 1 mm layer of silicone (Neves e Neves, Trofa, Portugal). All coupons were cleaned by immersion in ultrapure water for 2 h, followed by immersion in ethanol 50% (v/v) for 4 h. After rinsing with ultrapure water and air-drying, the coupons were autoclaved for 15 min at 121°C.

### *Adhesion assay*

Yeast cells were suspended in PBS to final concentrations of $10^4$, $10^5$, $10^6$, $10^7$ and $10^8$ yeast/mL. Then, 3 mL each cell suspension was added to each well of the plate for tissue culture containing either a confluent layer of epithelial cells or the silicone coupon. After incubation (2 h, 120 rpm, 37°C) the wells were washed (x2) with PBS to remove unattached yeasts. Yeast cells were quantified using the CV staining method and also light microscope observation. All procedures were repeated in triplicate in three separate assays.

### *Crystal violet assay*

Crystal violet (3 mL, 1% [w/v] in water) was added to each well containing the epithelial cells with adherent yeasts and allowed to stain for 5 min. The wells were then washed (x3) with PBS. To remove CV from the epithelial cells, 3 mL ethanol:acetone (1:1) was added to the wells and removed immediately. Acetic acid (33%, 3 mL) was added to each well and absorbance was read at 570 nm. Wells containing epithelial cells without yeasts were used as controls. Mean absorbance of yeasts was expressed as absorbance per area of each well.

*Candida* adherent to silicone were quantified according to the method of Henriques *et al*.[13] Briefly, the coupons containing adherent yeasts were removed from each well and immersed for 5 min in a new well plate containing 3 mL methanol. After discarding the methanol, the coupons were allowed to dry at room temperature. Crystal violet (3 mL) was added to each well and allowed to stain for 5 min. Coupons were then removed to a new well, washed with ultrapure water and immersed in 3 mL acetic acid (33%) to dissolve the stain. Coupons without yeasts were used as controls. Absorbance of the resultant acetic acid solution was read at 570 nm. Mean absorbance of yeasts was expressed as absorbance per unit area of the coupon.

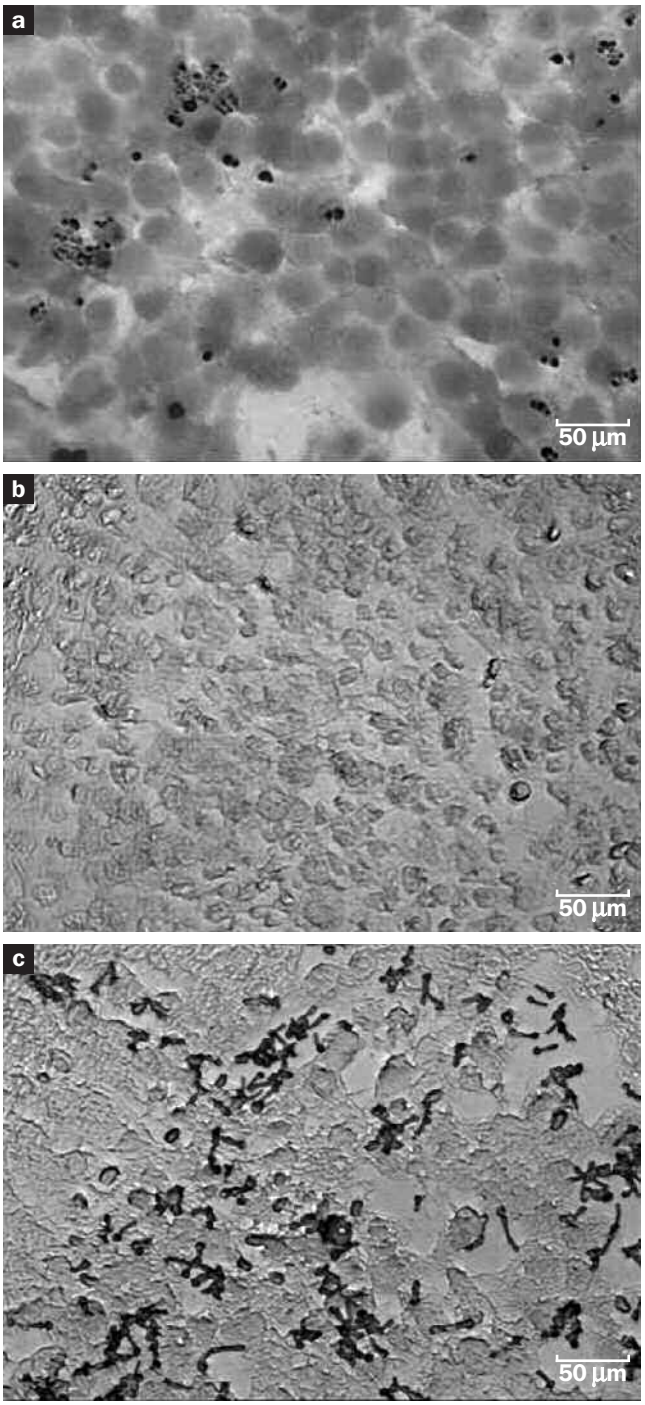


**Fig. 1.** Phase contrast images of the steps of the proposed method: a) yeasts and TCC-SUP cells stained with CV only; b) TCC-SUP cells destained with ethanol and acetone; c) Yeast cells stained strongly with crystal violet and TCC-SUP cells destained with ethanol and acetone (original magnification x200).

### *Microscope observation*

Epithelial cells and coupons with adherent yeasts were treated as described above, but without acetic acid. A duplicate of each plate was performed. *Candida* attached to TCC-SUP were quantified using an inverted light

microscope (Nikon Diaphot, x 400 magnification). Ten fields were randomly observed in each well. As the samples were set up in triplicate for each experiment, the mean number of yeasts per 30 fields was expressed as number of cells per unit area of the well. *Candida* cells with small daughter cells were regarded as one cell.

***Statistical analysis***

Results obtained were analysed using the SPSS (Statistical Package for the Social Sciences) program. One-way ANOVA with Bonferroni test was used to compare the number of adherent cells of the four strains. $P<0.05$ was considered significant.

## Results

The method proposed in this study involves CV staining of *Candida* adherent to an epithelial cell monolayer. As cells, yeasts and epithelium are all stained with CV (Fig. 1A) it was necessary to develop a technique (using a mixture of ethanol/acetone) that permitted the removal of CV from epithelial cells (Fig. 1B), allowing it to remain in the *Candida* cells (Fig. 1C). It was then possible, using acetic acid, to remove CV from the *Candida* and read the absorbance of the solution obtained.

After the confirmation, by microscopy, of the applicability of this method, it was necessary to validate the technique by assessing and comparing adhesion using traditional enumeration of adherent yeast cell by microscope observation. The relationship between CV absorbance and the number of *Candida* attached to epithelial cells is presented in Figure 2. The values obtained were $r^2 = 0.9995$ for *C. albicans*, $r^2 = 0.9997$ for *C. tropicalis*, $r^2 = 0.9724$ for *C. glabrata* and $r^2 = 0.9997$ for *C. parapsilosis*.

The method proved adequate for the detection of *Candida* attachment at high yeast numbers, specifically above $1 \times 10^5$ cell/cm$^2$ for *C. albicans* (Fig. 2A), $2 \times 10^5$ cell/cm$^2$ for *C. tropicalis* (Fig. 2B), $3 \times 10^4$ cell/cm$^2$ for *C. glabrata* (Fig. 2C) and $1 \times 10^4$ cell/cm$^2$ for *C. parapsilosis* (Fig 2D).

Different *Candida* species have different sizes and absorb distinct amounts of dye, which does not allow comparison of the level of adhesion through direct CV absorbance readings. However, standardisation of the results is possible using respective equation curves for each species.

After the implementation of the methodology, the extent of adhesion of the different *Candida* spp. to TCC-SUP epithelial cells and also to silicone was determined (Fig. 3). As shown in Figure 3A, there were significant differences in the number of yeasts adherent to epithelial cells among the different concentrations tested for each *Candida* sp., except for *C. parapsilosis*. On silicone, the differences were significant for all *C. albicans* and *C. parapsilosis* inocula concentrations but only for the highest values in *C. glabrata*

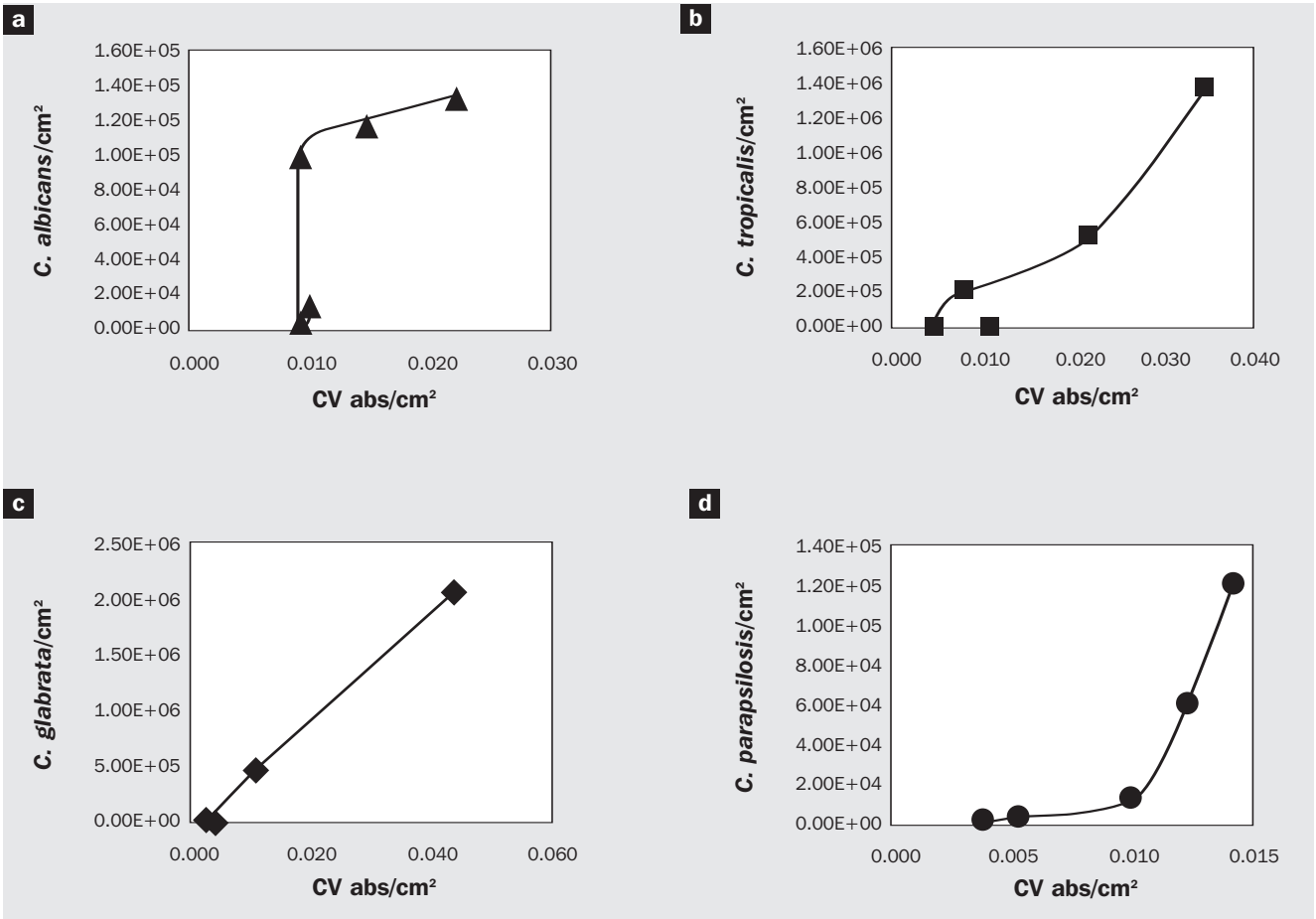


**Fig. 2.** Relationship between the number of a) *Candida albicans*, b) *C. tropicalis*, c) *C. glabrata* and d) *C. parapsilosis* adherent to TCC-SUP epithelial cells, and the corresponding CV absorbance (CV abs) at 570 nm. The adherent *Candida* spp. were expressed as yeast number or CV absorbance per area of each well. All procedures were performed in triplicate in three separate assays

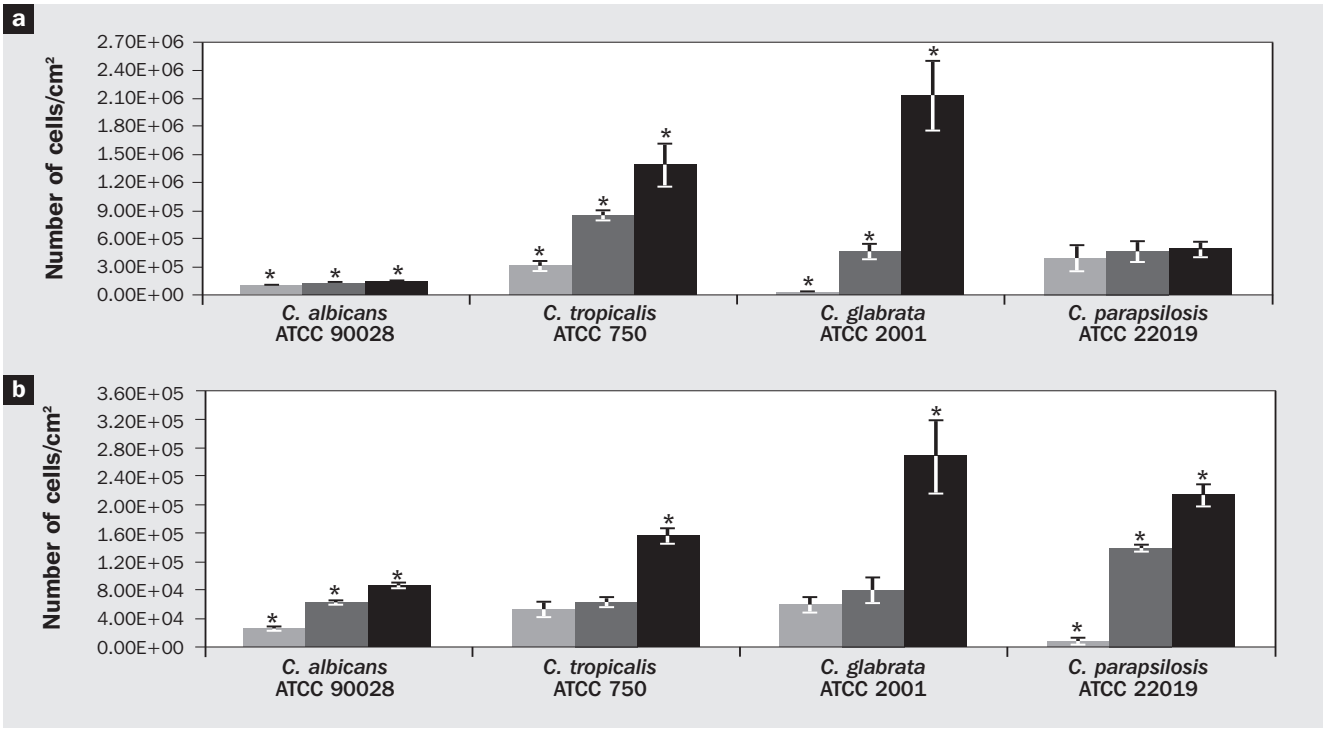


**Fig. 3.** *Candida* spp. cells adherent to a) TCC-SUP epithelial cells and b) silicone measured by crystal violet absorbance reported as cell/cm$^2$. Data are the average of three measurements (+SD). The initial cell density: $10^6$ (■), $10^7$ (■) and $10^8$ cell/mL (■). *$P<0.05$ between the different inoculums for the same species.

and *C. tropicalis* (Fig. 3B). All *Candida* species adhered to a greater extent to epithelial cells than to silicone.

Considering the difference between species, it is possible to observe (Fig. 3) that *C. glabrata* ($P<0.05$) adhered significantly more than other *Candida* spp. (initial cell density: $10^7$ and $10^8$ yeast/mL) to epithelial cells, and *C. tropicalis* (initial cell density: $10^6$ and $10^7$ yeast/mL; $P<0.05$) to silicone. *C. albicans* adhered less than other yeasts to both surfaces at $10^8$ yeasts/mL ($P<0.05$).

The methodology proposed proved efficient in demonstrating the *in vitro* adherence of *C. albicans* (ATCC 90028), *C. glabrata* (ATCC 2001), *C. parapsilosis* (ATCC 22019) and *C. tropicalis* (ATCC 750) to TCC-SUP.

## Discussion

Most *Candida* infections are associated with adhesion to implanted medical devices or to host epithelial cell surfaces.[14] *In vitro* adherence studies of *Candida* on different surfaces are well established.[3,4,8,10,15] One quantification method is direct enumeration by microscopy, which has been used widely to determine the extent of yeast adhesion to epithelial cells.[16] Although this technique permits visualisation of the yeast cells adherent to individual epithelial cells, it is very time-consuming.[11] Other techniques described to evaluate *Candida* adhesion to biological and inert surfaces include indirect immunofluorescence,[17] fluorescence-labelled cytometry,[18] radioisotope analysis[19] and photometric quantification.[20] However, most of these do not balance accuracy, speed, reproducibility and cost-effectiveness.[6]

The method described here is based on CV staining to quantify microbial adhesion and biofilm formation on inert surfaces.[13,21] However, the application of this method to assess adhesion to epithelial cells is not straightforward as both epithelial and yeast cells absorb CV dye (Fig. 1A). It is possible to circumvent this problem by using a mixture of ethanol/acetone to remove the stain from the epithelial cells (Fig. 1B) but not the *Candida* cells (Fig. 1C).

Figure 2 shows that it is possible to establish a direct relationship between the number of *Candida* adherent to TCC-SUP cells and CV absorbance, and were obtained for initial inocula concentrations of $10^6$, $10^7$ and $10^8$ yeast/mL. According to Henriques *et al.*,[11] there is no detectable yeast attachment at concentrations below $10^4$ yeast/mL, and $10^7$ yeast/mL is the most frequently used *Candida* inoculum concentration.[16,22] The high correlation between CV absorbance values and the number of *Candida* cells obtained by microscopy confirms the utility of determining attached cell numbers through the corresponding absorbance reading.

The extent of adhesion of *C. albicans* 90028, *C. glabrata* 2001, *C. parapsilosis* 22019 and *C. tropicalis* 750 to TCC-SUP cells and to silicone was also determined using the proposed method (Fig. 3). As *Candida* spp. differ on size, their absorbance value was standardised (according to each species curve) in order to permit comparison. It was noticed that different inoculum concentrations gave significantly different ($P<0.05$) numbers of *Candida* adherent to the epithelial cells; an observation made by others[23–25] who report that the attachment of *Candida* species to epithelial cells gradually increases as the ratio of yeasts to epithelial cells, in incubation mixtures, is raised from 10:1 to 10000:1.

Differences were detected in the adherence of *Candida* spp. to TCC-SUP cells and silicone. All *Candida* were more prone to adhere to TCC-SUP than to silicone (Fig. 3). Sohn *et al.*[3] described the comparison of adhesion of *C. albicans* to the

human colorectal carcinoma cell line Caco-2 and to epidermoid vulvovaginal A431 cells and to polystyrene, reporting that *C. albicans* adherence is high to polystyrene and both epithelia.

*C. albicans* and *C. parapsilosis* adhered to a similar extent on both surfaces at an initial concentration of $10^8$ cells/mL, but less than the other yeasts studied. Tamura *et al*.[26] investigated the adherence of *C. albicans* and *C. parapsilosis* to urinary catheters made of latex or silicone and found that adhesion of *C. albicans* was significantly greater than *C. parapsilosis* on latex, but similar on silicone. Furthermore, De Bernardis *et al*.[1] observed that from all the non-*C. albicans* species (NCAC) studied, *C. parapsilosis* seemed to be the least virulent, which might be consistent with lower adherence to epithelial cells.

Owing to the increasing prevalence of NCAC species, especially in immunosuppressed patients, more insight about virulence factors associated with these species is required. However, relatively little is known about the mechanisms of NCAC adhesion to epithelium or about factors affecting the adhesion process.[11] *C. tropicalis* is a common species related to nosocomial candidaemia and candiduria, and *C. glabrata* is now emerging as an important agent in both mucosal and bloodstream infections.[14,27] Nevertheless, the adhesion mechanism of these species to different surfaces remains unclear.[11,14]

From Figure 3A, it is possible to see that *C. glabrata* ($P<0.05$) adhered in greater numbers than did other yeasts (initial cell density: $10^7$ and $10^8$ cell/mL) to epithelial cells, and *C. tropicalis* ($P<0.05$) adhered in considerable numbers to silicone (initial cell density: $10^6$ and $10^7$ cell/mL).

Strain differences were noted in the ability of *C. glabrata* to adhere to oral epithelial cells, synthesise phospholipases and trigger cytokine responses.[28–30] According to Shin *et al*.,[31] biofilm occurred most frequently in isolates of *C. tropicalis*, followed by *C. parapsilosis, C. glabrata* and *C. albicans*. Virulence of *C. tropicalis* may be due to its greater adhesion to different surfaces and its ability to secrete moderate amounts of proteinase and filamentous forms compared to other NCAC species.[14,19,32]

In conclusion, the proposed methodology is both easy to execute and cheap, and is reproducible in assessing *Candida* adhesion to TCC-SUP cells. It is a valuable methodology to discriminate the adhesive capacity of different *Candida* spp. isolates to different epithelial cells, and may contribute to research on the virulence of *C. albicans* and NCAC species. □

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