

Full Length Research Paper

## Comparison of the adhesion ability of *Candida albicans* strains to biotic and abiotic surfaces

Noumi Emira<sup>1,2\*</sup>, Snoussi Mejd<sup>1,3</sup>, Kammoun Dorra<sup>4</sup>, Bakhrouf Amina<sup>1</sup> and Valentin Eulogio<sup>2</sup>

<sup>1</sup>Laboratoire d'Analyse, Traitement et Valorisation des Polluants de l'Environnement et des Produits, Faculté de Pharmacie, Rue Avicenne, Monastir 5000, Tunisie.

<sup>2</sup>Departamento de Microbiología y Ecología, Facultad de Farmacia, Universidad de Valencia, Burjassot, Valencia, Spain.

<sup>3</sup>Laboratoire de Traitement des Eaux Usées, Centre de Recherches et des Technologies des Eaux (CERTE), Technopole de Borj-Cédria, BP 273- Soliman 8020, Tunisie.

<sup>4</sup>Laboratoire de Biomateriaux et Biotechnologie, Faculté de Médecine Dentaire, Monastir, Tunisie.

Accepted 2 November, 2010

The purpose of the present study is to investigate the ability of oral *Candida albicans* strains to adhere to Caco-2 and Hep-2 epithelial cells, to produce slime using Congo red and Safranin methods and to form a biofilm on polymethylmethacrylate. A total of 20 *C. albicans* strains were tested in the present work. The biofilm formed by *C. albicans* isolates on acrylic denture was measured *in vitro* using the colorimetric method based on the reduction of the tetrazolium salt (XTT) and dry weight measurement. Our results showed that oral *C. albicans* strains were able to adhere to epithelial cell lines and biomaterials with different degree. In fact, more than 61% of the tested strains were adhesive to Hep-2 and 83% to Caco-2 cells. Strong slime production after safranin stain was found in 40% of strains. Slime producer *C. albicans* strains (16 strains) tested by safranin staining method were highly adhesive to Hep-2 (62.5%) and Caco-2 (87.5%) monolayers and biofilm formed on polystyrene and poly-methyl methacrylate (PMMA) surfaces (100%, respectively).

**Key words:** *Candida albicans*, biofilm, polymethylmethacrylate, Hep-2, Caco-2 cells, slime production.

### INTRODUCTION

The oral cavity is an open system in which *Candida* cells are mixed with saliva and can adhere and grow. *Candida albicans* is a commensal pathogen that lives on the skin and mucosal surfaces of the genital and intestinal tracts as well as in the oral cavity (Calderone and Fonzi, 2001). This yeast is the principal etiological agent of oral candidal infection (Budtz-Jorgensen, 1990; Nikawa et al., 1998; Webb et al., 1998). *Candida* strains have been isolated from 93% of patients with denture stomatitis

which are now considered to be the commonest form of oral candidosis (Budtz-Jorgensen et al., 1975). The adherence of *C. albicans* to host cells or polymers such as denture acrylic resin and soft lining materials is an essential and necessary first step in successful colonization and the development of pathogenesis and infection (Gristina et al., 1993; Cannon and Chaffin, 1990). Several studies have been conducted on the adhesion, biofilm formation and colonization of *C. albicans* to a range of dental materials such as oral prostheses (Edgerton et al., 1993; Goldman and Pier, 1993).

The adhesion of *C. albicans* cells to acrylic resin dentures (polymethylmethacrylate) directly or via the salivary pellicle is a first step to developing an infectious process (Edgerton et al., 1993; Chandra et al., 2001; Ramage et al., 2004; Avon et al., 2007). Attachment to cells is followed by proliferation and biofilm formation (Hawser and Douglas, 1994; Baillie and Douglas, 2000). Several factors implicated in the adhesion of *Candida* to

\*Corresponding author. E-mail: emira\_noumi@yahoo.fr; Tel: + 216 73 461000; Fax: + 216 73 461830;

**Abbreviations:** CSH, Cell surface hydrophobicity; YPD, yeast peptone dextrose; YNB, yeast nitrogen base; PBS, phosphate buffered saline; MEM, minimum essential medium; NA, non adhesive; W, weak adhesion; M, medium adhesion; S, strong adhesion; MIC, minimum inhibitory concentration.

the acrylic resin base have been reported from *in vitro* studies (Moura et al., 2006; Pereira-Cenci et al., 2006; Noumi et al., 2009). Surface structure, properties and composition of biomaterials, hydrophobicity and roughness influence the adhesion of microorganisms on biomaterials (Busscher et al., 1992; Verheyen et al., 1993). Some previous studies have demonstrated that components of the acrylic resin may reduce the adhesion and inhibit the growth of *Candida* (Waltimo et al., 1999). Although the strongest mechanism for adherence involves a mannoproteins adhesion on *C. albicans*, cell surface hydrophobicity (CSH) has been described by many investigators as involved in adherence (Hazen, 1989; Cannon and Chaffin, 1999). Hydrophobic proteins, however, embedded in the matrix of the *C. albicans* cell wall beneath the fibrillar layer provide the hydrophobic interactions needed to turn this initial attachment between the fungus and the surface into a strong bond (Rosenberg et al., 1983; Jose et al., 1991; Mary Ann et al., 2001).

Adhesion ability of *C. albicans* to epithelial cells has been frequently tested on HeLa, human embryonic kidney epithelial cells, fibroblasts and Hep-2 cells (Bektic et al., 2001). Cotter and Kavanagh (2000) reported that adhesion to buccal epithelial cells is an essential condition to oral candidiasis. Moreover, when adhered to epithelial cells and biomaterials, *Candida* produces large quantities of extracellular polymeric substance in solutions enriched with glucose. Slime is considered as a virulent factor produced by *Candida* strains related with their persistence and colonization of the host tissues (Vinitha and Ballal, 2007).

The aim of the present work is to study the cell surface hydrophobicity and to compare the adhesion ability of oral *C. albicans* strains to biotic surface (epithelial cells: Caco-2 and Hep-2) and abiotic surface (polystyrene and acrylic denture: polymethylmethacrylate) and their capacity to produce exopolysaccharide using Safranin staining method.

## MATERIALS AND METHODS

### Patients

Clinical *C. albicans* strains were associated with oral candidiasis and isolated from patients frequenting the Dental Hospital of Monastir town (Tunisia). The symptoms associated with oral *Candida* infection were ranged from none to a painful burning sensation that may interfere with the ability to swallow (dysphagia) and ability to take in nutrition. Patients used demonstrated erythematous, pseudomembranous, and denture stomatitis. This oral candidiasis was often associated with diabetes, smoking, prosthesis and poor oral hygiene.

### Clinical strains, media, growth conditions and enzymatic characterization

A total of 17 clinical *C. albicans* isolates and three *C. albicans* type strains were used in the present study (Table 1). Samples were collected from the oral cavity, the gingival sulci and the pharyngeal

portion, using a swabbing method. Yeast strains were cultured into sabouraud chloramphenicol agar (Bio-rad, France) for 48 h at 35°C. All oral isolates were identified by standard microbiological methods: macroscopic test on sabouraud chloramphenicol agar while microscopic test was by induction of hyphal form on Lee's medium. The carbohydrates assimilation was tested using the ID 32°C system (bio-Mérieux, Marcy l'Étoile, France) according to the manufacturer's specification and the results were read using an automated microbiological mini-API (bio-Mérieux). *C. albicans* ATCC 90028, *C. albicans* ATCC 2091 were kindly provided by Professor Stefania Zanetti (University of Sassari, Italy) and *C. albicans* SC 5314 were used in this study as type culture strains (Table 1). The genomic confirmation of all *C. albicans* strains tested at the species level was done according to the protocol described by Baquero et al. (2002) by amplification of a *CaYST1* gene intron fragment.

### Cell surface hydrophobicity (CSH)

The hydrophobicity of *Candida* strains was measured according to the protocol described by Rosenberg et al. (1983) which consists in measuring the adherence of the yeast to hydrocarbons, such as cyclohexane or xylene.

The tested strains were grown overnight in 5 ml of yeast peptone dextrose (YPD) or yeast nitrogen base (YNB) broth at 28°C. Cells were washed with phosphate buffered saline (PBS) and concentrated to obtain a solution corresponding to  $OD_{600} = 1$ . For adhesion assays, 3 ml of the cell suspension were mixed with 150  $\mu$ l of cyclohexane or xylene in an acid-washed glass tube. The sample was vigorously mixed using vortex for 1 min. After 20 to 60 min at room temperature, the absorbance at 600 nm of the aqueous phase ( $A_1$ ) was measured and compared with that obtained prior to the mixing procedure ( $A_0$ ). The percentage of cells in the cyclohexane / xylene layer (adhered cells) was used to estimate the hydrophobicity using the following formula:

$$\text{Percent cell adhesion} = (A_1/A_0) \times 100$$

All tests were run in duplicate. The results shown represent the mean of two consecutive experiments.

### Qualitative detection of exopolysaccharide (slime) production

Slime production was determined using the safranin method described for coagulase negative staphylococci briefly modified by Christensen et al. (1982) and Davenport et al. (1986). Briefly, a loopful of organisms from the surface of a Sabouraud dextrose agar plate was inoculated into a tube containing 10 ml of Sabouraud broth supplemented with glucose (final concentration, 8%). The tubes were incubated at 35°C for 24 h and examined for the presence of a viscid slime layer. Slime production by each isolate was scored as negative, weak (1+), moderate (2+), or strong (3+). Each isolate was tested at least three times and read independently by two different observers.

### Adherence assay to human epithelial cells lines: Hep-2 and Caco-2

Adhesion test was carried out in the Department of Biomedical Sciences, University of Sassari, Italy. Two human epithelial cell lines Hep-2 (cells from human laryngeal carcinoma) and Caco-2 (cells from human colon adenocarcinoma) were used in this study. For thus, the epithelial cells were grown, maintained in Eagle's minimum essential medium (MEM) medium (Gibco BRL, Life Technologies, Rockville, MD, USA) supplemented with 10% fetal bovine serum (Gibco, BRL) 1000 IU/ml of both penicillin and

**Table 1.** Cell surface hydrophobicity, adhesive properties and biofilm formation of *C. albicans* on polystyrene microtiter plates, polymethylmethacrylate biomaterial, and epithelial cells.

Strain/ Origin	Hydrophobicity (%)		Polystyrene (mean OD <sub>492</sub> ± SD)	PMMA (mean OD <sub>492</sub> ± SD)	Dry weight (mg ± SD)	Safranin assay	Epithelial cells	
	X	C					Hep-2	Caco-2
R <sub>1</sub> (ATCC 90028)	88.4	84.9	0.26 ± 0.014	0.356 ± 0.006	3.46 ± 0.046	+++	NA	W
R <sub>i</sub> (ATCC 2019)	*	*	0.246 ± 0.021	0.264 ± 0.009	1.84 ± 0.054	++	*	*
V <sub>1</sub> (SC 5314)	97.4	92	0.255 ± 0.019	0.354 ± 0.011	1.69 ± 0.02	+++	*	*
3 <sup>1</sup> Tunisia	71.8	70.5	0.233 ± 0.028	0.335 ± 0.011	4.81 ± 0.0451	+++	S	S
4 Tunisia	98	96.2	0.224 ± 0.023	0.395 ± 0.006	3.79 ± 0.046	+++	S	W
6 Tunisia	97.3	97.4	0.229 ± 0.027	0.329 ± 0.008	2.43 ± 0.03	++	NA	S
7 Tunisia	98.8	93	0.234 ± 0.019	0.402 ± 0.008	2.77 ± 0.035	++	NA	M
9 Tunisia	62.8	71.1	0.242 ± 0.008	0.317 ± 0.009	1.53 ± 0.019	++	S	W
10 Tunisia	69.9	73.2	0.213 ± 0.02	0.377 ± 0.019	3.88 ± 0.081	+++	S	S
11 Tunisia	40.2	46.5	0.234 ± 0.045	0.356 ± 0.01	2.0 ± 0.022	+	NA	NA
13 Tunisia	88.1	86.9	0.233 ± 0.036	0.348 ± 0.005	1.68 ± 0.029	+++	NA	NA
14 Tunisia	91	81.1	0.223 ± 0.019	0.342 ± 0.009	1.98 ± 0.006	++	S	S
15 <sub>B</sub> Tunisia	92.4	94.8	0.287 ± 0.101	0.353 ± 0.004	2.12 ± 0.074	++	S	M
16 Tunisia	81.4	61.6	0.264 ± 0.045	0.351 ± 0.003	4.03 ± 0.016	+++	S	S
17 Tunisia	90.7	79	0.27 ± 0.005	0.342 ± 0.017	0.92 ± 0.057	++	S	S
17 <sub>R</sub> Tunisia	66.3	82.6	0.266 ± 0.013	0.336 ± 0.02	2.97 ± 0.031	++	NA	M
18 Tunisia	81.6	82.3	0.245 ± 0.004	0.255 ± 0.029	1.86 ± 0.044	++	S	W
21 Tunisia	77	72.7	0.278 ± 0.014	0.343 ± 0.005	1.79 ± 0.042	+++	S	S
65 Tunisia	95.1	74	0.262 ± 0.01	0.37 ± 0.02	3.76 ± 0.051	+	S	S
I <sub>2</sub> Italy	*	*	0.266 ± 0.013	0.36 ± 0.03	2.54 ± 0.059	++	NA	NA
% of positive tests	82.68 %	79.96 %					61.1	83.3

X, Xylen; C, cyclohexane; PMMA, polymethylmethacrylate; NP, slime non-producer; Hep-2, cells from human laryngeal carcinoma; Caco-2, cells from human colon adenocarcinoma; NA, none adhesive; W, weak adhesion; M, medium adhesion; S, strong adhesion; +, slime weak positive; ++, moderate positive; +++, strong positive; SD, standard deviation; \*, not tested strain.

streptomycin. Twenty-four well tissue trays (Falcon) were seeded with the two cells lines (10<sup>3</sup> cells/well). Plates were incubated for 18 h at 37°C in a humidified atmosphere with 5% of CO<sub>2</sub>. The semiconfluent monolayers were washed with fresh MEM containing 1% foetal bovine serum. Adhesion of *Candida* strains to Hep-2 and Caco-2 cells was tested as described previously by Holmes et al. (2002). *Candida* strains were grown at 37°C in YPD medium (10 g of yeast extract, 10 g of peptone and 10 g of glucose in 1000 ml of distilled water).

For adherence assay, 100 µl of 10<sup>7</sup> cells/ml were added to Hep-2 and Caco-2 cells, transferred to sterile 24-well plates (Nalgene Nunc, Rochester, NY, USA) and incubated at 37°C for 3 h in 5% CO<sub>2</sub>. After being washed three times with phosphate-buffered saline (PBS, pH 7.4), *Candida* cells bound to cells were fixed with methanol, stained with Giemsa stain and examined microscopically (magnification x40). Uninoculated cell lines served as negative controls. The number of yeasts adhering to each of cell lines was counted. All organisms were tested three times. The adhesion index was assayed: NA = non adhesive (0 to 10 *Candida*/cells); W = weak adhesion (10 to 20 *Candida*/cells); M = medium adhesion (20 to 50 *Candida*/cells); S = strong adhesion (50 to 100 *Candida*/cells).

#### Biofilm assay on polystyrene and polymethylmethacrylate

In the present study, biofilms were produced on commercially

available presterilized polystyrene flat-bottom 96-well microtiter plates (Iwaki, Tokyo, Japan) for 66 h on yeast nitrogen base (YNB). Measurement of biofilm formation was studied by the 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) reduction assay according to the method described by Kuhn et al. (2002). The denture base materials used is Paladur-B (Translucent pink, Heraeus Kulzer GmbH). This biomaterial is usually used in the preparation of dental prosthesis in our country. The strips were made according to the method of Samaranyake and MacFarlane (1980).

The ability of *C. albicans* strains to form a biofilm was tested according to the protocol described by Chandra et al. (2001). For thus, cells were grown for 24 h at 37°C in yeast nitrogen base containing 50 mM of glucose. Batches of medium were inoculated with overnight yeast cultures and incubated at 37°C in an orbital shaker operating at 150 rpm. Cells were harvested after 24 h (stationary growth phase), washed once with phosphate-buffered saline (PBS, pH 7.2), and standardized to a density at 1 × 10<sup>7</sup> cells/ml. A volume of 80 µl of the standardized *C. albicans* cells suspensions was applied to polymethylmethacrylate strips (1.5 Cm<sup>2</sup>) placed in a 12-well tissue culture plate. The cells were allowed to adhere for 90 min at 37°C (adhesion phase). Non-adherent cells were removed from the strips by being gently washed with 5 ml PBS. Strips were then submerged in 4 ml of YNB containing 50 mM of glucose. Strips to which no cells were added served as negative controls. Control and experimental strips

were incubated at 37°C for 72 h (biofilm growth phase).

#### Quantitative measurement of biofilm

We have adopted two protocols previously described by Chandra et al. (2001) to quantify the biofilm formation by *C. albicans* strains: Dry-weight analysis was used to determine the total biofilm mass, while a colorimetric method was applied to determine the mitochondrial dehydrogenase activity of the metabolic fungal cells. For dry-weight determination, the biofilms were scraped from each strip into 4 ml PBS and transferred to a tube. The strip was vortexed to remove any remaining residual biofilm material. The pooled suspension was filtered through a pre-weighed filter (0.45 µm pore size), washed with PBS, air-dried at 35°C for 24 h, and weighed.

The colorimetric assay investigates metabolic reduction of 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenyl amino) carbonyl]-2H-tetrazolium hydroxide (XTT) to a water-soluble brown formazan product. Strips with biofilms were transferred to 12-well tissue culture plates containing 4 ml PBS/well. Fifty microliters of XTT (1 mg/mL in PBS) and 4 µl of menadione solution (1 mM in acetone) were added to each well. Plates were incubated at 37°C for 5 h. The entire contents of the well were transferred into a tube and centrifuged (5 min, 6000 g). XTT formazan in the supernatant was determined spectrophotometrically at 492 nm. Experiments were performed in triplicate.

#### Statistical analysis

The data were analyzed using the statistical program SPSS version 14.0 (Statistical Package for the Social Sciences). Since low samples numbers contributed to uneven variation, the adhesion results were analyzed by the nonparametric Wilcoxon test. Statistical calculations were based on a confidence level  $\geq 95\%$  ( $P < 0.05$  was considered statistically significant).

## RESULTS

### Yeast characterization

All *C. albicans* strains tested were successfully identified using ID 32°C strips. This biochemical system was previously used with success to identify *Candida* strains isolated from different body sites (Cardenas-Perera et al., 2004; Noumi et al., 2009). All strains tested were genetically confirmed to belong to *C. albicans* species as they amplify the 310-pb size fragment corresponding to the *CaYST1* gene intron fragment. The three *C. albicans* reference strains tested (ATCC 90028, ATCC 2019 and SC 5314) produce a 310-pb region specific for this species (Noumi et al., 2009).

### Adhesion to solvent

The results of measuring the adherence of *Candida* cells to liquid hydrocarbons (Xylen and cyclohexane) are summarized in Table 1. The two tested hydrocarbons were able to bind *Candida* cells with different degrees function of the species. In fact, the optimal adherence was observed in the presence of xylen: 82.68% for *C.*

*albicans*; 85.23% for *C. parapsilosis*; 76.45% for *C. glabrata*; 79.07% for *C. kefyr*; 74.3% for *C. holmii* and 40.2% for *C. sake*. Adherence to cyclohexane was lower than that observed for xylen. This sequence of affinity for the tested hydrocarbons has been observed with the six tested *Candida* species especially for *C. holmii* (74.3 % for xylen and 53.7% with cyclohexane) and may be related to the relatively high viscosity of cyclohexane.

Our results showed that *C. albicans* is the most hydrophobic specie using Xylen and cyclohexane as liquid hydrocarbons.

### Slime production

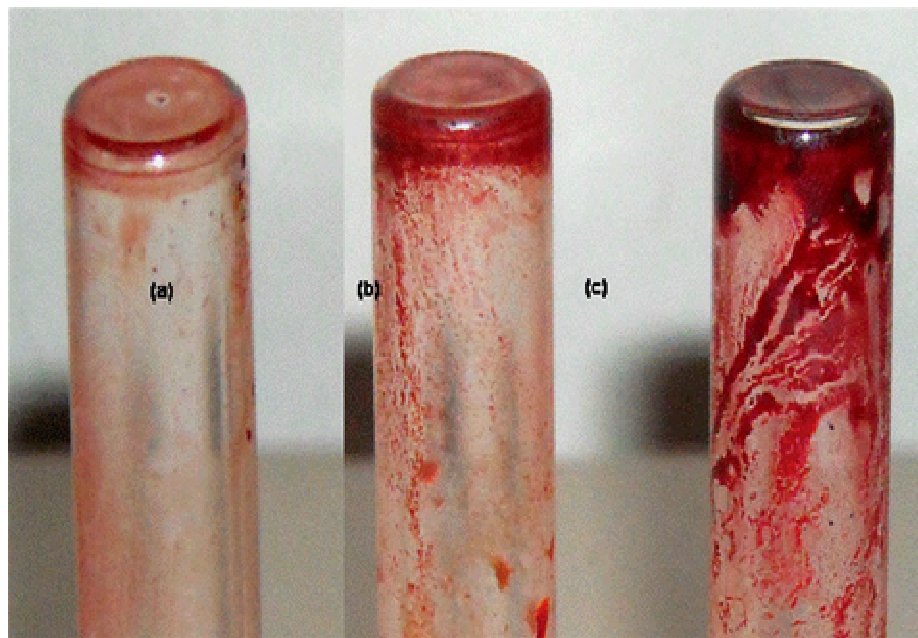
Figure 1 shows the different ranges of slime production by *C. albicans* strains on glass tubes stained with 1% safranin. Using this method, slime production was demonstrated in all of *C. albicans* isolates tested (Table 1). The majority of isolates (90%) and standard strains showed moderate to high slime production. Strong slime production was found in eight strains ( $R_1$ ,  $V_1$ ,  $3^1$ , 4, 10, 13, 16 and 21). Two isolates (10%) were weakly positive slime producer. Five *C. albicans* strains ( $R^1$ ,  $V^1$ , 4, 10 and 13) having the same morphotype (orange) obtained on CRA plates were strong slime producers tested with safranin staining method (Table 1).

### Adherence to Hep-2 and Caco-2 epithelial cells

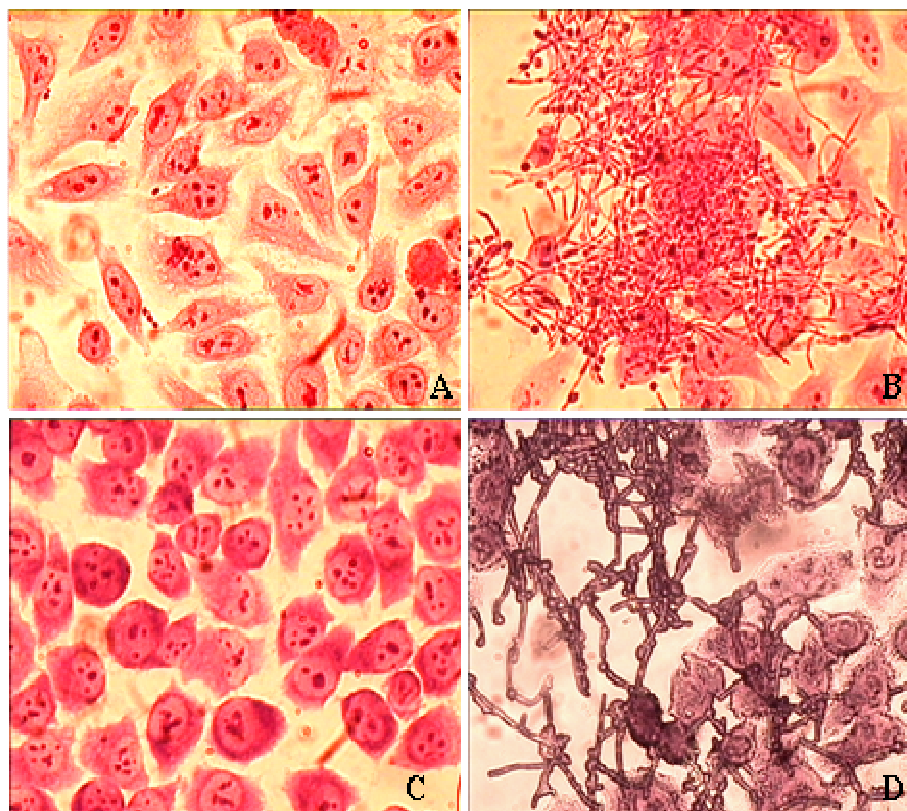
The *C. albicans* strains were able to adhere to the two cell lines used in this study with different degree: 83% to human colon adenocarcinoma cells (Caco-2 cells) and 61% to human laryngeal carcinoma cells (Hep-2). All these data are summarized in Table 1. Seven strains (38.8%) were unable to adhere to Hep-2 cells and three (16.6%) to Caco-2 cells. Eleven strains (61.1%) were able to adhere strongly to Hep-2 cells and eight (44.4%) to Caco-2 cells. Seven *C. albicans* strains (strains  $3^1$ , 10, 14, 16, 17, 21 and 65) isolated from oral cavity of patients suffering from denture stomatitis were strong adhesive to the two cells lines. Figure 2 presents the Optic microscopy of *C. albicans* (strain n° 16) showing strong adherence ability to epithelial cells.

### Quantitative biofilm formation by *C. albicans* strains on polystyrene

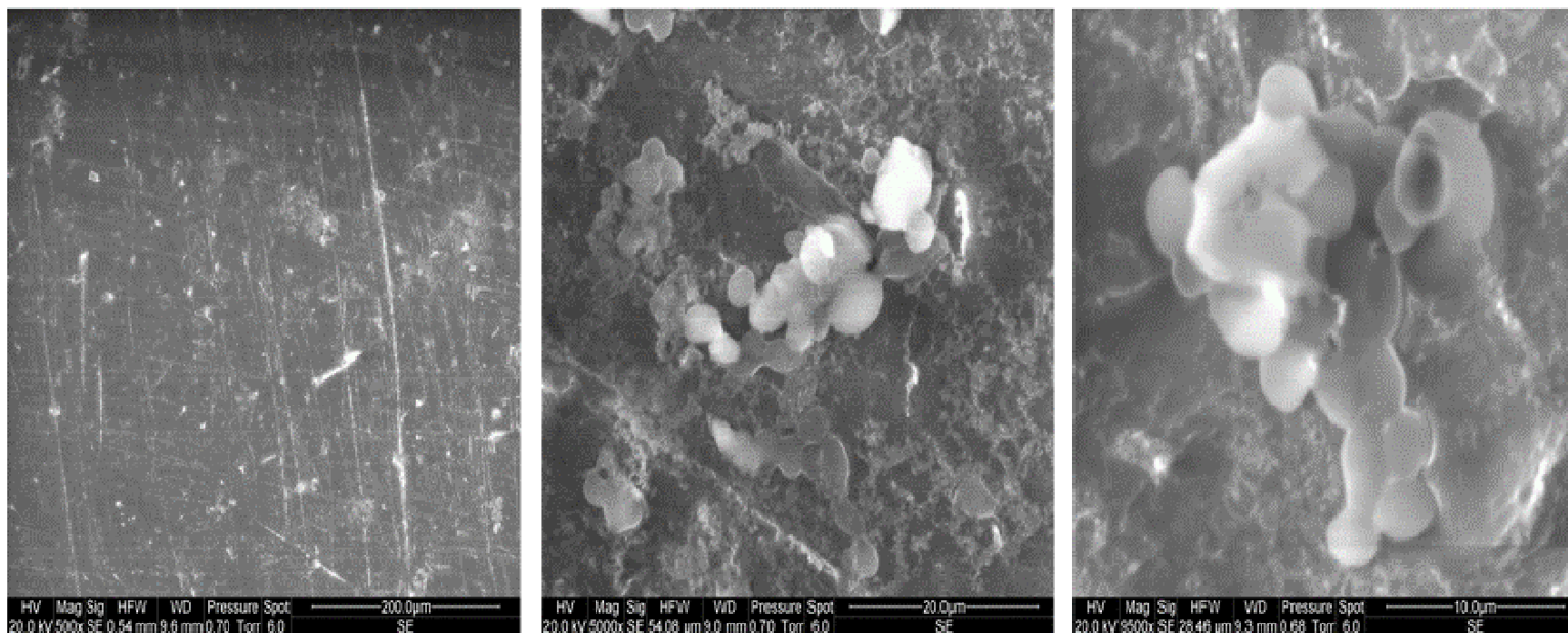
All *C. albicans* strains were adhesive to polystyrene 96-well microtiter plate at different degrees. The optical density values of XTT reduction estimated at 492 nm were ranging from 0.213 to 0.287 (Table 1). Three *C. albicans* strains (15<sub>B</sub>, 17 and 21) strongly adhesive to Hep-2 cells also showed high potency to adhere to polystyrene plates. Also, the oral *C. albicans* strain 16 with high ability to adhere to polystyrene was strong



**Figure 1.** Different ranges of slime production by *C. albicans* strains on glass tubes stained with 1% safranin. (a) Weak slime production (*C. albicans*: strain 65); (b) moderate slime production (*C. albicans*: strain 17); (c) strong slime production (*C. albicans* ATCC 90028).



**Figure 2.** Optic microscopy of *C. albicans* (strain n° 16) showing strong adherence ability to epithelial cells. A and C: negative controls, B and D: Adhesion to Hep-2 and Caco-2 epithelial cells respectively. The adherence assay was performed as described in the text. Giemsa stain: magnification ( $\times 40$ ).



**Figure 3.** SEM of a *C. albicans* mono-species biofilm formed on PMMA surface showing fungal cells.

slime producer when tested with safranin stain.

#### **Quantitative biofilm formation by *C. albicans* strains on PMMA**

In this study, we determined whether the metabolic activity and total biomass of *C. albicans* biofilms, as determined by reduction of XTT dye and dry weight measurement in the presence of glucose as a carbon source. We found that metabolic activity of *C. albicans* biofilm formed on polymethylmethacrylate did not differ between all tested strains (Table 1). Out of seven strains able

to adhere strongly to the two epithelial cell lines (Caco-2 and Hep-2), only two strains (4 and 65) were highly adhesive to polymethyl-methacrylate surface. The two *C. albicans* strong slime producers (4 and 10) obtained with safranin stain showed high ability to form biofilm on PMMA (Table 1).

Figure 3 showed the morphology of *C. albicans* biofilm formed on PMMA strips visualised by scanning electron microscopy. The *C. albicans* reference strain ATCC 2019 was weak biofilm forming strain on polystyrene plates and polymethylmethacrylate surface and also slime moderate positive strain (Table 2).

#### **DISCUSSION**

This study represents the first attempt to study the adhesion ability of clinical *C. albicans* strains associated with oral candidiasis and isolated from patients frequenting the Dental Hospital in Monastir (Tunisia) during year 2007. Oral *C. albicans* strains were identified on the basis of their biochemical activities tested on ID 32°C strips and their exoenzymes profile. These strains were biochemically heterogeneous. All *C. albicans* strains were genetically confirmed at the species level as they amplify a 310-pb fragment as described previously by Baquero et al. (2004).

**Table 2.** Comparison of slime production with adhesion to epithelial cells and ability to form a biofilm on polystyrene and PMMA surfaces.

Parameter	Cell	Slime (+) using safranin method 16 (88.88%)
Adhesion to epithelial cells	Hep-2	10/16 (62.5%)
	Caco-2	14/16 (87.5%)
Biofilm on polystyrene		16 (100%)
Biofilm on PMMA		16 (100%)

Slime+: Total slime positivity (weak positive, moderate positive and strong positive).

Slime production is considered as a virulent factor of *Candida* strains responsible of infection dissemination in susceptible hosts, their persistence and colonization of the host tissues (Ramage et al., 2001a; Ramage et al., 2001b, Ramage et al., 2005). Slime production of a *Candida* isolate is highly correlated with other properties responsible for its firm adherence such as hydrophobicity (Li et al., 2003). In fact, the ability of *C. albicans* strains to produce slime was tested by safranin stain. We founded that 90% of isolates showed moderate to high slime production. Indeed, six oral *C. albicans* strains, *C. albicans* ATCC 90028 and SC 5314 were strong slime producers and only two isolates (10%) were weak slime producing strains. Our results are similar to those reported by Ozkan et al. (2005) who founded that 82.8% of *C. albicans* strains were slime positive and 11.43% were strongly slime producer strains.

Kalkanci et al. (1999) investigated the correlation between the *Candida* strains and clinical isolation areas for slime production. Weak slime production was found in 8% and strong slime production was found in 4% of the strains. Slime positivity for *C. albicans* was 9.3%; on the other hand it was 25% for non-*albicans* strains. The slime factor is more important for non-*albicans* strains (*C. kefyr*, *C. tropicalis*, *C. rugosa*, *C. krusei*).

Yücesoy et al. (2003) have investigated the biofilm production of various *Candida* strains and compared this activity with fluconazole and amphotericin B susceptibility. There was no statistically significant difference between *Candida* strains biofilm activity and susceptibilities to amphotericin B. However, with *Candida* strains, statistically significant difference was determined between biofilm activity and susceptibilities to fluconazole by tube adherence method.

Ozkan et al. (2005) studied the slime and proteinase activity of 54 strains consisting of 19 *C. parapsilosis* and 35 *C. albicans* strains isolated from blood samples and compared Ketoconazole, amphotericin B, and fluconazole susceptibility of *Candida* species with slime production and proteinase activity. For both *Candida* species, no correlation was detected between the slime activity and minimum inhibitory concentration (MIC) values of the three antifungal agents and between the proteinase activity and the MIC values of amphotericin B, and fluconazole; however, statistically significant difference was determined between the proteinase activity

and MIC values of ketoconazole.

We studied the ability of *C. albicans* isolates to form biofilm on different surfaces. Two operational steps are involved in the biofilm formation: adhesion and biofilm growth and maturation (Baillie and Douglas, 2000) which are associated with the ability to cause infections and is considered as an important virulence factor during candidiasis. Our *Candida* strains were able to adhere to polystyrene microtiter plates. We noted that adhesion ability differed from strain to strain. Statistical analysis showed that there is no correlation between the slime producing ability tested with safranin stain and the adhesion power developed on polystyrene material ( $P > 0.05$ ). In fact, some weak slime producer strains were adhesive to polystyrene microtiter plates ( $OD_{492} = 0.278$ ) after determination of metabolic reduction of XTT.

Acrylic dentures play an important role by increasing the risk of *Candida* colonization, by acting as reservoirs of micro-organisms (Edgerton et al., 1993). In our work, only the polymethylmethacrylate was tested because it is the material most commonly used for the fabrication of dental prostheses. Previous studies demonstrated that the metabolic activity of *C. albicans* grow as a biofilm increased with time and XTT reduction assay (Chandra et al., 2001). Our results demonstrated that metabolic activity of *C. albicans* biofilm formed on polymethylmethacrylate did not differ between tested strains. Also, strong slime producing strains showed high ability to form biofilm on PMMA because the production of a slime layer (biofilm) has been associated with the ability of some organisms to adhere to the surfaces (Franson et al., 1984).

The process of candidal adhesion to acrylic resins is complex. Previous studies have shown that a number of factors including poor oral and denture hygiene, low pH under dentures, intake of dietary carbohydrates, *Candida* cell surface mannoprotein, and surface free energy of the resin strips modify the adhesion and colonisation of *Candida* (He et al., 2006). Biswas and Chaffin (2005) reported that anaerobically grown *C. albicans* SC 5314 was unable to form a true biofilm on three different substrata namely, polymethylmethacrylate strips, glass cover slips, and polystyrene tissue culture plate. The penetration of yeasts at the acrylic-liner junction point may contribute to deterioration of the prosthesis lining and function (Bulad et al., 2004).



In this study, overall tested *C. albicans* isolates were able to adhere to the two epithelial cell lines with different degree. These strains were more adhesive to Caco-2 cells (83.3%) than Hep-2 cells (61.1%). These data are in accordance with the results shown by Holmes et al. (2002) who studied the effect of saliva on the adherence of *C. albicans* to human epithelial cells. These researchers reported that *C. albicans* adhered to monolayers of all three epithelial cell lines (A549, Hep-2, and HET-1A). In fact, adhesion of *C. albicans* isolates to epithelial cells is an initial event in the development of candidiasis and pathogenesis (Cannon and Chaffin, 1999; Willis et al., 2000; Ueta et al., 2000). *Candida* strains adhere to several cell types, such as oral epithelial cells (Willis et al., 2000) and cultured epithelial cell lines (Ueta et al., 2000). Of the 16 slime positive strains, 62.5% were found to be adherent to Hep-2 cells and 87.5% were adherent to Caco-2 monolayers. All *C. albicans* slime positive strains were biofilm producers on both polystyrene and PMMA surfaces (Table 2).

## Conclusion

In this work, the majority of oral *C. albicans* isolates were able to adhere to epithelial cell lines and biomaterials with different degree. The Congo red method is not an appropriate method to discriminate between slime positive and slime negative *C. albicans* strains. Adhesive properties of *C. albicans* strains allow this yeast to survive in oral cavity, to colonize and damage tissues and cells.

## ACKNOWLEDGMENTS

This work was supported by the Spanish Ministry of Science and Technology (BFU2006-08684) and University of Valencia (UV-AE-10-24011). We gratefully acknowledge Prof. Hajer Hentati (Service d'Odontologie, Clinique Dentaire de Monastir, Tunisie) for her help in the collection of the oral *C. albicans* strains. We also thank Professor Stefania Zanetti (University of Sassari, Italy) for providing the ATCC strains of *C. albicans*.

## REFERENCES

- Avon SL, Goulet JP, Deslauriers N (2007). Removable acrylic resin disk as a sampling system for the study of denture biofilms *in vivo*. *J. Prosthet. Dent.* 97: 32-38.
- Baillie GS, Douglas LJ (2000). Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. *J. Antimicrob. Chemother.* 46: 397-403.
- Baquero C, Montero M, Sentandreu R, Valentin E (2002). Identification of *Candida albicans* by polymerase chain reaction amplification of a *CaYST1* gene intron fragment. *Rev. Iberoam. Micol.* 19: 80-83.
- Bektic J, Lell CP, Fuchs A, Stoiber H, Speth C, Lass-Flörl C (2001). HIV protease inhibitors attenuate adherence of *Candida albicans* to epithelial cells *in vitro*. *FEMS Immunol. Med. Microbiol.* 31: 65-71.
- Biswas SK, Chaffin WL (2005). Anaerobic growth of *Candida albicans* does not support biofilm formation under similar conditions used for aerobic biofilm. *Curr. Microbiol.* 51: 100-104.
- Budtz-Jorgensen E (1990). *Candida*-associated denture stomatitis and angular cheilitis. In: Oral candidosis. Samaranyake LP, MacFarlane TW, editors. London: Wright; pp 156-183.
- Budtz-Jorgensen E, Stenderup A, Grabowski M (1975). An epidemiologic study of yeasts in elderly denture wearers. *Community Dent. Oral. Epidemiol.* 3: 115-119.
- Bulad K, Taylor RL, Verran J, McCord JF (2004). Colonization and penetration of denture soft lining materials by *C. albicans*. *Dent Mat.* 20: 167-175.
- Busscher HJ, Cowan MM, van der Mei HC (1992). On the relative importance of specific and non-specific approaches to oral microbial adhesion. *FEMS Microbiol. Rev.* 8: 199-209.
- Calderone RA, Fonzi WA (2001). Virulence factors of *Candida albicans*. *Trends Microbiol.* 9: 327-350.
- Cannon RD, Chaffin WL (1999). Oral colonization by *Candida albicans*. *Crit. Rev. Oral. Biol. Med.* 10: 359-383.
- Cardenes-Perera CD, Torres-Lanaab A, Alonso-Vargasc R, Moragues-Tosantasc MD, Ponton-San Emeterioc J, Quindos-Andres G, Arevalo-Moralesa MP (2004). Evaluation of API ID 32C® and VITEK-2® to identify *Candida dubliniensis*. *Diag. Microbiol. Infect. Dis.* 50: 219-221.
- Chandra J, Mukherjee, PK, Leidich SD, Faddoul FF, Hoyer LL, Douglas LJ, Ghannoum MA (2001). Antifungal resistance of candidal biofilms formed on denture acrylic *in vitro*. *J. Dent. Res.* 80: 903-908.
- Christensen GD, Simpson WA, Bisno AL, Beachey EH (1982). Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. *Infect. Immun.* 37: 318-326.
- Cotter G, Kavanagh K (2000). Adherence mechanisms of *Candida albicans*. *Br. J. Biomed. Sci.* 57: 241-249.
- Davenport DS, Massanari RM, Pfaller MA, Bale MJ, Streed SA, Hierholzer WJ (1986). Usefulness of a test for slime production as a marker for clinically significant infections with coagulase-negative staphylococci. *J. Infect. Dis.* 153: 332-339.
- Edgerton M, Scannapieco FA, Reddy MS, Levine MJ (1993). Human submandibular-sublingual saliva promotes adhesion of *Candida albicans* to polymethylmethacrylate. *Infect. Immun.* 61: 2644-2652.
- Franson TR, Sheth NK, Rose HD, Sohnle PG (1984). Scanning electron microscopy of bacteria adherent to intravascular catheters. *J. Clin. Microbiol.* 20: 500-505.
- Goldmann DA, Pier GB (1993). Pathogenesis of infections related to intravascular catheterization. *Clin. Microbiol. Rev.* 6: 176-192.
- Gristina AG, Giridhar G, Gabriel BL (1993). Cell biology and molecular mechanisms in artificial device infections. *Int. J. Artif. Organs.* 16: 755-763.
- Hawser SP, Douglas LJ (1994). Biofilm formation by *Candida* species on the surface of catheter materials *in vitro*. *Infect. Immun.* 62: 915-921.
- Hazen KC (1989). Participation of yeast cell surface hydrophobicity in adherence of *Candida albicans* to human epithelial cells. *Infect. Immun.* 57: 1894-1900.
- He XY, Meurman JH, Kari K, Rautemaa R, Samaranyake LP (2006). *In vitro* adhesion of *Candida* species to denture base materials. *Mycoses.* 49: 80-84.
- Holmes AR, Bandara BMK, Cannon RD (2002). Saliva promotes *Candida albicans* adherence to human epithelial cells. *J. Dent. Res.* 81(1): 28-32.
- Jose LLR, Manuel C, Jose PM, Rafael S (1991). Characterization of Cell Wall Proteins of Yeast and Hydrophobic Mycelial Cells of *Candida albicans*. *Infect Immun.* 59 (7): 2324-2332.
- Kalkanci A, Yalynay ÇM, Mansuroglu H, Kustimur S (1999). *Candida* türlerinde slaym faktör belirlenmesi. *Türk. Mikrobiyol. Cem. Derg.* 29: 183-185.
- Kuhn DM, Chandra J, Mukherjee PK, Ghannoum MA (2002). Comparison of biofilms formed by *Candida albicans* and *Candida parapsilosis* on bioprosthetic surfaces. *Infect Immun.* 70: 878-888.
- Li X, Yan Z, Xu J (2003). Quantitative variation of biofilms among strains in natural populations of *Candida albicans*. *Microbiology.* 149: 353-362.
- Mary Ann JR, William AF, William GM, Abdullahel AMAB, Jacqueline IK, Timothy FM (2001). Cell surface hydrophobicity-associated adherence of *Candida dubliniensis* to human buccal epithelial cells.



- Rev. Iberoam Micol. 18: 17-22, 17.
- Moura JS, Silva WJ, Pereira T, Del Bel Cury AA, Rodrigues Garcia RC (2006). Influence of acrylic resin polymerization methods and saliva on the adherence of four *Candida* species. J. Prosthet. Dent. 96: 205-211.
- Nikawa H, Hamada T, Yamamoto T (1998). Denture plaque-past and recent concerns. J. Dent. 26:299-304.
- Noumi E, Snoussi M, Saghrouni F, Ben Said M, Del Castillo L, Valentin E, Bakhrouf A (2009). Molecular typing of clinical *Candida* strains using random amplified polymorphic DNA and contour-clamped homogenous electric fields electrophoresis. J. Appl. Microbiol. 107: 1991-2000.
- Ozkan S, Ayse-Kalkanci FK, Semra Kustimur UA (2005). Slime production and proteinase activity of *Candida* species isolated from blood samples and the comparison of these activities with minimum inhibitory concentration values of antifungal agents. Mem. Inst. Oswaldo. Cruz. 100 (3): 319-324.
- Pereira-Cenci T, Cury AA, Cenci MS, Rodrigues-Garcia RC (2007). *In vitro* *Candida* colonization on acrylic resins and denture liners: influence of surface free energy, roughness, saliva, and adhering bacteria. Int. J. Prosthodont. 20: 308-310.
- Ramage G, Stephen Saville P, Derek PT, Lopez- Ribot JL (2005). *Candida* biofilms; an update. Eukaryotic Cel. 4: 633-638.
- Ramage G, Tomsett K, Wickes BL, Lopez-Ribot JL, Redding SW (2004). Denture stomatitis: a role for *Candida* biofilms. Oral. Surg. Oral. Med. Oral. Pathol. Oral. Radiol. Endod. 98: 53-59.
- Ramage G, Walle KV, Wickes BL, Lopez-Ribot JL (2001 b). Standardized method for *in vitro* antifungal susceptibility testing of *Candida albicans* biofilms. Antimicrob. Agents. Chemother. 45: 2475-2479.
- Ramage G, Walle KV, Wickes BL, Lopez-Ribot JL (2001a). Biofilm formation by *Candida dubiliensis*. J. Clin. Microbiol. 39: 3234-3240.
- Rosenberg M, Judes H, Weiss E (1983). Cell Surface Hydrophobicity of Dental Plaque Microorganisms. Infect Immun. 42 (2): 831-834.
- Samaranayake LP, MacFarlane TW (1980). An *in vitro* study of the adherence of *Candida albicans* to acrylic surfaces. Arch Oral Biol. 25: 603-606.
- Ueta E, Tanida T, Doi S, Osaki T (2000). Regulation of *Candida albicans* growth and adhesion by saliva. J. Lab. Clin. Med. 136 (1): 66-73.
- Verheyen CC, Dhert WJ, De Bleeck-Hogervorst JM, van der Reijden TJ, Petit PL, de Groot K (1993). Adherence to a metal, polymer and composite by *Staphylococcus aureus* and *Staphylococcus epidermidis*. Biomaterials. 14: 383-391.
- Vinitha M, Ballal M (2007). Biofilm as virulence marker in *Candida* isolated from blood. World J. Med. Sci. 2(1): 46-48.
- Waltimo T, Tanner J, Vallittu P, Haapasalo M (1999). Adherence of *Candida albicans* to the surface of polymethylmethacrylate- E glass fiber composite used in dentures. Int. J. Prosthodont. 12: 83-86.
- Webb BC, Thomas CJ, Willcox MD, Harty DW, Knox KW (1998). *Candida*-associated denture stomatitis. Aetiology and management: a review. Part 2. Oral diseases caused by *Candida* species. Aust. Dent. J. 43: 160
- Willis AM, Coulter WA, Hayes JR, Bell P, Lamey PJ (2000). Factors affecting the adhesion of *Candida albicans* to epithelial cells of insulin-using diabetes mellitus patients. J. Med. Microbiol. 49: 291-293.
- Yücesoy M, Karaman M (2003). *Candida* türlerinin biyofilm üretimi ve antifungal duyarlılık paternleri.. Ulusal Mantar Hastalıkları ve Klinik Mikoloji Kongresi. 24:360.