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Biofilm formation and adhesive/invasive properties of *Candida dubliniensis* in comparison with *Candida albicans*

Research Article

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Abstract: *Candida dubliniensis* and *Candida albicans* are closely related spp. exhibiting differences in their virulence potency. This study compared clinical isolates of *C. dubliniensis* with *C. albicans* from HIV patients with oropharyngeal candidiasis (OPC) and standard strains in power to form biofilm and their adhesive and invasive properties. Members of both spp. were able to form strong biofilms. However, SEM microscopy confirmed that *C. albicans* undergoes the more effective yeast-to-hyphae transition than *C. dubliniensis* with prevalent yeast form and limited ability to form filaments. Kinetic patterns indicated that while the first 30 min are critical for sufficient attachment to a polystyrene surface, adhesion to human carcinoma cell lines (Caco-2 and TR 146) needs additional time with maximal saturation observed at 240 min for both spp. The invasion process was tested on 3D RHE (reconstituted human epithelium) with Caco-2 or TR 146 on the collagen surface. *C. albicans* rapidly produced hyphae that penetrated the tissue layer, demonstrating substantive invasion within 21 h. In contrast, *C. dubliniensis* attached to the tissue surface and proliferated, suggesting the formation of a biofilm-like structure. After 21 h, *C. dubliniensis* was able to penetrate the RHE layer and invade unusually, with a cluster of the yeast cells.

Keywords: *Candida dubliniensis* • *Candida albicans* • *Adhesive and invasive properties* • *Biofilm* • *RHE*

1. Introduction

C. albicans is a commensal microorganism found in the oral cavity and small intestine flora. It is also one of the most common opportunistic pathogens known to be recovered from almost any part of the human body, while closely-related *C. dubliniensis* has a more limited array of colonization sites [1-3]. However, both spp. are frequent sources of oropharyngeal candidiasis (OPC) in HIV-infected individuals [4]. Some authors have already reported that *C. dubliniensis* and *C. albicans* possess a similar spectrum of putative virulence factors, including germ tube and hyphae formation [5], chlamydospore production [6], an ability to attach to many substrates, and to form biofilms [7-9]. It is of interest that while *C. albicans* prefers the formation of true hyphae during prolonged tissue colonization and penetration, *C. dubliniensis* occurs in the yeast or reduced pseudohyphal form rather than mycelial form [10,11]. Adhesion to the epithelium and the ability to invade the gingival conjunctive tissue [12] as well as subgingival biofilm formation [13] seem to have a direct impact on oral *C. dubliniensis* and *C. albicans* infections. Some authors proved that those strains of both spp. with a decreased ability to form hyphae exhibited poor adherence and reduced tissue penetration [10,14,15]. Generally, *C. dubliniensis* is considered to be less pathogenic than *C. albicans* [10,11]. On the other hand, *C. dubliniensis* is able to replace *C. albicans* during fluconazole treatment [16] and rapidly develops resistance to this drug [17]. This observation, as well as the frequent occurrence of *C. dubliniensis* in HIV-positive patients, underlies the interest in this *Candida* sp., although its incidence in clinical samples is relatively low and this species has not occupied a “headline” position in epidemiological statistics. This study was focused on an investigation of

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the differences between *C. dubliniensis* and *C. albicans* clinical isolates recovered from HIV-infected patients with OPC in biofilm formation, and adhesive, and invasive properties. Additionally, the next focus was on the ability of *C. dubliniensis* to invade the reconstituted human epithelium (RHE) in comparison with *C. albicans* and whether the yeast-to-hyphae transition is necessary for this process.

2. Experimental Procedures

2.1 Strain identification

For this study, clinical isolates of *C. dubliniensis* and *C. albicans* recovered from HIV positive patients with OPC were tested: *C. dubliniensis* 539 isolated at The Dublin Dental School and Hospital, kindly provided by Dr. D. Sullivan from Trinity College, Dublin, Ireland [18] and *C. albicans* 64-1 isolated at The Clinic of Infectology and Geographic Medicine, Bratislava, Slovakia; kindly provided by Dr. M. Mokráš. The isolates were selected because of their strong biofilm formation (A. Kolecka, personal communications). Effect of pH, glucose concentration and fluconazole on cell surface hydrophobicity in *Candida albicans* and *Candida dubliniensis* isolated from HIV patients with oral candidiasis. *C. dubliniensis* CBS 7987 (CBS Fungal Biodiversity Centre, Utrecht, Netherlands) and *C. albicans* SC 5314 [19] were used as standard strains. All *Candida* strains were reidentified by their growth on CHROMagar Candida (Becton Dickinson) at 37°C for 24-48 h as well as distinguished by Polymerase Chain Reaction using primers (Ca-INT-L and Ca-INT-R) designed for the group I intron in the 25S rRNA, as described previously [20,21]. Strains were grown on YPD plates (2% yeast extract, 2% mycological peptone, 2% glucose, 2% agar; Biomark Laboratories) at 30°C for 24-48 h. Isolates were kept in glycerol/YPD at -80°C until used.

2.2 *In vitro* biofilm formation – XTT reduction assay

The inoculums for biofilm formation were prepared for each of the tested strains and cultivated on YPD agar plates (Biomark Laboratories) at 30°C for 24 h. From each strain, a loop of cells was transferred into YNB medium with amino acids (YNB broth, Difco). The medium was supplemented with *D*-glucose (Applichem) to a concentration of 0.9% (w/v). After overnight cultivation at 37°C, cells were centrifuged (4500x*g*, 5 min) and washed twice with 0.5 ml of PBS (136.7 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.2). Harvested cells were resuspended in 1.5 ml of fresh YNB medium (supplemented as indicated above). The final cell suspension was adjusted to 1.0 (OD_{600}).

Polystyrene 96-well microplates (flat bottom; Sarstedt) were used for the static model of biofilm formation according to the standard protocol [22]. From each strain, 100 µl of the cell suspension with OD_{600} 1.0 was transferred into wells and incubated for 90 min to allow the cells to attach to the polystyrene surface. Afterwards, medium containing non-adherent cells was removed and the wells were gently washed twice with 150 µl of sterile PBS. For mature biofilm development, 100 µl of fresh YNB medium was added to each well; the plates were covered and incubated at 37°C. After 48 h, medium containing dispersal cells was discarded and the wells were washed three times with 200 µl of sterile PBS. Biofilm formation was estimated as the viability of the cells forming the biofilm by quantifying their mitochondrial dehydrogenase activity by their reduction of XTT ([2,3-bis(2-methoxy-4-nitro-5-sulphenyl)-2H-tetrazolium-5-carboxanilide] sodium salt (Sigma-Aldrich) to formazan [22]. The final colorimetric reaction was determined at 490 nm in a microplate reader (MRX Microtiter plate absorbance reader, Dynex Technologies) after a 3 h incubation at 37°C in the dark. Three independent experiments were performed with each strain cultivated in three parallel wells. YNB medium containing no inoculum (no biofilm) was used as the negative control. Results were expressed as an average ± standard deviation (SD).

2.3 *In vitro* biofilm formation – Scanning Electron Microscopy (SEM)

An SEM technique was applied to analyze the architecture and morphology of the biofilm formed on sterile 13-mm-diameter Thermanox Plastic Cover slips (Nunc). Briefly, coverslips were placed in polystyrene 24-well plates (Sarstedt) and inoculated with 300 µl of cell suspension (OD_{600} 1.0) in YNB medium containing 0.9% *D*-glucose (as described previously for the XTT assay) and incubated at 37°C for 90 min. Later, medium with non-adherent cells was removed and the cells attached to the coverslips were washed twice with 500 µl of sterile 1xPBS. The coverslip in each well was then overlaid with 300 µl of fresh YNB medium and incubated at 37°C. After 48 h, the biofilms were gently washed with 250 µl of sterile distilled water three times, transferred to sterile Petri dishes (Sarstedt), coated with a drop of distilled water and frozen at -80°C. Next, the biofilms were dried by overnight lyophilization and an SEM examination was performed using a LEO 1530 VP microscope (LEO Electron Microscopy Ltd.).

2.4 Preparation of the monolayer with Caco-2 and TR 146 cell lines

For the adhesion assay and reconstituted human intestinal and oral epithelia models, a confluent layer of the two carcinoma cell lines was employed: a monolayer of the colorectal epithelial cell line Caco-2 (ATCC HTB-37) and the stratified epithelium of neck-node metastasis of the human buccal carcinoma cell line TR 146 (kindly provided by Imperial Cancer Research Technology, London, UK to IGB Fraunhofer Institute). Cell lines were grown in 75 cm^2 tissue culture flasks (Greiner Bio-One) in D-MEM (Dulbecco's modified Eagle's Medium, Gibco) containing 4.5 g/l of glucose supplemented with 10% heat inactivated fetal bovine serum (w/v, FBS, Gibco) and 1 mM sodium pyruvate, and 1% gentamicin (Sigma-Aldrich). Cells were cultivated at 37°C under 5% CO_2 and at saturated humidity up to 80% confluence. Cell cultures were then split 1:5 using standard methods [23,24] and distributed into the 24-well plates for the adhesion assay, as well as plating onto collagen-coated surfaces for the adhesion assay and invasion experiment, as described below.

2.5 Adhesion assay and adhesion kinetics

A quantitative adhesion assay and an investigation of adhesion kinetics was performed in polystyrene 24-well plates (ThinCert-24well-Multiwell-Platte, Greiner Bio-one) with 5 selected time points (0, 30, 60, 120, 240 min; time point 0 min was used as the background control), according to the published protocol of Sohn *et al.* [23]. For this experiment, a polystyrene plastic surface and RHE were used as described by Dieterich *et al*. [14] with the minor modifications reported by Sohn & Rupp [25].

After every time point, 24 well plates were initially incubated for 2 min in a flat horizontal shaker (200 rpm) and then 300 µl of medium containing non-adherent cells were placed on YPD agar plates. The attached cells were washed with 300 µl of CMF-PBS (calcium- and magnesium-free phosphate buffered solution; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 1 mM EDTA, pH 7.2) and then after the addition of 300 µl of fresh CMF-PBS, cells were scraped, plated on YPD agar plates, and incubated at 30°C for 24 h. Attached and non-attached cells were counted as the colony-forming units (CFU). The percentage of adherent cells was calculated according to the formula: [(adherent cells)/(adherent cells + non-adherent cells)]x100 for each time point. Two independent experiments, with two parallels were performed for each strain. Mean was finally calculated from 4 samples.

2.6 Invasion assay

The invasion assay was performed on an *ex vivo* model designed as a three dimensional model employing reconstituted human epithelium as described previously by Hernandez & Rupp [24]. Firstly, polystyrene inserts (ThinCertTM, Greiner Bio-One) with a bottom of microporous polyethylene terephthalate membrane (pore diameter 0.4 µm) were placed in polystyrene 24-well plates (ThinCertTM-24well-Multiwell-Platte, Greiner Bio-One). Collagen gel matrix, as the supporting interface between the membrane and cell line, was prepared from rat-tail collagen solution mixed with gelification solution (Fraunhofer Institute in Stuttgart, Germany) transferred into each insert and incubated in the 24-well plates for 10 min at 37°C for gelling.

For the *ex vivo* invasion model, the yeast cell suspensions with OD_{600} 1.0 (YPD medium, 28°C, with shaking) were prepared and 15 µl of each strain suspension was used to inoculate each insert with the cell line followed by incubation at 37°C with 5% CO_2 and saturated humidity for 7 h or 21 h. The progress of *Candida* invasive growth was monitored by histological examination. For this reason, inserts were first fixed in Bouin's solution (Sigma-Aldrich). After 50 min, the filters with the sample were removed from the insert and placed between filter papers in the tissue-embedding cassette. Afterwards, the cassettes were placed in the container and washed with tap water for at least double the time used for fixation. Cassettes were then transferred to a tissue processor for embedding in paraffin with the 12 reagent containers of the Shandon Citadel 1000 Tissue Processor containing the following: Water I/ Water II/ 70% Ethanol/ 90% Ethanol/ 96% Ethanol/ Isopropanol I/ Isopropanol II/Xylol:Isopropanol (1:1)/ Xylol I/ Xylol II/ Paraffin I/ Paraffin II. Paraffin blocks were prepared manually; the tissue sample was placed into the embedding mold and covered with melted paraffin to form a block. Once cool, the blocks were used for sectioning. The paraffin blocks with embedded infected tissue were then cut with a rotary microtome (Leica RM2145). Slices of 5 µm thick sections were mounted on glass slides and stained. Histological staining was performed successively with the PAS staining protocol (chemicals as Periodic Acid solution and Schiff reagent, Fluka) that enables all components of the model system to be distinguished [14]. Final confirmation of the development of the invasion process was documented with images taken with the Olympus BX 60 microscope (Olympus).

2.7 Data analyses

The final data was estimated from three independent experiments with minimal three parallels in every

experiment with biofilm formation and in two independent biological replicas with two parallels for each strain in adhesion/invasion assays. The data was calculated for each strain as means ± SD. Statistical significance in the differences between strains and spp. was determined using the standard Student's *t*-test. Results were considered statistically significant with $P<0.05$.

3. Results

3.1 Biofilm formation

Biofilm formation was evaluated by XTT reduction assay ($A_{490\ nm}$) after 48 h. The biofilm cells of *C. dubliniensis* 539 and *C. albicans* 64-1 clinical isolates formed stronger biofilms (0.600±0.096 and 0.517±0.1463, respectively) than those estimated for the standard strains of *C. dubliniensis* CBS 7987 (0.218±0.105) and *C. albicans* SC 5314 (0.156±0.019). While the differences in activity of biofilm between *Candida* spp. were not significant ($P>0.05$, the detailed structure of the *in vitro* mature biofilm (48 h) evaluated by SEM (Figure 1) proved divergence. The images revealed morphological heterogeneity and the presence of an external polymeric material - extracellular matrix. As indicated in the figure, the majority of the biofilm formed by *C. dubliniensis* was constructed of the yeast cells; but some filaments were also found (Figure 1 C-1, C-2; D-1, D-2). Additionally, the density of *C. dubliniensis* cells attached to the surface seemed to be reduced when compared with the *C. albicans* biofilm, which was composed of a mass of hyphae and only a few yeast cells (Figure 1 A-1, A-2; B-1, B-2).

3.2 Adhesion to abiotic and biotic surfaces and its kinetics

Biofilm formation is a biological process that not only depends on the adhesive properties of the fungal cells and the physical characteristics of the surface, but is also time-dependent. In this context, differences in the kinetics of the adhesion pattern to an abiotic as well as to a biotic surface between both spp. were studied during the first four hours of interaction (30, 60, 120, 240 min) (Figure 2). While the strains of both spp. adhered to the plastic surface to a similar extent within 30 min, after 60 and 120 min of interaction, the *C. dubliniensis* 539 clinical isolate diverged from that pattern with a lower adherence capability ($P<0.001$).

The adhesion assay employing two *ex vivo* models (gastrointestinal Caco-2 cell line and buccal TR146 cell line) showed that both spp. proceeded rapidly within the first 30 min (Figure 2), just as in the experiment on the plastic surface. However, the general number of adherent cells was much lower. The *C. dubliniensis* 539 clinical isolate confirmed discrepancy in attachment at all time points tested using both cell lines, the percentage of viable attached fungal cells was significantly lower ($P<0.001$). With Caco-2 the *C. dubliniensis* cells saturated the surface within 120 min, but the overall number of recovered adherent cells decreased by 240 min, while both *C. albicans* strains had reached their maximum surface saturation level by this time. With TR 146 cell line, the strains of both *Candida* spp. maximally adhered within 120 min, while the number of adhered cells decreased by 240 min.

3.3 Invasion of RHE with Caco-2 and TR 146 cell lines

To assess the invasion ability of both *Candida* spp., two three-dimensional *ex vivo* models were used, consisting of the confluent Caco-2 or TR 146 cell layer seeded on a collagen matrix (Figure 3). After 7 h of interaction, both *C. albicans* spp. were able to produce hyphae rapidly and penetrate the Caco-2 layer. In addition, these hyphae were found in the collagen matrix

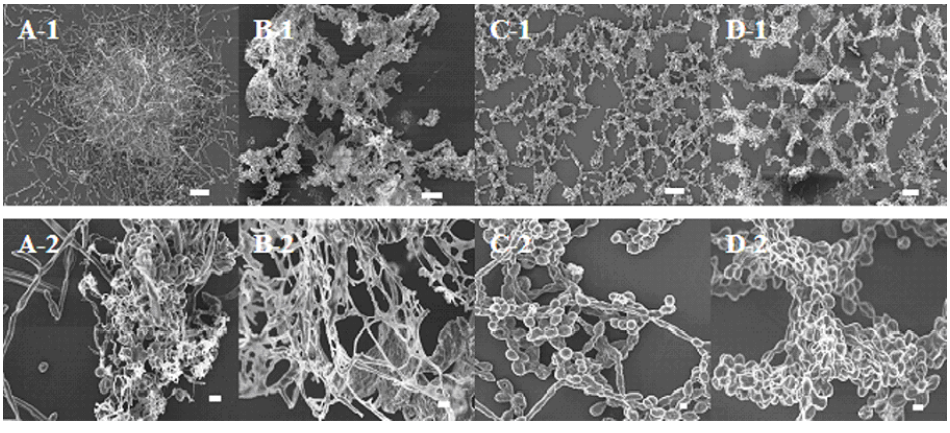

Figure 1. Scanning electron microscopy of *in vitro* developed biofilms after 48 h at 37°C. The images showed the detailed structure of the mature biofilm formed by *C. albicans* SC 5314 (A-1, A-2) *C. albicans* 64-1 (B-1, B-2), *C. dubliniensis* CBS 7987 (C-1, C-2), and *C. dubliniensis* 539 (D-1, D-2). The dimension bar in the upper row represents 20 μm and in the lower row represents 2 μm.

(Figure 3 A-1, B-1). Both strains exhibited a very destructive and powerful invasion within 21 h of inoculation (Figure 3 A-2, B-2). The hyphal form was dominant and seemed to elongate along the tissue layer, initiating serious damage of the surface and entering deeply into the collagen layer. In contrast to the aggressive *C. albicans* invasion, both tested *C. dubliniensis* strains adhered, but did not form filaments. After inoculation for 7 h both *C. dubliniensis* strains only colonized the upper part of the Caco-2 monolayer, proceeding in clusters of the yeast form (Figure 3 C-1, D-1). Occasionally, some elongated hyphae of *C. dubliniensis* CBS 7987 were able to enter the tissue surface and penetrate, as they were detected in the collagen layer (Figure 3 C-1). Microscopic examination of histological sections at 21 h after inoculation indicated that both *C. dubliniensis* exhibited proficiency in tissue penetration, but mainly in the yeast form (Figure 3 C-2, D-2). The yeasts of the *C. dubliniensis* 539 clinical isolate penetrating through the Caco-2 cell line remained, surprisingly, between Caco-2 and the collagen surface (Figure 3 D-2).

As the clinical isolates of *C. dubliniensis* and *C. albicans* caused OPC, their ability to penetrate through stratified epithelium was tested on the oral model employing a TR146 cell line. The same time points were used as for the intestinal model with the Caco-2 cell line. After inoculation for 7 h, no tested strains exhibited any significant penetration through the layers of TR146 (Figure 3 A-3, B-3, D-3) with the exception of the *C. dubliniensis* CBS 7987 standard strain (Figure 3 C-3). After 21 h, *C. albicans* strains again demonstrated their superiority over *C. dubliniensis* strains in their capacity to produce hyphae and to invade epithelial cells and collagen layers (Figure 3 A-4, B-4). The invasion of

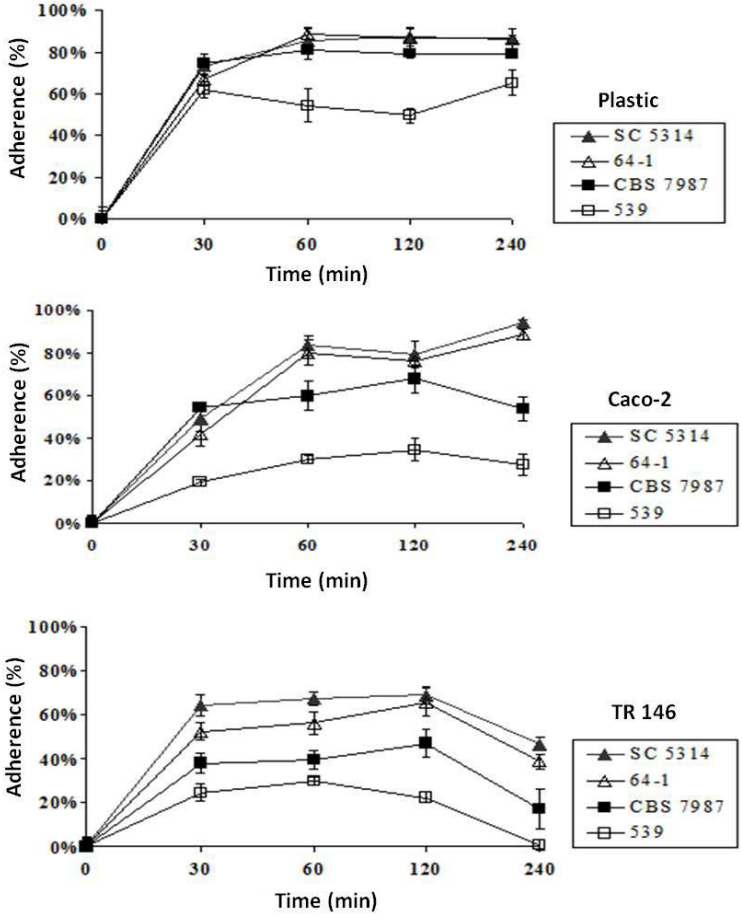

Figure 2. Quantitative assay determining the adhesion efficiency and kinetic profiles of *C. albicans* SC 5314 (black triangle), *C. albicans* 64-1 (white triangle), *C. dubliniensis* CBS 7987 (black square), *C. dubliniensis* 539 (white square). Various surfaces were used: the plastic surface represented by polystyrene, the colorectal cell line Caco-2 and the oral epithelial surface represented by the TR 146 cell line. All strains were incubated with D-MEM +10% FBS at 37°C with 5% of CO_2 and saturated humidity. Error bars indicate the SD for the average of two independent experiments prepared in duplicate. The maximum error was 8% and minimum error was 1% for the plastic model; the maximum error was 7% and minimum error was 1% for the Caco-2 model; the maximum error was 9% and minimum error was 2% for the TR 146 cell line.

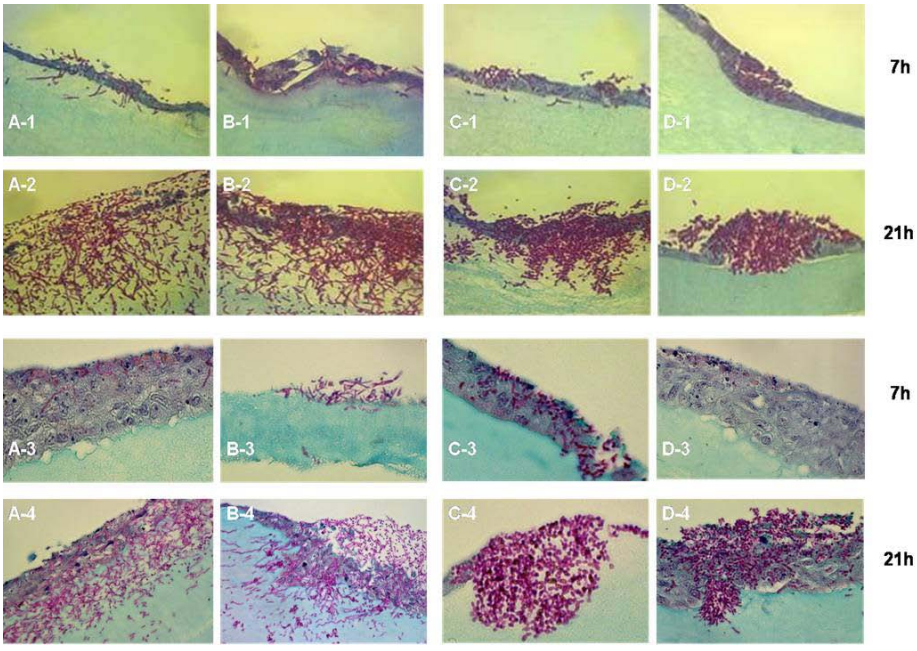

Figure 3. Invasion profile of *C. albicans* and *C. dubliniensis* strains were characterized on representative slices of reconstituted human epithelium after histological staining. The first model employed the interaction of *Candida* spp. with the Caco-2 cell line: *C. albicans* SC 5314 (A-1, A-2), *C. albicans* 64-1 (B-1, B-2), *C. dubliniensis* CBS 7987 (C-1, C-2), *C. dubliniensis* 539 (D-1, D-2) after 7 h and after 21 h of interaction with the Caco-2 cell line. The second model utilised interaction with the TR 146 cell line: *C. albicans* SC 5314 (A-3, A-4), *C. albicans* 64-1 (B-3, B-4), *C. dubliniensis* CBS 7987 (C-3, C-4), *C. dubliniensis* 539 (D-3, D-4) after 7 h and 21 h of interaction.

C. dubliniensis observed with the TR 146 RHE model was very similar to that with Caco-2 (Figure 3 C-4, D-4). It is of interest that *C. dubliniensis* strains were not only able to penetrate the upper stratum of the TR 146 tissue layer with a higher amount of the yeast form, but also invade the collagen matrix with clusters of the yeast cells.

4. Discussion

C. dubliniensis is a frequent pathogen causing OPC in HIV-positive individuals. It has already been described as closely related to *C. albicans* in phenotype and genotype [10,26]. However, comparisons of both spp. in genomes confirmed some principal differences described in the putative ortholog genes from the *SAP* and *ALS* families, known to play a significant role in adhesion [11,27]. Additionally, both genomes vary in their telomere associated genes (*TLO*, family of putative transcription factors) as well as in their leucine rich repeat protein genes (*IFA*, family of putative transmembrane proteins) that may explain the diminished pathogenicity of *C. dubliniensis*.

In this work, we compared the biofilm formation, adhesion and invasion characteristics of selected *C. dubliniensis* and *C. albicans* strains, employing *in vitro* and *ex vivo* models. An *in vitro* statically formed biofilm was evaluated *via* XTT reduction assay, which enables the study of the intact biofilms without their disruption [28]. This method describes the differences in colorimetric signals that reflect the metabolic activity of the biofilm cells, but say nothing about the actual structure of the biofilm. Therefore, a combination with microscopy is necessary. As expected, all tested strains were able to form a biofilm, but this ability proved to be strain-dependent for both *C. albicans* as ell as *C. dubliniensis*, as was previously described by Borecká-Melkusová *et al.* [29]. It is of interest that while *C. dubliniensis* was able to form as vital biofilm as *C. albicans* on a polystyrene surface, SEM microscopy revealed a morphological diversity between both spp. of tested strains with mainly the yeast form observed in *C. dubliniensis*, in contrast to *C. albicans*, where the mycelial form was more prevalent. Adhesion to unrelated substrates has already been noted as an important step not only in biofilm formation [30-32], but can also be essential for colonization [3]. However, the degree of colonization of any surface is not only dependent on the adhesive properties of individual strains, but the duration and kinetics of adhesion can also be important. The context of the interactions between *Candida* cells and their adhesion to various surfaces has recently been investigated [32-35]. The results of our study do not indicate a significant difference in adhesion to a polystyrene surface between the tested clinical and

nonclinical strains, with the exception of *C. dubliniensis* 539. Taking into consideration the results summarizing the degree of attached cells and kinetic pattern of adherence to polystyrene, the first 30 min of interaction seems to be equally critical for the cell attachment of tested strains of both spp. The observation that the first 30 min are critical for adhesion confirmed results previously described by Bujdakova *et al.* [32] in biofilm model with the *C. albicans* standard strain SC 5314 and other clinical isolate than tested in this study.

It has already been mentioned that both *C. dubliniensis* and *C. albicans* are human commensal yeasts [15]. Therefore, an *ex vivo* adhesion was tested on two cell lines; on Caco-2, since *C. albicans* is the general commensal of the small intestine; and on TR 146, because the tested clinical isolates were recovered from patients with OPC. Considering the kinetic pattern of adherence on the Caco-2 cell line, differences in cell attachment were observed between the tested spp. *C. dubliniensis* strains adhered poorly compared to *C. albicans* strains using both cell lines. All tested strains achieved sufficient attachment and surface saturation within 60 or at most within 120 min, thus the 2-h adhesion phase could be considered critical for stabilization of interaction between *Candida* and the animate surface formed by both cell lines. However, the tested strains adhered to the oral epithelial line of TR 146 cells with a much lower intensity. Moreover, *C. dubliniensis* strains exhibited a limited adhesion of only up to 20-40%. We hypothesize that the generally observed limitation in the yeast-to-hyphae transition observed in *C. dubliniensis* [10] could contribute to a reduction in their interaction with animate surfaces, since when *C. albicans* blastospores make contact with epithelial cells, they rapidly switch to the hyphal mode [15].

After adhesion, invasion is the next step in the progress of infection. The 3D model combined with a series of histological and microscopic approaches was used to investigate the migration, invasion and RHE destruction by the tested strains. Regardless of the cell line used, no significant RHE damage was observed in the early phase of interaction (after 7 h). However, after 21 h, *C. albicans* strains began to produce many filaments that damaged the Caco-2 layer. On the other hand, hyphae only penetrating the top layer of TR 146 were unable to invade deeply. It has already been mentioned that in terms of the invasion process, only filamentous forms have been assumed to penetrate tissue cells layers (epithelial or endothelial) and disseminate using thigmotropism or physical forces as the dominant mode of invasion [15,36]. Contrary to previous implication, we found that *C. dubliniensis* invaded the collagen matrix in both RHE models with the yeast cells organized in clusters.

In summary, the kinetics of adhesion confirmed a similar time limitation for the surface saturation of biotic as well as abiotic surfaces by colonizing yeast cells of the tested spp. However, there is the implication that switching from the the yeast to the hyphal form being one of the major virulence factors supporting adhesive properties during biofilm formation and invasiveness is not an absolute rule. We have presented evidence that the adhered yeast form of *C. dubliniensis* can also take part in penetrating RHE, leading to its damage.

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