J Oral Pathol Med (2006) 35: 484–91

www.blackwellmunksgaard.com/jopm

Quantitative evaluation of tissue invasion by wild type, hyphal and *SAP* mutants of *Candida albicans*, and non-*albicans Candida* species in reconstituted human oral epithelium

J. A. M. S. Jayatilake[1,2], Y. H. Samaranayake[2], L. K. Cheung[3], L. P. Samaranayake[2]

[1]*Division of Microbiology, Faculty of Dental Sciences, University of Peradeniya, Peradeniya, Sri Lanka;* [2]*Oral Biosciences, Faculty of Dentistry, University of Hong Kong, Hong Kong;* [3]*Oral and Maxillofacial Surgery, Faculty of Dentistry, The Prince Philip Dental Hospital, Sai Ying Pun, Hong Kong*

BACKGROUND: Oral candidiasis is a common problem in compromised patients. Although several non-*albicans* *Candida* species have emerged as pathogens the majority of candidal infections are caused by *Candida albicans*. Morphogenesis from the blastospore to filamentous phase, and production of secretory aspartyl proteinases (SAP) are two major virulence attributes of these opportunistic yeast. Histopathology of oral candidiasis is characterized by fungal invasion of the superficial epithelium although the invasive potentials of different *Candida* species vary. Computerized image analysis systems (IAS) utilizing immunohistochemistry have been successfully employed for quantification of such histopathological features. The purpose of this study was to evaluate quantitatively the *in vitro* invasive potential of *C. albicans* and its hyphal and *SAP* mutants, and five other non-*albicans* *Candida* species using a computerized IAS.

METHODS: *In vitro* human oral candidiasis was produced using five wild type and one reference *C. albicans* isolates, hyphal and *SAP* mutants of *C. albicans* SC 5314, and one wild type and one reference isolate each of *C. tropicalis*, *C. dubliniensis*, *C. glabrata*, *C. parapsilosis* and *C. krusei* in a reconstituted human oral epithelium (RHOE) model. The infected tissues were examined histologically at 12, 24 and 48 h. Invading fungal elements were visualized by periodic acid-Schiff (PAS) staining and quantitatively evaluated as a percentage of total tissue invasive area, using a computerized IAS.

RESULTS: All *C. albicans* isolates including hyphal mutant *cph1*/*cph1* and *SAP* mutants; *sap 1–3*, *sap 4–6* produced hyphae and differentially ($P < 0.05$) invaded the tissue over 48 h. The invasive potential of hyphal mutant *cph1*/*cph1* and *SAP* mutants (*sap 1–3*, *sap 4–6*) were similar to the parent wild-type isolate at 12 h although after 24 h their invasion was dissimilar ($P < 0.05$). Non-*albicans* *Candida* species and hyphal mutants; *efg1*/*efg1*, *efg1*/*efg1* *cph1*/*cph1* were all non-invasive.

CONCLUSIONS: RHOE model in combination with computerized image analysis permits for the first time, the assessment of invasive potential of *Candida* species in a quantitative manner. The differential tissue invasive patterns of various *C. albicans* isolates, their mutants and other *Candida* species are also described.

J Oral Pathol Med (2006) **35**: 484–91

Keywords: *Candida* species; image analysis; oral candidiasis; reconstituted human oral epithelium

Correspondence: Prof. L. P. Samaranayake, Oral Biosciences, Faculty of Dentistry, The University of Hong Kong, 34 Hospital Road, Hong Kong. Tel: (+852) 28590480. Fax: (+852) 25476133. E-mail: lakshman@hku.hk
Accepted for publication February 16, 2006

Introduction

Many *Candida* species inhabit human mucosal surfaces as commensals. However, they cause opportunistic infections that are increasingly recognized especially in compromised patients with HIV infection, diabetes mellitus, and those receiving steroid, broad-spectrum antibiotics and cytotoxic drug therapy (1). Of about 200 species of *Candida*, *Candida albicans* is the most frequent pathogen (2), although several other species, such as *C. tropicalis*, *C. dubliniensis*, *C. glabrata*, *C. parapsilosis* and *C. krusei* have emerged as important organisms in this context. For instance, in one clinical trial where 224 invasive candidiasis patients participated, it was found that 45% of the infections were caused by *C. albicans*, while the majority 55%, were caused by non-*albicans* *Candida* species (3). In the United States, 47% of nosocomial blood stream infections caused by *Candida* are due to non-*albicans* *Candida* species (4).

Pathogenesis of mucosal candidiasis has been investigated by several workers using animal models (5) and

tissue culture techniques. Biopsy specimens from humans (6, 7) as well as animals (8) and chick chorioallantoic membranes (9–11) have been used for such *in vitro* experimental candidiasis. These studies have been recently extended, and multilayer, tissue culture-based, reconstituted human epithelia appear to fulfill this purpose with very satisfactory results (12–18).

Histopathologically both acute and chronic oral candidal infections are characterized by yeast and hyphal forms invading the superficial parakeratotic epithelium (19). Despite extensive qualitative studies on mucosal candidiasis (20–22), quantitative data on the invasive growth of *Candida* species into the superficial epithelium are lacking. Such data will help better understand the pathogenesis of this relatively common disease as the epithelium is the front line defense barrier against deep or disseminated candidiasis. The invasive behaviour of *C. albicans* is often attributed to its ability to form hyphae and the production of extracellular enzymes particularly the secretory aspartyl proteinase (SAP) and phospholipases. Although several workers have demonstrated the differential hyphal interactions between *C. albicans* and the host in tissue culture as well as in animal experimental models (12, 13, 23) no quantitative data on hyphal invasion of human epithelium are available.

Therefore, in this study we attempted to determine the invasive characteristics of different *Candida* species, including hyphal and *SAP* mutants of *C. albicans*, in a reconstituted human oral epithelium (RHOE) model (12, 14–18). For this purpose we used periodic acid-Schiff (PAS)-stained histological sections of *Candida*-infected RHOE as described previously in quantitative studies of candidal invasion in experimental keratitis in rabbits (23, 24).

Materials and methods

Organisms

The following *Candida* isolates were used in the study.

Candida albicans: Well characterized wild-type SC 5314 and its hyphal and *SAP* mutants (HLC52: *efg1*/*efg1*, HLC54: *efg1*/*efg1 cph1*/*cph1*, JKC19: *cph1*/*cph1*) *sap 1–3* and *sap 4–6* (graciously provided by Prof. N. A. R. Gow, University of Aberdeen, UK), four *C. albicans* wild-type isolates; HK 36Aa, HK 38Hd, HK 39Dd, HK 46Cd (obtained from HIV-infected patients attending the Queen Elizabeth Hospital, Hong Kong) and a reference isolate *C. albicans* American Type Culture Collection (ATCC) 90028.

Non-*albicans Candida*: One reference isolate and one wild-type isolate each of the following *Candida* species: *C. tropicalis* (ATCC 13803, HK 192552), *C. dubliniensis* (ATCC MYA646, HK 186434), *C. glabrata* (ATCC 90030, HK 205734), *C. parapsilosis* (ATCC 22019, HK 205373) and *C. krusei* (ATCC 6258, CAML27b). All non-*albicans*, wild-type isolates were obtained from patients attending the Prince Philip Dental Hospital (Hong Kong) using the oral rinse technique (25). ATCC strains were obtained from ATCC (Gaitherburg, MD, USA).

All *Candida* isolates were subcultured from thawed suspensions of pure isolates stored at the Oral Biosciences Laboratory (Prince Philip Dental Hospital, University of Hong Kong, Hong Kong). Their identities were reconfirmed using the standard germ tube test, and fermentation reactions in commercially available API 20C auxonogram strips (Biomerieux, Marcy I'Etoile, France).

Culture media and growth conditions

Inocula for the infection of RHOE were prepared as described previously (15). Briefly, yeast cells were cultured for 24 h at 37°C on Sabouraud dextrose agar (Difco, Hampshire, UK). The resultant harvest was washed thrice in 0.9% NaCl and an inoculum of approximately 2×10^5 cells were suspended in 10 ml of YPG medium (1% yeast extract, 2% peptone and 2% glucose; Difco, Detroit, MI, USA). This suspension was cultured for 16 h at 25°C with orbital shaking and the cells so harvested, were washed in 0.9% NaCl and a sample of cells was resuspended in fresh medium in shaking for 24 h at 37°C. Afterwards the cells were harvested by centrifugation and the inocula were prepared in phosphate-buffered saline (PBS; pH 7.2).

Reconstituted human oral epithelium

Commercially obtained human oral epithelium (human keratinocytes derived from squamous cell carcinoma of the buccal mucosa, cell line TR146; Skinethic Laboratory, Nice, France) was reconstituted by incubating in serum-free, MCDB 153-defined medium (Clonetics, San Dieg, CA, USA), containing 5 μg/ml insulin, 1.5 mM $CaCl_2$, 25 μg/ml gentamicin and 0.4 μg/ml hydrocortisone in 6-well tissue culture plates (Corning Inc., Corning, New York, NY, USA) according to the manufacturer's instructions. The *in vitro* model and all culture media were prepared without antimycotics. After reconstitution, a 0.5 cm^2 segment of tissue was inoculated with 2×10^6 yeast cells in 50 μl of PBS (pH 7.2). Control samples were inoculated with 50 μl PBS only. Infected tissue cultures were incubated at 37°C, at 5% CO_2 and saturated humidity for up to 48 h. They were harvested at 12, 24 and 48 h time intervals for histopathological evaluations as described below. The medium was replenished once in every 24 h.

Light microscopy

Harvested tissue samples were washed gently with PBS to remove free fungal elements and fixed in 10% formalin in PBS, for at least 2 h at room temperature. The circular piece of tissue was bisected along the diameter into approximately two equal halves (Fig. 1a). After a series of dehydrations, they were embedded in paraffin. Series of 3–4 μm thick sections were cut at 20 μ intervals from the centre of the tissue (Fig. 1b) outwards using a Leica RM 2155 rotary microtome (Nussloch, Germany). Afterwards, 10 representative sections selected by systematic random sampling (approximately every 20th section derived by the serial sectioning of the tissue block) were processed for staining (14). The sections were stained with PAS reagent and counterstained with Myers Hematoxylin (Dako, Carpinteria, CA, USA) for quantitative image analysis.

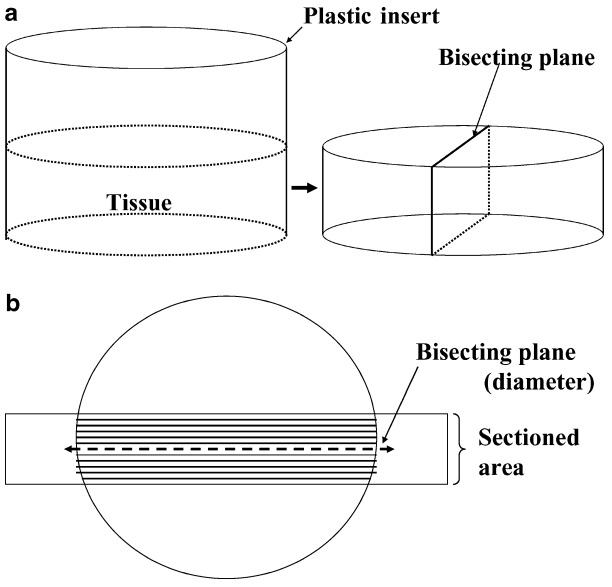

Figure 1 A schematic representation of (a) the harvesting of the tissue for histopathology and (b) sectioning the tissue for quantitative analysis.

Quantitative image analysis

To quantify the invasive fungal elements within RHOE, a computer-assisted image analysis system (IAS) was utilized. PAS-stained areas were detected and analysed using image analysis software (Leica Qwin, version 2.4, Leica Microsystems Imaging Solutions Ltd, Cambridge, UK).

For this purpose a charged coupled device (3CCD) Red, Green, Blue (RGB) colour video camera (Leica DC 300 V. 2.0, Wetzlar, Germany; 2088 × 1550 pixel) was attached to a Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany), and digitized binary colour microscopic images of sections were captured at ×360 magnification. Illumination provided by a halogen light source connected to a stabilized power supply, was kept constant throughout the evaluation process. Entire area of the selected sections, i.e. 8–12 microscopic fields per section, was studied. For each section, total PAS-stained area of fungal elements were quantified, then expressed in a percentage of the whole tissue surface area. Each experiment with all isolates was repeated on two different occasions with duplicate samples on each occasion.

Statistical analysis

The Wilcoxon signed rank non-parametric test was used for the statistical comparison of the percentage values of PAS-positive areas in the tissue sections, infected by six *C. albicans* isolates, *cph1*/*cph1* hyphal mutant, *sap 1–3* and *sap 4–6* mutants at 12 and 24 h. The PAS-positive area percentages of each *C. albicans* isolate at 12 and 24 h were compared within the group using Friedman non-parametric test. The PAS-positive area percentages of parent wild-type isolate SC 5314 and invasive mutants (*cph1*/*cph1*, *sap 1–3* and *sap 4–6*) at 12 and 24 h were also compared using Friedman test. All *P*-values were two-tailed and the differences were considered significant at $P < 0.05$.

Results

Histological evaluation of control samples

Reconstituted human oral epithelium consisted of 10–12 layers of stratified squamous keratinized epithelial cells (Fig. 2a). The tissue was devoid of stratum corneum, whilst mitotic figures and dyskeratotic cells could be occasionally observed.

Histological evaluation of Candida albicans-infected samples

All *C. albicans* isolates-infected RHOE demonstrated classic histological signs of hyphal invasion, which intensified with the period of incubation. On visual examination *C. albicans* isolates appeared more invasive than non-*albicans Candida* isolates. After 12 h of infection clusters of *C. albicans* blastospores were attached to the superficial cell layers and initial germ tubes were seen penetrating the tissue (Fig. 2b). Both blastospores and hyphal forms were noted during the early phase of infection although hyphal phase became predominant over time (Fig. 2c).

At 48 h, when invasion of hyphae were more extensive, disorganization of the epithelial structure was evident (Fig. 2d). At this stage detached superficial cell layers and candidal hyphae appeared to constitute a pseudomembrane, which was extremely fragile. Invasiveness of individual isolates appeared to be different on gross microscopic examination (Figs 2b and 3). When compared with non-*albicans* isolates, all *C. albicans* isolates (wild types and reference isolate) after 48 h of incubation demonstrated a greater depth of hyphal penetration leading to progressive disorganization of RHOE.

Histological evaluation of Candida albicans hyphal and SAP mutants-infected samples

In contrast to the wild-type parent isolate *C. albicans* SC 5314, we found that single hyphal mutant, *efg1*/*efg1* and double mutant *efg1*/*efg cph1*/*cph1* were unable to invade RHOE even after 48 h of incubation. However, *cph1*/*cph1* single hyphal mutant was invasive. Both *sap 1–3* and *sap 4–6* mutants also produced hyphae and invaded the artificial epithelium akin to the parent isolate. Histopathological changes accompanying the invasion of these hyphal and *SAP* mutants were also similar to that of the parent isolate.

The extent of *C. albicans* hyphal invasion was isolate-specific and was reproducible on repeat experiments on separate occasions. Accordingly the pattern of invasion was not identical among isolates and two distinct forms of invasion were noted; focal invasion at the early stage of infestation (12–24 h) and then generalized invasion at a later stage (24–48 h). Initial focal invasion was mainly localized where hyphae invaded distinct areas of RHOE (Fig. 3). In contrast, subsequent uniform invasion was characterized by the regular distribution of invading fungal elements throughout the epithelial cell layers.

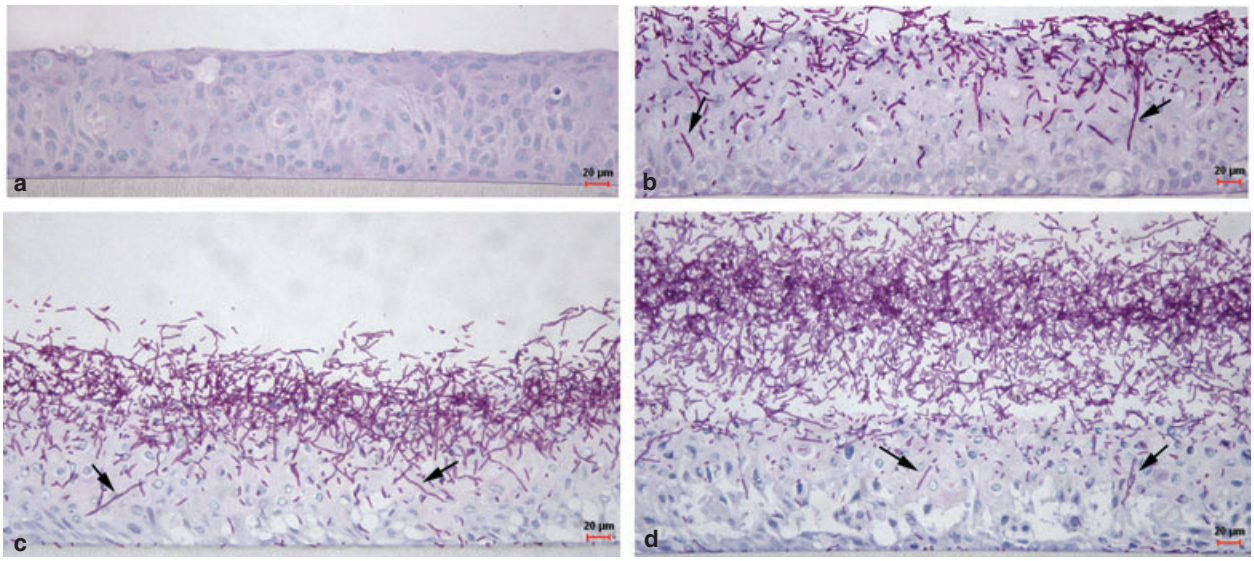

Figure 2 Light micrographs of reconstituted human oral epithelium (RHOE; stained with periodic acid-Schiff stain and invading fungal hyphae are arrowed). (a) Uninfected (control) after 12 h culture. Stratified keratinocytes with 10–12 cell layers are seen. (b) Adherence and invasion of *Candida albicans* SC 5314 yeast and hyphal forms through the superficial cell layers after 12 h of incubation. (c) Progressive and generalized tissue invasion of *C. albicans* SC 5314 after 24 h incubation; the tissue structure of RHOE is still intact. (d) Extensive tissue invasion of *C. albicans* SC 5314 at 48 h leading to disorganization of RHOE with intercellular oedema, exfoliation of the superficial cell layers and detachment of the basal cell layers.

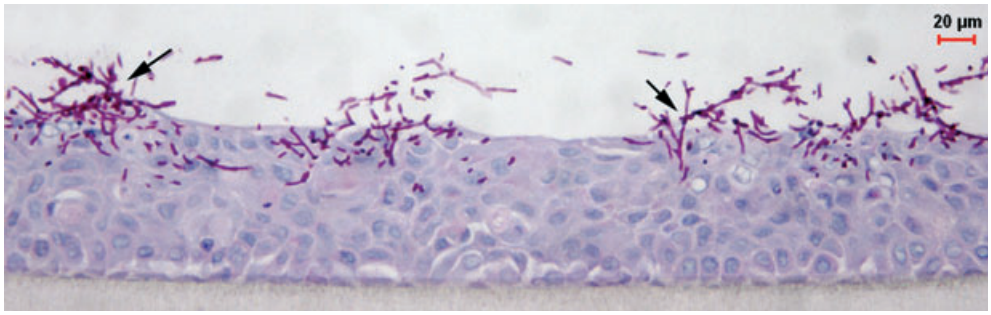

Figure 3 A light micrograph of *Candida albicans* HK 36Aa-infected reconstituted human oral epithelium (RHOE) after 12 h incubation (periodic acid-Schiff stain). Adherence and focal invasion (arrowed) of yeast and hyphal forms through the superficial cell layers are seen.

However, at the latter stages of infection (48 h) fungal elements were seen throughout the entirety of the tissue.

Histological evaluation of non-albicans Candida species-infected samples

Macroscopic observations revealed that all non-*albicans Candida* species (reference and the wild-type isolate of *C. tropicalis*, *C. dubliniensis*, *C. glabrata*, *C. parapsilosis* and *C. krusei*) used in the study grew well over the epithelial surface under the culture conditions used. However, on microscopic examination, these yeast except *C. dubliniensis* HK 186434 wild-type isolate, did not elicit hyphal forms nor did they invade the tissue and were present in the blastospore form throughout the 48-h incubation period. *C. dubliniensis* HK 186434 produced sparse hyphal elements (Fig. 4a) with a few blastospores. These results indicate that RHOE offers resistance to invasion by many of the non-*albicans Candida* species tested compared with *C. albicans*. Nonetheless, non-*albicans Candida* species also produced a variable degree of pathological tissue changes (compared with the uninfected controls), such as intercellular oedema and disorganization of the tissue structure at 48 h time point, resembling *C. albicans*-induced histopathology (Fig. 4a,b).

Quantitative evaluation of tissue invasion

In order to quantify the degree of invasion by different *C. albicans* isolates, in RHOE model of oral candidiasis we used a novel computer-assisted IAS. For this purpose, PAS-stained histological sections of the infected tissue were used. These representative tissue sections were selected by systematic random sampling technique (26) to ensure uniform and unbiased sample distribution within the tissue.

In our hands the fungal elements including both hyphae and blastospores that were strongly PAS-positive could be easily visualized, detected and quantified. The quantitative image analysis data of PAS-stained

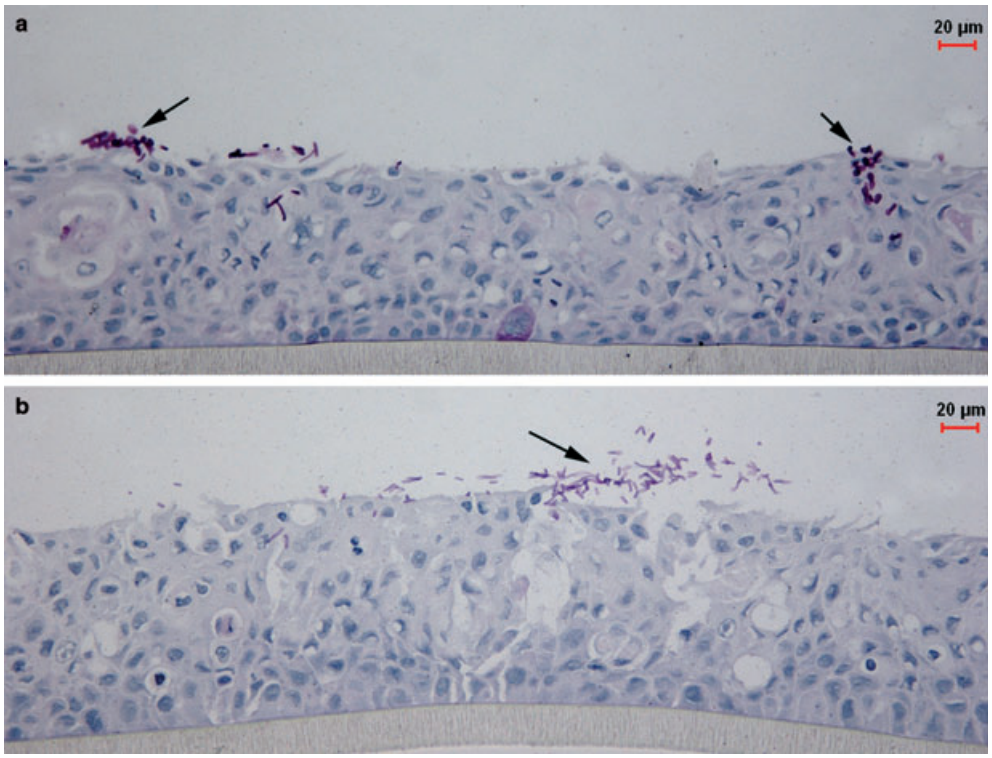

Figure 4 Light micrographs of (a) *Candida dubliniensis* HK 186434 and (b) *C. glabrata* HK 205734-infected reconstituted human oral epithelium (RHOE) after 48 h incubation (periodic acid-Schiff stain). Few blastospores are attached to the superficial cell layer (arrowed). Intercellular oedema is present within the basal cell layers.

areas that exemplify the degree of invasion by *C. albicans* wild-type isolates, and their hyphal and *SAP* mutants are shown in Fig. 5a,b and Table 1.

In all samples with fungal invasion, the percentage PAS-positive areas increased towards the latter stages of the infection (24–48 h). However, 48 h time point was not included in our quantitative analyses due to extensive disorganization of the tissue caused by the fungal invasion (Fig. 2d). Statistical analysis (Friedman test) was used to compare the degree of invasion by all invasive *C. albicans* isolates (wild types and the reference isolate). Their invasion was significantly different ($P < 0.05$) from each other at both time points (12 and 24 h) as demonstrated by the percentage PAS-positive area. When the invasion of individual *C. albicans* isolates at 12 and 24 h time points were compared (Wilcoxon signed ranks test), there were significant differences ($P < 0.05$) in invasion at 12 and 24 h time points for each isolate.

We also compared the invasion of mutant isolates (*cph1*/*cph1*, *sap 1–3*, *sap 4–6*) with the parent wild-type *C. albicans* isolate SC 5314. At 12 h time point there was no significant difference in the degree of tissue invasion among the parent *C. albicans* isolate SC 5314 and its hyphal mutant, *cph1*/*cph1* and *SAP* mutants, *sap 1–3* and *sap 4–6*. However, after 24 h their invasive behaviours were significantly different ($P < 0.05$) from each other with hyphal mutants, *cph1*/*cph1* and *SAP* mutant, *sap 1–3* showing less tissue penetration compared with the parent isolate.

Discussion

Candidiasis models based on *in situ* animals or explant culture techniques are difficult to standardize and are in general, rather laborious. For instance in explant cultures *Candida* can invade all exposed sites including the explant periphery in addition to the epithelial surface thus leading to spurious results (8). Reconstituted human oral epithelium on the other hand, permits the direct penetration of the yeast only through the superficial epithelial surface and no lateral invasion is possible as it is contained within a plastic insert. Furthermore, the uniform and standardized structural features of RHOE provide comparability among replicate experiments and tissue samples allowing assessment of the invasive capability of either different *Candida* isolates or species, tested. Other advantages of this model are: (i) its multilayer structure (up to 10–12 cell layers) closely resembling the oral epithelium that contrasts with the monolayer traditional tissue culture systems, (ii) the ability to artificially reproduce the internal milieu of the oral cavity within a pH range of 7.0–7.4, (iii) its ready 'off-the-shelf' use for time-limited experiments. Previous studies have shown that, histologically, RHOE resembles the normal human oral epithelium and the pathological changes that accompany candidal invasion are akin to human disease (12, 16). Hence we believe that RHOE model is, by far, the most suitable current system available to study *in vitro* the yeast invasion of human epithelia in a quantitative manner. However, the

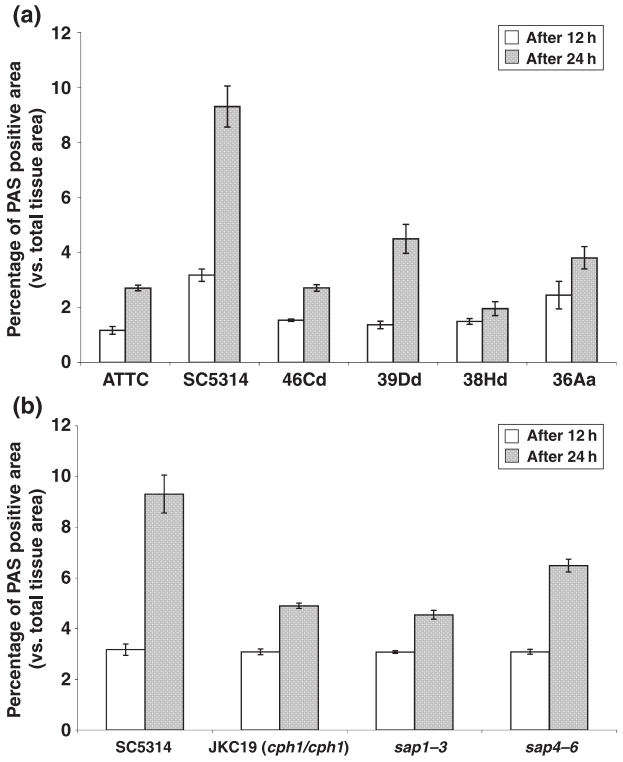

Figure 5 (a) Quantification of *Candida albicans* invasion (six isolates) evaluated using a computerized image analysis system (IAS). Columns represent mean values (±SD) of periodic acid-Schiff (PAS)-positive surface area, expressed as a percentage of total tissue surface area. (b) Quantification of *C. albicans* SC 5314 and mutant isolates; hyphal mutant JKC19: *cph1*/*cph1* and *SAP* mutants: *sap 1–3* and *sap 4–6*, invasion using a computerized IAS. Columns represent mean ± SD of PAS-positive surface area, expressed in percentage of total tissue surface area.

absence of a connective tissue support is a limitation of this model when compared with its natural counterpart.

One drawback of RHOE model in relation to the quantification of the area percentages of the invading fungal elements is that the tissue structure deteriorates because of intercellular oedema and detachment of the superficial cell layers at latter stages of the infection (48 h). However, RHOE model did provide satisfactory stability at 12 and 24 h (Fig. 2b,c). Despite significant difference in patterns of tissue invasion between different *C. albicans* isolates it was evident that all invaded RHOE at 12 h. The IAS reproducibly quantified 12 h tissue invasion but a reduction of the sensitivity of the method was seen at 6 h because of minimal tissue invasion at the early phase of infection. Hence, we used 12 h as the first time point for the assessment of invasion. The sequential examination of the invasion of each isolate tested exhibited significant difference between 12 and 24 h time points suggesting that the progressive invasion relates to the period of incubation. Repeat experiments produced consistent results and reconfirmed the suitability of this RHOE model for the purpose. Our findings of two different invasive patterns, viz. focal and generalized, also support the previous reports of Bartie et al. (12).

Table 1 Quantification of invasion by *Candida albicans* isolates, hyphal and *SAP* mutants, evaluated using a computerized image analysis system

	Mean (%) ± SD of PAS-positive area	
C. albicans isolates	*After 12 h*	*After 24 h*
ATCC 90028	1.1662 ± 0.1391	2.7052 ± 0.1024
SC 5314	3.1728 ± 0.2231	9.3079 ± 0.7467
HK 46Cd	1.5332 ± 0.0475	2.7086 ± 0.0117
HK 39Dd	1.3630 ± 0.1353	4.4934 ± 0.5286
HK 38Hd	1.4891 ± 0.1080	1.9586 ± 0.2555
HK 36Aa	2.4473 ± 0.5011	3.8038 ± 0.4049
JKC19 (*cph1*/*cph1*)	3.0861 ± 0.1133	4.8994 ± 0.1061
sap 1–3	3.0773 ± 0.0549	4.5463 ± 0.1732
sap 4–6	3.0843 ± 0.0966	6.4880 ± 0.2518

ATCC, American Type Culture Collection; PAS, periodic acid-Schiff.

Image analysis techniques are increasingly used in the assessment and evaluation of histopathological changes in affected tissues including microbial infections (27, 28). Some investigators (27, 28) have utilized computer-assisted image analysis methods to determine the area percentages of positively stained structures after immunohistochemical staining of pathological tissues. Periodic acid-Schiff stain, traditionally used for the identification of *Candida* species in histopathological sections (29), provides good contrasting visuals that can be readily detected by image analysis. Therefore, we hypothesized that this method could also be used for the determination of the invasive potential of different *Candida* species in our experimental candidiasis model. We found that the invasive profile of different *C. albicans* strains or other *Candida* species could be readily and reproducibly quantified using computer-assisted image analysis method described here. For instance, PAS-positive area percentage of SC 5314-infected tissues increased markedly from 12 to 24 h, in agreement with the histological evidence that more invasion takes place with time (Figs 2b,c and 5a). And also, the significant difference in invasive potentials of SC 5314 and HK 36Aa at 12 h time point is exemplified by the differences in PAS-positive area percentages (Fig. 5a) and the histopathological observations (Figs 2b and 3). However, it must be emphasized that it is critical to establish a standard protocol including the staining period, section thickness, lighting intensity and quantification yardsticks in order to yield reproducible data from one experiment to another.

Differential invasion of *Candida* species have been investigated using various *in vitro* candidiasis models including explants and tissue culture systems. In a pioneering study, Howlett (8) demonstrated using a rat tongue mucosal explant culture over a 45-h incubation period that *C. albicans* developed hyphal elements and was more invasive than *C. tropicalis, C. krusei, C. parapsilosis* and *C. guillerimondii*. In another model using chick chorioallantoic membrane and six different *Candida* species (*C. albicans*, *C. tropicalis*, *C. pseudotropicalis*, *C. parapsilosis*, *C. krusei* and *C. guillerimondii*), Partridge et al. (9) found that *Candida* species could be graded according to their relative pathogenicity and they identified *C. albicans* and *C. tropicalis* to be the most

invasive of the six species studied. In a recent *in vivo* experimental study in mice, Arendrup et al. (30) observed remarkable differences in pathogenicity amongst eight *Candida* species, and categorized them into three groups in terms of decreasing pathogenicity. The most pathogenic group comprised *C. albicans* and *C. tropicalis*, followed by an intermediate group with *C. glabrata*, *C. lusitaniae* and *C. kyfyr* and a least pathogenic group of *C. parapsilosis*, *C. krusei* and *C. guillerimondii*.

According to Hurley and Stanley (31) who studied the cytopathic effects of different *Candida* species on murine renal epithelial cells the destruction of host cells correlates with the yeast to hyphal transition as well as their growth rate. However in the present study, of the six *Candida* species evaluated, the only invasive yeast was *C. albicans* despite the fact that we used germ tube-positive wild-type isolates of *C. tropicalis* and *C. dubliniensis*. Of the non-*albicans* strains *C. dubliniensis* and *C. glabrata* could adhere to the epithelial surface after 48 h (Fig. 4a,b) while *C. tropicalis*, *C. krusei* and *C. parapsilosis* were unable to do so even after prolonged incubation. These results are in agreement with those of Schaller et al. (18) who reported that of *C. albicans*, *C. tropicalis* and *C. glabrata* the only invasive species was *C. albicans*. To conclude then, our findings are in agreement with the previous similar observations in *in vitro* culture models that *C. albicans* is the most invasive compared with other *Candida* species (8, 9, 18, 30, 31). On the other hand, the potential of RHOE to withstand candidal invasion differentially seems to imply that it is not merely an aggregation of cells serving as a passive growth medium for the fungi, but on the contrary a cellular community that closely mimics the epithelial barrier, still retaining the characteristics of the latter.

As all six *C. albicans* isolates used in our study differentially invaded RHOE. It is reasonable to conclude that isolate-specific differences exist in the invasive ability of *C. albicans* comparable with its other virulence attributes, such as adherence, and extracellular enzyme production. Interestingly many of the non-*albicans Candida* species tested invaded RHOE model. Although non-invasive, these species produced pathogenic effects, such as intercellular oedema and disorganization of the tissue architecture with progressively increasing incubation period. This perhaps may be attributable to the action of extracellular enzymes, such as phospholipases and SAPs released by these yeast.

We also compared the invasive ability of the three congenic hyphal mutants of *C. albicans* isolates deleted for *CPH1, EFG1* or both transcription factors with the parent isolate SC 5314. It was observed that the single mutant *efg1/efg1* and the double mutant *efg1/efg1 cph1/cph* neither produced hyphae nor penetrated RHOE successfully supporting the theory that the hyphal formation in *C. albicans* is a critical determinant of invasive pathogenesis especially in comparison with other non-*albicans Candida* species. Of these two non-invasive mutant isolates *efg1/efg1* showed limited attachment to the superficial epithelial cell layers at 48 h time point whilst the double mutant could not do so implies that the two transcription factors act synergistically during candidal invasion. In contrast the single mutant *cph1/cph1* could invade the epithelium resembling the parent isolate. These results are in agreement with the previous observations of Dieterich et al. (32) where a single mutant *cph1/cph1* behaved in a similar manner to the parent clinical isolate while *efg1/efg1* single mutant and *efg1/efg1 cph1/cph* double mutant were non-invasive. Taken together these findings suggest that *EFG1* has more significant effect than *CPH1* in candidal morphogenesis and tissue invasion. However, additional studies are required to validate this hypothesis.

We also found that *sap 1–3* and *sap 4–6* mutants are indistinguishable from the parent isolate SC 5314 in their invasive potential at the initial stage (12 h) although their invasion appeared different from each other after 24 h. These results support the observations of Gow et al. (11) who found that there is no detectable change in both invasion and pathogenicity in *sap 1–3*, and *sap 4–6* mutants of *C. albicans* compared with the parent isolate SC 5314 during infection of chick chorioallantoic membrane, over 24 h. Interestingly, after 24 h we found that *sap 4–6* mutant was more virulent than *sap 1–3* mutant in RHOE model. These findings are comparable with that of Schaller et al. (17) who described an enhanced virulence of *sap 4–6* mutant compared with individual *SAP* mutants; *sap 1*, *sap 2*, *sap 3* and double mutant *sap 1/3* in a similar RHOE model. These investigators concluded that *sap 1–3*, but not *sap 4–6* are important in *in vitro* pathogenesis. In contrast, in an experiment to study the pathogenesis of disseminated candidiasis in mice Felk et al. (33) demonstrated that *sap 4–6* mutants are less invasive on parenchymal tissues although they still produce hyphae, and argued that *sap 4–6* are important in invasive pathogenesis. Thus, the precise roles and functions of the different SAPs during human infections are still unclear (34) and further studies are warranted to identify the role of *SAP* subfamilies in candidal invasion.

To conclude, our findings support the hypothesis that the conversion from the blastospore phase to the hyphal phase is an important virulence attribute of *C. albicans*, in comparison with non-*albicans Candida* species. We reconfirm the fact that RHOE is a suitable *in vitro* model to assess the invasive ability of different *C. albicans* isolates. Furthermore, this model in combination with image analysis permits ready quantitative evaluation of tissue invasion by *Candida* species. This tool could be potentially valuable to assess the morphogenic changes in terms of filamentation, following the exposure of *Candida* species to antifungals, proteinase inhibitors and cytokines.

References

1. Samaranayake LP. Host factors and oral candidosis. In: Samaranayake LP, Macfarlane TW, eds. *Oral candidosis*. London, UK: Butterworth & Co (Publishers) Ltd, 1990; 66–103.
2. Arendorf TM, Walker DM. The prevalence and intra-oral distribution of *Candida albicans* in man. *Arch Oral Biol* 1980; **25**: 1–10.

3. Colombo AL, Perfect J, DiNubile M, et al. Global distribution and outcomes for *Candida* species causing invasive candidiasis: results from an international randomized double-blind study of caspofungin versus amphotericin B for the treatment of invasive candidiasis. *Eur J Clin Microbiol Infect Dis* 2003; **22**: 470–4.
4. Edmond MB, Wallace SE, McClish DK, Pfaller MA, Jones RN, Wenzel RP. Nosocomial bloodstream infections in United States hospitals: a three-year analysis. *Clin Infect Dis* 1999; **29**: 239–44.
5. Samaranayake YH, Samaranayake LP. Experimental oral candidiasis in animal models. *Clin Microbiol Rev* 2001; **14**: 398–429.
6. Pemberton J, Turner EP. Organ culture model to study tissue invasion by *Candida albicans*. *J Dent Res* 1973; **52**: 948.
7. Borg M, Ruchel R. Expression of extracellular acid proteinase by proteolytic *Candida* spp. during experimental infection of oral mucosa. *Infect Immun* 1988; **56**: 626–31.
8. Howlett JA. The infection of rat tongue mucosa in vitro with five species of *Candida*. *J Med Microbiol* 1976; **9**: 309–16.
9. Partridge BM, Ather MA, Winner HI. Chick embryo inoculation as a pathogenicity test for *Candida* species. *J Clin Pathol* 1971; **24**: 645–8.
10. Shimizu K, Kondoh Y, Tanaka K. Proteinase production and pathogenicity of *Candida albicans*, invasion into chorioallantoic membrane by *C. albicans* strains of different proteinase activity. *Microbiol Immunol* 1987; **31**: 1045–60.
11. Gow NAR, Knox Y, Munro CA, Thompson WD. Infection of chick chorioallantoic membrane (CAM) as a model for invasive hyphal growth and pathogenesis of *Candida albicans*. *Med Mycol* 2003; **41**: 331–8.
12. Bartie KL, Williams DW, Wilson MJ, Potts AJC, Lewis MAO. Differential invasion of *Candida albicans* isolates in an in vitro model of oral candidosis. *Oral Microbiol Immunol* 2004; **19**: 293–6.
13. Bernhardt J, Herman D, Sheridan M, Calderone R. Adherence and invasion studies of *Candida albicans* strains using in vitro models of esophageal candidiasis. *J Infect Dis* 2001; **184**: 1170–5.
14. Bonowitz A, Schaller M, Laude J, Reimer K, Korting HC. Comparative therapeutic and toxic effects of different providone iodine (PVP-1) formulations in a model of oral candidosis based on in vitro reconstituted epithelium. *J Drug Target* 2001; **9**: 75–83.
15. Jayatilake JAMS, Samaranayake YH, Samaranayake LP. An ultrastructural and a cytochemical study of candidal invasion of reconstituted human oral epithelium. *J Oral Pathol Med* 2005; **34**: 240–6.
16. Schaller M, Schafer W, Korting HC, Hube B. Differential expression of secreted aspartyl proteinases in a model of human oral candidosis and in patient samples from the oral cavity. *Mol Microbiol* 1998; **29**: 605–15.
17. Schaller M, Korting HC, Schafer W, Bastert J, Chen WC, Hube B. Secreted aspartic proteinase (Sap) activity contributes to tissue damage in a model of human oral candidosis. *Mol Microbiol* 1999; **34**: 169–80.
18. Schaller M, Mailhammer R, Grassl G, Sander CA, Hube B, Korting HC. Infection of human oral epithelia with *Candida* species induces cytokine expression correlated to the degree of virulence. *J Invest Dermatol* 2002; **118**: 652–7.
19. Butz-Jogensen E. Histopathology, immunology and serology of oral yeast infections: diagnosis of oral candidosis. *Acta Odontol Scand* 1990; **48**: 37–43.
20. Sobel JD, Muller G, McCormick JF. Experimental chronic vaginal candidosis in rats. *Sabouraudia* 1985; **23**: 199–206.
21. Cole GT, Seshan KR, Pope LM, Yancey RJ. Morphological aspects of gastrointestinal tract invasion by *Candida albicans* in the infant mouse. *J Med Vet Mycol* 1988; **26**: 173–85.
22. Allen CM, Paulson R, Duncan R. Clinical, histologic and scanning electron microscopic study of the development of chronic candidiasis of the rat tongue. *J Oral Pathol Med* 1989; **18**: 352–9.
23. O'Day DM, Head WS, Csank C, et al. Differences in virulence between two *Candida albicans* strains in experimental keratitis. *Invest Ophthalmol Vis Sci* 2000; **41**: 1116–21.
24. O'Day DM, Head WS, Robinson RD, Yang R, Shetlar D, Wang MX. Contact lens-induced infection – a new model of *Candida albicans* keratitis. *Invest Ophthalmol Vis Sci* 1999; **40**: 1607–11.
25. Samaranayake LP, MacFarlane TW, Lamey PJ, Ferguson MM. A comparison of oral rinse and imprint sampling techniques for the detection of yeast, coliform and *Staphylococcus aureus* carriage in the oral cavity. *J Oral Pathol* 1986; **15**: 386–8.
26. Weibel ER. *Stereological methods, practical methods for biological morphometry*, Vol. 1. London, UK: Academic press Inc. (London) Ltd, 1979.
27. Lepidi H, Fournier PE, Raoult D. Quantitative analysis of valvular lesions during *Bartonella* endocarditis. *Am J Clin Pathol* 2000; **114**: 880–9.
28. Lepidi H, Fenollar F, Gerolami R, et al. Whipple's disease: immunospecific and quantitative immunohistochemical study of intestinal biopsy specimens. *Hum Pathol* 2003; **34**: 589–96.
29. Stevens A, Francis RF. Micro-organisms. In: Bancroft J, Stevens A, eds. *Theory and practice of histological techniques*. New York, USA: Churchill Livingstone, 1996; 291–308.
30. Arendrup M, Horn N, Frimodt-Moller N. In vivo pathogenicity of eight medically relevant *Candida* species in an animal model. *Infection* 2002; **30**: 286–91.
31. Hurley R, Stanley VC. Cytopathic effects of pathogenic and non-pathogenic species of *Candida* on cultured mouse epithelial cells: relation to the growth rate and morphology of the fungi. *J Med Microbiol* 1969; **2**: 63–74.
32. Dieterich C, Schandar M, Noll M, et al. In vitro reconstructed human epithelia reveal contributions of *Candida albicans EFG1* and *CPH1* to adhesion and invasion. *Microbiology* 2002; **148**: 497–506.
33. Felk A, Kretschmar M, Albrecht A, et al. *Candida albicans* hyphal formation and the expression of the Efg1-regulated proteinases Sap4 to Sap6 are required for the invasion of parenchymal organs. *Infect Immun* 2002; **70**: 3689–700.
34. Naglik JR, Challacombe SJ, Hube B. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol Mol Biol Rev* 2003; **67**: 400–28.

Acknowledgements

The authors thank Prof. N. A. R. Gow (The University of Aberdeen, UK) for providing *C. albicans* SC 5314 strain, hyphal and *SAP* mutants, and Prof. A. B. M. Rabie (The University of Hong Kong, Hong Kong) for making the image analysis system available for our study. This study was supported by the Research Grants Council of the University of Hong Kong (Grant No. HKU/7339/02M).