Oral Microbiology Immunology 2007: 22: 188–194
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY
AND IMMUNOLOGY

Characterization of *Candida albicans* infection of an *in vitro* oral epithelial model using confocal laser scanning microscopy

S. Malic[1], K. E. Hill[1], J. R. Ralphs[2], A. Hayes[2], D. W. Thomas[1], A. J. Potts[1], D. W. Williams[1]
[1]Oral Surgery, Medicine & Pathology, School of Dentistry, Heath Park, Cardiff, UK, [2]School of Biosciences, Cardiff University, Cardiff, UK

Malic S, Hill KE, Ralphs JR, Hayes A, Thomas DW, Potts AJ, Williams DW. Characterization of Candida albicans *infection of an* in vitro *oral epithelial model using confocal laser scanning microscopy.*
Oral Microbiol Immunol 2007: 22: 188–194. © 2007 The Authors. Journal compilation © 2007 Blackwell Munksgaard.

Introduction: Oral candidosis presents as several distinct forms and one of these, chronic hyperplastic candidosis, is distinguished by penetration of the epithelium by *Candida*. The aim of this study was to use confocal laser scanning microscopy to examine invasion of the oral epithelium by *Candida albicans* from different oral conditions and to determine whether inherent strain differences exist that could relate to infection type. Reverse transcription–polymerase chain reaction was also used to detect products from virulence gene families.
Methods: *C. albicans* ($n = 19$) was used to infect reconstituted human oral epithelium, which was incubated for 12 h. One half of the reconstituted human oral epithelium was then fixed and stained with concanavalin A–Alexa 594, pan-cytokeratin antibody–Alexa 488 and Hoechst nucleic acid dye. RNA was extracted from the remaining tissue for reverse transcription–polymerase chain reaction targeting secreted aspartyl proteinase, phospholipase and agglutinin-like sequence genes of *C. albicans*.
Results: Confocal laser scanning microscopy revealed strain-dependent tissue invasion, with differences evident in surface colonization, *C. albicans* morphology and the extent and pattern of tissue penetration. Hyphae were seen to directly penetrate epithelial cells and migrate between keratinocytes with yeast budding also evident in the reconstituted human oral epithelium. A relationship between 'high tissue invasion' and expression of secreted aspartyl proteinase genes 4–6 was noted. Interestingly, four of the five 'high invaders' originated from chronic hyperplastic candidosis.
Conclusions: Confocal laser scanning microscopy permitted high resolution analysis of reconstituted human oral epithelium invasion by *C. albicans* and identified strain differences in the invasion process. Association between extensive hyphal morphology, direct epithelial penetration and high surface colonization were made with the 'highly invasive' strains.

Key words: *Candida albicans*; confocal laser scanning microscopy; oral epithelial model; virulence factors

D. W. Williams, Oral Surgery, Medicine & Pathology, School of Dentistry, Heath Park, Cardiff, CF14 4XY, UK
Tel.: +44 29 2074 4252;
fax: +44 29 2074 2442;
e-mail: williamsdd@cardiff.ac.uk
Accepted for publication October 13, 2006

Candida albicans is an important cause of superficial infections of mucosal membranes and of systemic disease in humans (27). Four clinical forms of primary oral candidosis are recognized, namely, acute erythematous candidosis, pseudomembranous candidosis, chronic erythematous candidosis and chronic hyperplastic candidosis (CHC) (2). CHC is distinct, and is

characterized by hyphal penetration of the oral epithelium (28) and by the presence of an inflammatory cell infiltrate (32). Importantly, CHC is also associated with the development of squamous cell carcinoma at lesional sites (3, 13, 33). Host factors implicated in CHC include tobacco smoking, denture wearing and regular alcohol intake (1). However, it is also possible that *Candida* expresses specific virulence attributes that promote this infection. Since hyphal invasion of the epithelium is a distinguishing feature of CHC, it can be hypothesized that expression of certain *C. albicans* virulence factors associated with invasion may be enhanced for CHC strains compared with others.

In recent years, a commercially available reconstituted human oral epithelium (RHE; SkinEthic Laboratories, Nice, France) has successfully been used to investigate mechanisms of tissue degradation by *Candida* (11, 20–22) and to examine the response of tissue to infection (24). Indeed, previous studies using periodic acid Schiff staining to profile *C. albicans* RHE infection have suggested that strain differences exist between *C. albicans* isolates with respect to tissue invasion (4). Confocal laser scanning microscopy (CLSM) is a technique that enables the acquisition of high-resolution images and, by generating focused images through thick specimens at various depths, three-dimensional reconstructions. The use of CLSM on this RHE model would allow a more detailed examination of *C. albicans* invasion, allowing tracking of hyphae through the tissue as well as determining the extent of hyphal branching and yeast budding that may occur during the process. The primary focus of this study was therefore to use CLSM to characterize the interaction of keratinocytes and *C. albicans* during infection. In addition, the simultaneous detection of virulence gene products by reverse transcription–polymerase chain reaction (RT-PCR) was made to determine whether the presence of messenger RNA from the gene families correlated with invasion profiles. Comparisons between the *C. albicans* from different oral conditions were made to ascertain whether inherent strain differences existed.

Materials and methods

Isolation and culture of test isolates

Nineteen isolates of *C. albicans* from a range of oral conditions (normal oral mucosa, $n = 4$; non-CHC oral candidoses, $n = 4$; CHC, $n = 8$; squamous cell carcinoma, $n = 3$) were used. *C. albicans* identification was by standard morphological and biochemical profiling.

In vitro RHE infection model

To facilitate comparison between all 19 *C. albicans* strains, for any given experimental run, each strain was used to infect separate, single wells containing the RHE. The experiments were repeated on up to five separate occasions to allow intra-strain comparison. A non-infected control tissue was included with each experiment. *C. albicans* was cultured in yeast nitrogen base medium (BD Diagnostics, Cowley, UK) supplemented with 0.5% glucose (weight/volume) for 12 h at 37°C. Yeast cells were harvested by centrifugation and washed three times with phosphate-buffered saline. The resulting yeast cells were enumerated using a haemocytometer and a total of 2×10^6 yeast cells were added to the RHE (SkinEthic Laboratories). Infected tissue was incubated for 12 h at 37°C in a humidified atmosphere, enriched with 5% CO_2. After incubation, the tissue was rinsed twice in phosphate-buffered saline to remove non-adherent *C. albicans*, and then halved with one portion used for CLSM analysis and the other for RT-PCR.

Tissue processing for CLSM

Infected tissue was fixed in formalin and embedded in paraffin wax. Tissue sections (20 μm) were placed on microscope slides, de-waxed and rehydrated before fluorescent labelling. To visualize the infecting *C. albicans*, sections were directly labelled with 20 μl Alexa 594-conjugated concanavalin A lectin (Molecular Probes-Invitrogen, Paisley UK; 0.025% w/v in phosphate-buffered saline) for 20 min at room temperature. Keratinocytes within the RHE sections were labelled with a pan-cytokeratin antibody using an indirect immunofluorescent labelling procedure. Sections were first exposed to an antigen unmasking step in citrate buffer for 10 min at 95°C, washed in phosphate-buffered saline and blocked with normal goat serum (Sigma-Aldrich, Poole, UK) at a 20-fold dilution for 30 min, before the primary antibody step. Sections were then incubated overnight at 4°C with a mouse monoclonal pan-cytokeratin primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1: 50 dilution in phosphate-buffered saline. After washing, sections were fluorescently labelled with Alexa 488-conjugated goat anti-mouse secondary antibody (5 μg/ml; Invitrogen, Paisley, UK) for 30 min at room temperature. For nuclear context, sections were counterstained with Hoechst 33258 (1 μg/ml) for 20 min, before being washed in phosphate-buffered saline and mounted in Vectashield fade-retarding mountant (Vector Laboratories Ltd, Peterborough, UK).

Confocal laser scanning microscopy

Sections were viewed and analysed by CLSM using a Leica TCS SP2 AOBS spectral confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). Sections were scanned through the full depth of the tissue section using appropriate settings for fluorescence recordings. For multi-channel recordings, fluorochromes were scanned sequentially to eliminate spectral overlap between probes. Selected images were presented either as single confocal optical sections or as maximum intensity type reconstructions. Several characteristics of *C. albicans* invasion were determined, including the depth and pattern of invasion, proportions of hyphae to yeast, track of hyphae through the tissue and extent of hyphal branching or budding. Non-invading strains were classed as those that did not penetrate the RHE, 'low invaders' were those strains that did not pass completely through the tissue over the 12-h infection period, while high invaders penetrated completely through the tissue over the infection period.

RT-PCR

Tissue for RT-PCR was stored in a 2-ml microtube with RNA*later*® (Ambion Europe Ltd, Huntington, UK) solution before RNA extraction. RLT extraction medium (Qiagen, Crawley, UK) and glass beads (0.5 mm diameter, approx. 500 μl) were added to the tissue, which was homogenized twice for 30 s each, using a Mini-BeadBeater-8 (Stratech Scientific Ltd, Soham, UK). After tissue disruption, the RNeasy Mini Kit (Qiagen) was used to complete total RNA extraction according to the manufacturers' recommended protocol. Potential DNA contamination was removed by RNase-Free DNase I (Qiagen) treatment. Synthesis of complementary DNA involved the use an oligo-dT primer (Promega, Southampton, UK), with Moloney murine leukaemia virus-reverse transcriptase (Promega).

PCR targeted a number of virulence gene families including secreted aspartyl

proteinase, phospholipase and agglutinin-like sequence genes. For each gene a specific primer pair was used to yield an amplicon of distinct size. The primers and reaction conditions were as previously described for secreted aspartyl proteinases (14, 15), phospholipases (17, 30) and agglutinin-like sequence genes (9). Control S14 primers (5) were also used to detect human recombinant DNA and additional primers were employed for the amplification of the candidal elongation factor 1β RNA (26). These controls were used to confirm RNA recovery from the tissue. Elongation factor 1β provided an ideal control gene for RT-PCR because it is universally expressed by all forms of *C. albicans* (6). In addition, gene expression was also assessed for planktonic strains of *C. albicans* cultured in the tissue maintenance medium in an identical culture environment but in the absence of the RHE tissue.

PCR products (10 μl) were detected by standard gel electrophoresis with ethidium bromide staining. Controls for each reaction included tissue extracts not treated with reverse transcriptase (to establish that genomic DNA amplification was not occurring) and a water template control. Gel images were obtained using a Gel-Doc 2000 (Bio-Rad, Hemel-Hempstead, UK) and the average band densities for each PCR product were measured using the Discovery Series™ Quantity One® (Bio-Rad) software. For each strain, band density was expressed as a percentage relative to that of the matched elongation factor 1β amplicon.

Results

In vitro RHE infection model

The majority of *C. albicans* (17/19) invaded the RHE (Table 1) and CLSM showed the presence of both yeast budding and hyphae on the RHE surface and within the tissue. Hyphal growth forms were most frequently detected within tissues. Yeast predominated for 10 of the 14 isolates where little or no invasion was evident (Fig. 1A). This contrasted with 'high invaders', where extensive surface colonization and large numbers of hyphae occurred within the tissue (Fig. 1B). The extent of invasion was isolate-dependent and was consistent in repeat experiments for each given isolate. Overall, the ability of *Candida* to invade appeared to correlate with the isolates' capacity to exhibit the yeast-to-hypha transition. It was interesting to note that for 13 of the 19 strains tested, the morphological form was the same regardless of whether culture occurred in the presence or absence of the RHE (Table 1).

CLSM analysis of triple-stained tissues clearly demonstrated that hyphal invasion occurred both directly through keratinocytes (nuclear penetration was detected on occasions) (Fig. 1C) and also by passage via epithelial cell junctions (Fig. 1D). Furthermore, it was evident for some strains that yeast growth occurred within the RHE, originating from budding of hyphal branches (Fig. 1E,F). Another important feature was that while the majority of strains displayed a heterogeneous network of hyphal elements within the tissue (Fig. 2A), for a number of 'high invading' isolates (e.g. 135BM2/94, PTR/94, 1190/97), a more direct and vertical invasion profile occurred (Fig. 2B) suggesting that these strains were less impeded by the tissue. Significantly, the latter three isolates all originated from cases of CHC. Furthermore, it was also evident that four of the five strains that were classed as 'high invaders' also originated from CHC infection.

RT-PCR

RT-PCR analysis (Table 2) revealed a wide range of expression of secreted aspartyl proteinase genes (*SAP*) for the 19 *C. albicans* strains in the infected RHE model. *SAP1–3*, *SAP4–6* and *SAP8* transcripts were frequently detected, while *SAP7* was rarely encountered. The expression of *SAP4–6* in all high invading strains was evident. These genes were not, however, expressed by the two non-invasive strains (WK1/93 and 40/01). In contrast, phospholipase gene expression did not appear to readily correlate with the level of RHE invasion (Table 2) and was apparent for 11 out of 19 strains in the infection model. Similarly, associations between expression of the agglutinin-like sequence genes (*ALS*) and invasion were not evident, with four *ALS* genes (*ALS1*, *ALS2*, *ALS3* and *ALS4*) being detected in 14 of the 19 infected RHE samples (Table 2). Detection of *ALS5* (and to a certain extent of *ALS6*) was noticeably lower in infected

Table 1. Candida albicans invasion of reconstituted human oral epithelium as determined by confocal laser scanning microscopy

C. albicans strain	Isolate source	Surface colonization	Strain morphology in presence of tissue (without tissue)[1]	Invasion
135BM2/94	CHC-SCC, buccal mucosal	+++	Yeast and hyphae	High
970/00	CAC, oral mucosa	+++	Predominantly hyphae	High
PTR/94	CHC, buccal mucosa	+++	Yeast and hyphae	High
324LA/94	CHC, commissure	+++	Yeast and hyphae (predominantly yeast)	High
1190/97	CHC, buccal mucosa	+++	Yeast and hyphae	High
705/93	CHC, buccal mucosa	+++	Yeast and hyphae	Low
DW1/93	Normal oral mucosa	++	Yeast and hyphae	Low
PB1/93	Normal oral mucosa	++	Yeast and hyphae (predominantly yeast)	Low
243/00	Lichen planus	++	Yeast and hyphae	Low
455rgh/94	CHC, tongue	++	Predominantly yeast (yeast and hyphae)	Low
LR1/93	Normal oral mucosa	++	Predominantly yeast (yeast and hyphae)	Low
289T/00	SCC, tongue	++	Predominantly yeast	Low
480/00	SCC, oral mucosa	++	Yeast and hyphae	Low
819/99	Keratosis, sublingual	++	Predominantly yeast (yeast and hyphae)	Low
408/99	SCC, tongue	++	Predominantly yeast	Low
848/99	CHC, tongue	++	Predominantly yeast	Low
458R/94	CHC, buccal mucosa	++	Predominantly yeast	Low
WK1/93	Normal oral mucosa	+	Predominantly yeast	None
40/01	PMC, palate	+	Predominantly yeast (yeast and hyphae)	None

CHC, chronic hyperplastic candidosis; SCC, squamous cell carcinoma; CAC, chronic atrophic candidosis; PMC, pseudomembranous candidosis. +, sparsely colonized; ++, moderately colonized; +++, extensively colonized.
[1]Without tissue refers to the culture of *C. albicans* strains in the tissue maintenance medium devoid of the reconstituted oral epithelium. The morphological appearance of *C. albicans*, in the presence/absence of tissue, was the same unless indicated in parenthesis.

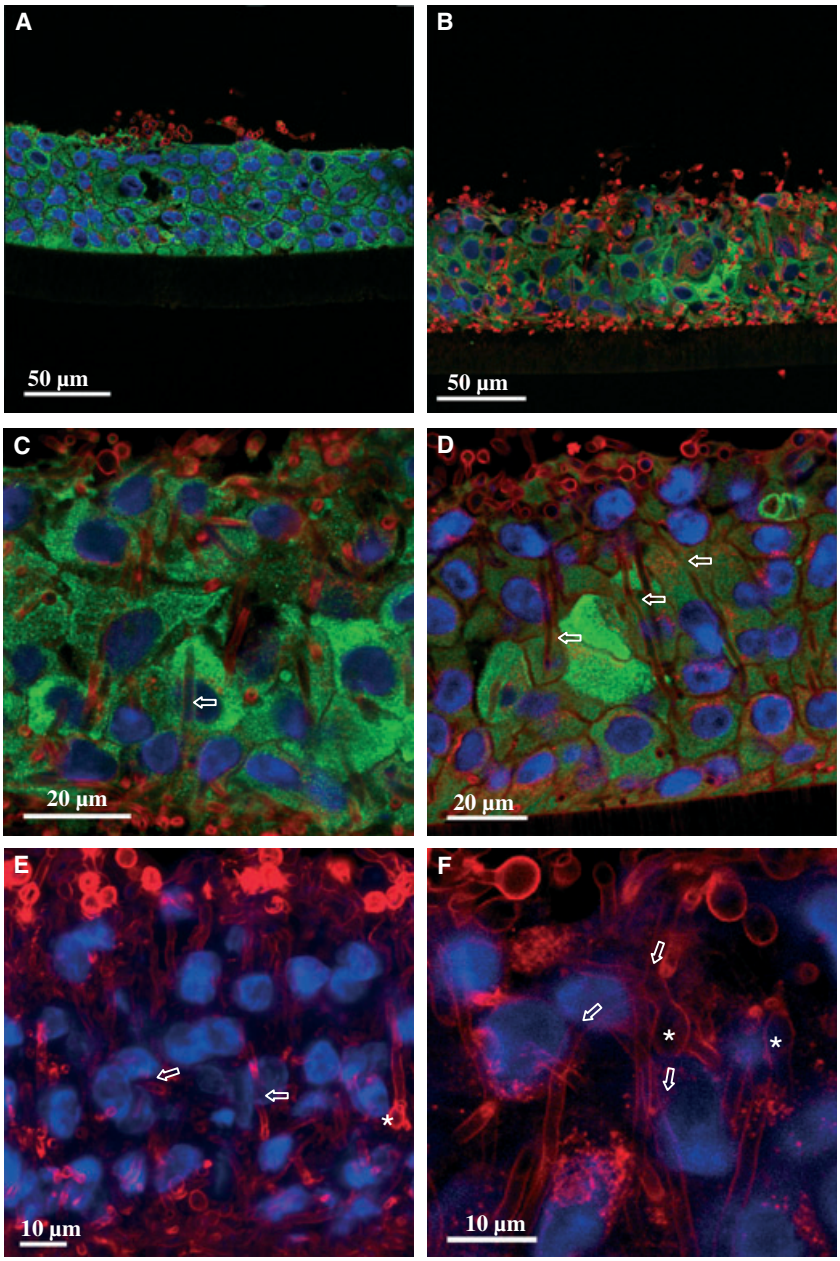

Fig. 1. Confocal laser scanning microscopy of *Candida albicans*-infected oral epithelium showing the pattern of hyphal invasion: (A) low invasion by strain 705/93; (B) high invasion by strain 135BM2/94; (C) epithelial penetration by hyphae of strain 135BM2/94; (D) interstitial hyphal penetration by strain PTR/94; (E) nuclear penetration by hyphae of strain 1190/97; (F) nuclear penetration and budding of yeast from hyphal branch by strain 135BM2/94. Arrows indicate the example of hyphal invasion type described for a particular figure; * indicates budding.

RHE compared with other members of the gene family. A comparison of gene expression in the infected RHE model with equivalent planktonic cultures revealed a largely consistent expression of secreted aspartyl proteinases between the two growth environments (Tables 2 and 3). Agglutinin-like sequence gene expression was similar between the two growth conditions but was perhaps slightly more expressed by strains grown with RHE, particularly evident with *ALS2*, *ALS6* and *ALS9*. This finding contrasted with phospholipase expression, which appeared to be more frequently detected in planktonic forms of the *Candida* isolates. When cultured in the presence of RHE, 23 phospholipase amplicons were evident compared with 42 amplicons in planktonic culture.

Semi-quantitative analysis did indicate varying quantities of messenger RNA for the expressing strains, although these differences could not be related to levels of *Candida* invasion within the model (Tables 2 and 3). For all the RT-PCRs control gene expression was routinely detected.

Discussion

A particular pathogenic feature of *C. albicans* that is essential to the development of CHC is its ability to invade the oral mucosa. Furthermore, this feature of mucosal invasion is notably distinct from other forms of oral candidosis and indeed serves as a diagnostic marker of CHC.

Compared with standard light microscopy, CLSM allows much more detailed information to be generated on *C. albicans* tissue invasion. Previous use of CLSM to assess the interaction of *C. albicans* with oral epithelium has been limited. Studies involving infection of the FaDu oropharyngeal epithelial cell line with *C. albicans* have previously described the use of CLSM in determining fungal–epithelial cell interaction mechanisms (18). Additional work examining endothelium infection by *C. albicans* using CLSM has elegantly revealed a self-induced endocytotic mechanism exhibited by candidal hyphae as a means of tissue invasion (19).

The commercially available RHE used in this present study has previously been used as a basis of *in vitro Candida* infection models. A possible limitation of such a system is the lack of certain host immune responses; although proinflammatory cytokine responses by *in vitro* epithelial cell lines, including RHE infected with *Candida*, appear to be maintained and actually have been shown to vary with *C. albicans* strains with different invasive potential and virulence (23, 31). The levels of candidal invasion reported in this present study were largely comparable to those previously found by our group using periodic acid Schiff histological staining (4) and this was reassuring, particularly as the methods of assessment between the two studies were different and were performed by separate operators. All isolates previously deemed to be 'high invaders' (4) were also identified as such in this present study. In addition, three of the four isolates originating from normal healthy oral mucosa exhibited low RHE invasion and two of these were also previously considered to be low invaders (4). This latter finding for commensal strains could indicate a limited ability of such isolates to produce the necessary virulence attributes required for invasion. This was also

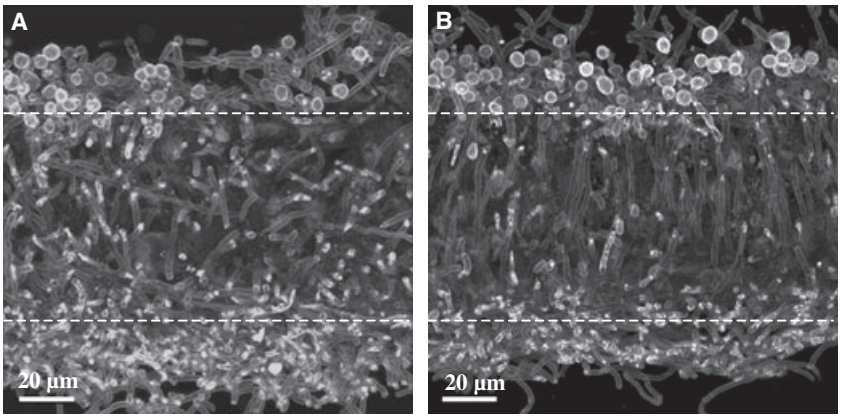

Fig. 2. Pattern of *Candida albicans* hyphal invasion of infected human oral epithelium. (A) Heterogeneous hyphal network during *C. albicans* infection of oral epithelium by strain 243/00; (B) vertical hyphal invasion during *C. albicans* infection of oral epithelium by strain 135BM2/94.

reflected by the relatively low level of colonization by these isolates together with an apparent reduced ability to produce hyphal forms. It is, however, worth noting that invasion, albeit at a low level, did occur with three of the four commensal isolates and it is therefore possible that given an appropriate oral environment, these 'commensal' isolates could instigate infection.

Four of the five strains classed in this study as 'high invaders' originated from CHC and might suggest an inherently greater ability of these strains to invade the oral epithelium. The fact that four other CHC strains were 'low invaders' further highlights the importance of additional host factors in the development of CHC infection. Indeed, studies performed by Soll (29) emphasize the importance of candidal adaptation to the host environments as being critical in determining whether commensalism or virulence results. Evidence from complex DNA profiling of *C. albicans* populations currently suggests the absence of true commensal and pathogenic strains, and advocates rapid phenotypic adaptation as a key component in promoting a particular oral status (29). The advantage of the current model would be that the strain differences can be ascertained under the standardized conditions used.

An ability to associate invasive features with clinical origin of the isolates would, however, be a significant advantage because it would enable the early identification of strains with the potential to cause CHC, and possibly influence patient management.

Interestingly, in all but one case, the 'high invaders' revealed a predominance of hyphae within the tissue. This was not surprising because movement of yeast from the surface of the RHE would have been physically difficult; traditionally, yeast cells are considered to lack the migratory ability possessed by hyphal elements (8). For a number of tissues, yeast predominated on the surface of the RHE, while hyphae predominated within and below the tissue. This would suggest either that the epithelium provided a trigger for morphological transition following contact or that only hyphae penetrated the tissue and thus predominated as a result of this selective process. To support the latter, previous work using CLSM to study endothelium invasion by *C. albicans* has shown that hyphae can induce their own endocytosis, an ability not possessed by yeast (19). For most strains used in the present study, the same morphological type was evident in either the presence or absence of tissue (Table 1), which would suggest that hyphal morphology is not induced through contact with the epithelium and supports the view that hyphae are the principal invasive forms of *C. albicans*.

Using conventional microscopy procedures, it has previously been difficult to ascertain the manner by which hyphae migrate through tissue. The CLSM approach used in this study conclusively demonstrated that, in addition to being

Table 2. Detection of gene families associated with pathogenesis during reconstituted human oral epithelium infection using RT-PCR

	Secreted aspartyl proteinase (*SAP1–9*)					Phospholipase genes (*B–D*)				Agglutinin-like sequence genes (*ALS1–7, ALS9*)							
C. albicans	*1–3*	*4–6*	*7*	*8*	*9*	*B1*	*B2*	*C*	*D*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*9*
135BM2/94[1]	47	150	37	1	0	0	0	0	0	53	17	95	182	0	72	121	32
970/00[1]	249	28	0	92	0	30	20	0	0	245	126	311	707	0	78	192	561
PTR/94[1]	34	108	0	57	0	0	0	0	0	60	22	143	0	0	0	0	0
324LA/94[1]	433	1384	0	134	0	122	70	0	14	0	22	137	0	0	0	0	0
1190/97[1]	163	211	0	111	569	0	0	0	0	108	21	196	172	0	38	205	219
705/93[2]	279	104	0	144	156	319	0	0	66	237	209	616	820	0	148	293	267
DW1/93[2]	0	110	0	62	48	0	6	0	0	54	40	85	125	0	53	91	56
PB1/93[2]	109	82	0	0	43	96	21	85	53	111	192	203	293	0	0	137	242
243/00[2]	201	717	0	207	1010	310	0	0	0	388	452	680	0	0	0	0	0
455rgh/94[2]	31	56	0	29	0	1	15	0	1	62	3	46	7	0	0	0	0
LR1/93[2]	236	768	0	345	0	61	210	18	35	254	132	403	832	0	0	263	106
289T/00[2]	23	22	51	19	0	5	0	0	0	60	60	71	172	20	0	60	67
480/00[2]	218	70	0	46	0	0	0	0	0	116	0	156	282	57	0	43	64
819/99[2]	147	0	0	96	0	0	0	0	0	240	154	325	630	0	0	249	0
408/99[2]	60	112	0	11	80	0	48	0	0	41	74	13	0	0	0	0	0
848/99[2]	127	0	0	181	0	110	0	0	0	27	77	336	631	0	0	0	0
458R/94[2]	216	0	0	177	0	0	0	0	0	687	367	628	595	0	0	377	0
WK1/933	69	0	36	6	0	0	0	0	0	71	4	143	26	0	11	6	0
40/01[3]	580	0	0	197	0	0	0	0	0	104	52	113	277	30	0	49	42

For all RT-PCRs control gene expression was routinely detected.
[1]Highly invasive strain, [2]low invading strain, [3]non-invasive strain. The numbers represent the relative quantity (as a percentage) of PCR amplicon compared with the candidal housekeeping gene product (elongation factor 1β).

Table 3. Detection of gene families associated with pathogenesis during planktonic culture using RT-PCR

	Secreted aspartyl proteinase (*SAP1–9*)					Phospholipase genes (*B–D*)				Agglutinin-like sequence genes (*ALS1–7, ALS9*)							
Candida albicans	*1–3*	*4–6*	*7*	*8*	*9*	*B1*	*B2*	*C*	*D*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*9*
135BM2/94	0	19	0	80	51	49	0	0	48	87	89	121	107	0	0	19	0
970/00	351	176	0	214	0	150	44	26	27	192	149	326	230	0	0	422	264
PTR/94	779	243	0	57	0	0	48	80	0	217	0	408	179	0	0	45	0
324LA/94	162	201	0	96	0	154	106	0	0	0	0	60	0	0	0	0	0
1190/97	642	233	0	85	0	0	0	0	0	664	136	467	312	0	0	0	0
705/93	842	348	0	504	0	245	96	605	95	490	178	1040	137	0	0	120	0
DW1/93	189	0	0	5	0	32	0	0	68	123	82	398	75	0	0	0	422
PB1/93	1157	414	0	717	704	292	116	378	397	976	0	925	359	0	0	0	0
243/00	143	84	0	102	49	85	39	94	27	67	72	105	77	0	56	29	125
455rgh/94	828	320	0	8	278	145	62	0	0	476	99	363	164	0	0	0	0
LR1/93	81	27	0	66	0	41	14	24	14	56	0	111	14	0	0	0	0
289T/00	45	59	0	20	0	0	0	0	0	23	0	69	24	0	0	0	0
480/00	56	0	0	2	0	0	0	0	0	141	113	199	172	0	0	121	0
819/99	67	55	0	96	36	76	30	0	38	30	0	0	0	0	0	0	0
408/99	140	43	0	81	0	25	0	57	0	68	60	125	87	0	0	14	0
848/99	381	0	0	180	37	76	59	0	0	174	103	265	45	0	0	30	0
458R/94	0	21	0	42	0	9	24	0	0	91	101	145	182	0	0	38	0
WK1/93	0	0	0	0	0	8	0	0	0	9	0	0	0	0	0	0	0
40/01	56	49	0	91	42	25	0	0	8	0	0	52	0	0	0	0	0

For all RT-PCRs control gene expression was routinely detected. The numbers represent the relative quantity (as a percentage) of PCR amplicon compared with the candidal housekeeping gene product (elongation factor 1β).

able to move between epithelial cell junctions, hyphae can track directly through keratinocytes. Epithelial cell penetration was also more strongly associated with strains that demonstrated the vertical invasion profile depicted in Fig. 2B compared with the less direct invasion profile seen in Fig. 2A.

The exact mechanism of hyphal penetration remains unclear. It may occur either through pressure exerted from the growing tip of the hyphae or as mentioned earlier by inducing an endocytotic process. Alternatively, invasion may be facilitated by extracellular hydrolytic enzymes promoting the passage of hyphae. The principal extracellular hydrolytic enzymes produced by *C. albicans* are secreted aspartyl proteinases, phospholipases and lipases (7, 16, 25). In this present study, a definitive role for the expression of secreted aspartyl proteinase and phospholipases in tissue invasion could not be demonstrated (Table 2). Semi-quantitative analysis of PCR products showed that strain differences occurred in the levels of obtained PCR products although these could not be related to invasion profiles.

It was apparent from the RT-PCR analysis that those strains categorized as 'high invaders' were consistent producers of *SAP4–6* despite the level of expression being strain variable. Furthermore, the *SAP4–6* genes were not detected for the two non-invading strains (WK1/93 and 40/01). *SAP4–6* are important for the yeast to hypha transition (16) and so an indirect role for these enzymes in tissue invasion is readily envisaged. These results might be considered to contrast with those of Korting et al. (12), who previously used *efg1*/*cph1* mutants of *C. albicans* SC5314 (exhibiting reduced expression of the hypha-associated genes *SAP4–SAP6*) to demonstrate that *SAP1* and *SAP3* were important causes of epithelial cell damage in the same RHE model. In the studies by Korting et al. (12), epithelial cell injury was determined by lactate dehydrogenase release and this correlated with the presence of *SAP1* and *SAP3* transcripts. However, while our present study focused on RHE invasion and did not incorporate the lactate dehydrogenase release assay, detection of *SAP1–3* transcripts by the majority of strains was evident and it may be the case that tissue damage was still occurring, even for strains where limited RHE invasion was detected.

Phospholipase expression did not appear to correlate with RHE invasion (Table 2), as exemplified by the fact that three highly invasive strains did not produce mRNA for any phospholipases, while the poorly invasive *C. albicans* PB1/93 and *C. albicans* LR1/93 were positive for expression of all four phospholipase genes. Furthermore, phospholipase gene expression actually seemed to be more frequently detected in the absence of the RHE (planktonic growth), which might suggest the possible induction of phospholipase genes through a starvation trigger as opposed to through contact with tissue. A relationship between RHE invasion and agglutinin-like sequence gene expression was similarly not evident. Although there may have been some up-regulation of agglutinin-like sequence genes for certain *C. albicans* strains when cultured in the presence of the RHE. The agglutinin-like sequence gene family, designated *ALS1–ALS7* and *ALS9*, has recently been implicated in facilitating candidal adherence to host tissues (9, 10). The expression of the *ALS* genes in this study agreed in part with previous reports by Green et al. (9), who also used an infected RHE model. Green et al. (9) found consistent expression of *ALS1*, *ALS2*, *ALS3* and *ALS4*, in contrast to the more sporadic detection of *ALS6* and *ALS7*. In our present investigation, a lower incidence of *ALS5* and *ALS6* was found (three of 19 and six of 19 strains respectively). However, only four *C. albicans* strains were used by Green et al. (9) compared to the 19 strains from specifically defined oral infections used in this study, which may account for these apparent differences.

In summary, this investigation has confirmed the effectiveness of the RHE as an *in vitro* model for studying the invasion of *C. albicans* strains as reported by others (12, 21). The CLSM approach has conclusively shown that *C. albicans* invade the oral epithelium by a combination of direct keratinocyte penetration and movement between cells. It is evident though, that heterogeneous behaviour for isolates of *C. albicans*, from a variety of clinical origins, exists. While correlation with CHC remains difficult to ascertain, some

strain types appear to be fundamentally more 'invasive' and therefore inherently better 'equipped' to instigate infections such as CHC.

Acknowledgments

We would like to thank Mrs Kath Allsop for processing and sectioning tissue samples. We are also grateful for the financial support of the School of Dentistry, Cardiff, UK.

References

1. Arendorf TM, Walker DM, Kingdom RJ, Roll JRS, Newcombe RG. Tobacco smoking and denture wearing in oral candidal leukoplakia. Br Dent J 1983: **155**: 340–343.
2. Axell T, Samaranayake LP, Reichart PA, Olsen I. A proposal for reclassification of oral candidosis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1997: **84**: 111–112.
3. Bánóczy J. Follow-up studies in oral leukoplakia. J Maxillofac Surg 1977: **5**: 69–75.
4. 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**: 293–296.
5. Cairns MT, Church S, Johnston PG, Phenix KV, Marley JJ. Paraffin-embedded tissue as a source of RNA for gene expression analysis in oral malignancy. Oral Dis 1997: **3**: 157–161.
6. 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**: 3689–3700.
7. Ghannoum MA. Potential role of phospholipases in virulence and fungal pathogenesis. Clin Microbiol Rev 2000: **13**: 122–143.
8. Gow NA, Brown AJ, Odds FC. Fungal morphogenesis and host invasion. Curr Opin Microbiol 2002: **5**: 366–371.
9. Green CB, Cheng G, Chandra J, Mukherjee P, Ghannoum MA, Hoyer LL. RT-PCR detection of *Candida albicans* ALS gene expression in the reconstituted human epithelium (RHE) model of oral candidiasis and in model biofilms. Microbiology 2004: **150**: 267–275.
10. Hoyer LL. The ALS gene family of *Candida albicans*. Trends Microbiol 2001: **9**: 176–180.
11. Jayatilake JA, Samaranayake Y-H, Samaranayake LP. An ultrastructural and a cytochemical study of candidal invasion of reconstituted human oral epithelium. J Oral Pathol Med 2005: **34**: 240–246.
12. Korting HC, Hube B, Oberbauer S et al. Reduced expression of the hyphal-independent *Candida albicans* proteinase genes *SAP1* and *SAP3* in the *efg1* mutant is associated with attenuated virulence during infection of oral epithelium. J Med Microbiol 2003: **52**: 623–632.
13. Krogh P, Hald B, Holmstrup P. Possible mycological etiology of oral mucosal cancer: catalytic potential of infecting *Candida albicans* and other yeast in production of *N*-nitrosobenzylmethylamine. Carcinogenesis 1987: **8**: 1543–1548.
14. Monod M, Togni G, Hube B, Sanglard D. Multiplicity of genes encoding secreted aspartic proteinases in *Candida* species. Mol Microbiol 1994: **13**: 357–368.
15. Monod M, Hube B, Hess D, Sanglard D. Differential regulation of SAP8 and SAP9, which encode two new members of the secreted aspartic proteinase family in *Candida albicans*. Microbiology 1998: **144**: 2731–2771.
16. Naglik JR, Challacombe SJ, Hube B. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. Microbiol Mol Biol Rev 2003: **67**: 400–428.
17. Niewerth M, Korting HC. Phospholipases of *Candida albicans*. Mycoses 2001: **44**: 361–367.
18. Park H, Myers CL, Sheppard DC et al. Role of the fungal Ras-protein kinase A pathway in governing epithelial cell interactions during oropharyngeal candidiasis. Cell Microbiol 2005: **7**: 499–510
19. Phan QT, Fratti RA, Prasadarao NV, Edwards JE, Jr, Filler SG. *N*-cadherin mediates endocytosis of *C. albicans* by endothelial cells. J Biol Chem 2005: **280**: 10455–10461.
20. Schaller M, Schaefer 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 Biol 1998: **29**: 605–615.
21. Schaller M, Korting HC, Schaefer W, Bastert J, Chen W, Hube B. Secreted aspartic proteinase (Sap) activity contributes to tissue damage in a model of human oral candidosis. Mol Microbiol 1999: **34**: 169–180.
22. Schaller M, Januscke E, Schackert C, Woerle B, Korting HC. Different isoforms of secreted aspartyl proteinases (Sap) are expressed by *Candida albicans* during oral and cutaneous candidosis *in vivo*. J Med Microbiol 2001: **50**: 743–747.
23. 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**: 652–657.
24. Schaller M, Korting HC, Borelli C, Hamm G, Hube B. *Candida albicans*-secreted aspartic proteinases modify the epithelial cytokine response in an *in vitro* model of vaginal candidiasis. Infect Immun 2005: **73**: 2758–2765.
25. Schaller M, Borelli C, Korting HC, Hube B. Hydrolytic enzymes as virulence factors of *Candida albicans*. Mycoses 2005: **48**: 365–377.
26. Schofield DA, Westwater C, Warner C, Nicholas PJ, Paulling EE, Balish E. Hydrolytic gene expression during oesophageal and gastric candidiasis in immunocompetent and immunodeficient gnotobiotic mice. J Infect Dis 2003: **188**: 591–599.
27. Sherman RG, Prusinski L, Ravenel MC, Joralmon RA. Oral candidosis. Quintessence Int 2002: **33**: 521–532.
28. Sitheeque MA, Samaranayake LP. Chronic hyperplastic candidosis/candidiasis (candidal leukoplakia). Crit Rev Oral Biol Med 2003: **14**: 253–267.
29. Soll DR. *Candida* commensalism and virulence: the evolution of phenotypic plasticity. Acta Tropica 2001: **81**: 101–110.
30. Sugiyama Y, Nakashima S, Mirbod F et al. Molecular cloning of a second phospholipase B gene, caPLB2 from *Candida albicans*. Med Mycol 1999: **37**: 61–67.
31. Villar CC, Kashleva H, Mitchell AP, Dongari-Bagtzoglou A. Invasive phenotype of *Candida albicans* affects the host proinflammatory response to infection. Infect Immun 2005: **73**: 4588–4595.
32. Williams DW, Potts AJC, Wilson MJ, Matthews JB, Lewis MAO. Characterisation of the inflammatory cell infiltrate in chronic hyperplastic candidosis of the oral mucosa. J Oral Pathol Med 1997: **26**: 83–89.
33. Williams DW, Bartie KL, Potts AJ, Wilson MJ, Fardy MJ, Lewis MA. Strain persistence of invasive *Candida albicans* in chronic hyperplastic candidosis that underwent malignant change. Gerodontology 2001: **18**: 73–78.