Genotypic variation of *Candida albicans* during orthodontic therapy

Wilson Lee[1], Bernard K.M. Low[1], Lakshman P. Samaranayake[2], Urban Hagg[1]

[1] *Orthodontics, Faculty of Dentistry, The University of Hong Kong, 34 Hospital Road, Hong Kong SAR,* [2] *Oral Bio-Sciences, Faculty of Dentistry, The University of Hong Kong, 34 Hospital Road, Hong Kong SAR*

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1. ABSTRACT

Candida is a common oral, opportunistic pathogen. The aim of this longitudinal study was to analyze the genotypes of sequential isolates of *Candida albicans* in a cohort of healthy consistent candidal carriers ('carriers') during fixed orthodontic appliance therapy. 11 of 97 subjects were 'carriers'. *Candida* isolates from baseline samples (T0) and sequential visits after insertion (T1 to T10) were speciated and RAPD fingerprint patterns of 101 sequential *Candida albicans* isolates were analyzed with PCR and gel electrophoresis. A similarity coefficient (S_{AB}) for each pair of strains was calculated and clusters of similar strains grouped using Dendrogram analysis of the RAPD gel profiles. The composite dendrogram of all isolates indicated that the *Candida* populations in this cohort are genotypically dissimilar although collected from the same geographic locale and, from a similar healthy, age group. Genotypes of a majority of the cohort (6 of 11 consistent carriers) show minor evolutionary variations in profile and genotypic 'shuffling', implying this may be a survival mechanism of this common, human, opportunist pathogen residing in a hostile oral environment.

2. INTRODUCTION

Insertion of an orthodontic appliance into the oral cavity vastly increases the number of plaque retention areas (1, 2) and may have an effect on the oral microbial ecosystem (3). While bacteria are the predominant commensals of the oral cavity, fungi belonging to the genus *Candida* are also found intraorally in some 50-60 % of the human population (4).

Candida is well recognized as an opportunistic pathogen which may cause superficial, mucosal or skin infections or deep infections of the human body, especially in immunocompromised patients. There has been an alarming rise in opportunistic fungal infections since the 1980's due to the exponential worldwide increase of HIV infected individuals, and those on radiation and immunosuppressive therapy. Infections caused by *Candida* – called candidiasis or candidosis are frequently seen on mucosal and cutaneous surfaces including the oral cavity, and *Candida albicans* is the most frequently isolated species from such lesions. Other *Candida* species isolated from oral lesions include *Candida parapsilosis, Candida tropicalis, Candida glabrata,*

Candida krusei, Candida kefir, Candida guilliermondii and *Candida dubliniensis*.

The prevalence of yeasts in the oral cavities of healthy Hong Kong Chinese was studied in 300 individuals and was found to be 24%, with *Candida albicans* forming 77% of all yeasts isolated (5). Subjects taking medication or wearing dentures had a significantly higher oral yeast prevalence of 37% and 45%, respectively. A longitudinal study of 22% yeast prevalence in an ethnic Chinese cohort wearing orthodontic appliances has also been reported but only limited to a three month period in 27 subjects (3).

There are only a few reported studies in the literature on genotypic profiles of *Candida* isolates from healthy individuals (6, 7). Most of the healthy subjects in these studies were in the control group and the duration of studies only a few months. In one longitudinal study of genotypic diversity of *Candida* species within healthy subjects, Kam and Xu (6) found that changes of species and genotypes over the study period within the same host, but the study lasted only four months. A recent study in Sichuan, China investigated the genotypic profiles of *Candida albicans* isolates from patients with erosive- and non-erosive oral lichen planus (7). Random Amplification of Polymorphic DNA (RAPD) analyses with a random primer revealed four different genotypes among all isolates, and there was significant difference in genotypes of the two groups. They concluded that some *Candida albicans* isolates with special genotypic profiles might contribute to the development and progression of oral lichen planus.

There have been longitudinal studies on the genotypic characteristics of oral *Candida albicans* isolates from immunocompromised patients (8-11) such as those with HIV disease. Other recent studies on genetic relatedness of oral yeasts within and between patients include investigations of marginal periodontitis patients and healthy controls (12, 13), and those with thrush and denture stomatitis (13).

It is important to study, longitudinally the phenotypes and genotypes variations of a fungal pathogen since it is of benefit for clinical and epidemiological studies to provide information on sources, carriage and transmission of infection, and on relations between strain types and properties such as antifungal resistance and virulence (14). *Candida albicans* with characteristic of high-frequency phenotypic switching and variation before first episode of oral thrush were far more antifungal resistant than commensal isolates from healthy individuals (15). Similarly, longitudinal genotypic variations have been studied extensively to better understand the antimicrobial susceptibility and epidemiology (10, 16-19). In general, there is greater genotypic diversity of *Candida albicans* in healthy states than in diseased states, particularly the immunocompromised patients (20). These studies have shown in general that there is no significant difference in the distribution of genotypes between immunocompromised and healthy patients (21) and the possible existence of minor shifts in genetic traits of sequential candidal isolates – so called genetic 'shuffling'. Understanding genetic characteristics of *Candida albicans*' increases our comprehension of the pathogenesis of candidosis and how these organisms persist in the oral cavity in an extremely hostile environment. Furthermore, sequential follow-up of genetic variation of these species over a period of time may yield clues to the evolution of drug resistance in *Candida* which is not infrequent.

To our knowledge there has been no study in the indexed literature on the genotypes of *Candida albicans* in healthy individuals wearing orthodontic appliances over a prolonged period. Thus, the objectives of this study were to evaluate the prevalence of *Candida* species in a group of consecutive patients undergoing fixed orthodontic appliances therapy (FOA); and then to investigate the genotype and phenotype variation of sequential isolates of *Candida albicans* in those who consistently carried the organism at each return visit over a period of 12 months.

3. MATERIALS AND METHODS

3.1. Subjects

A cohort of 112 ethnic Chinese patients were recruited from the Postgraduate Orthodontics clinic of the Prince Philip Dental Hospital, Sai Ying Pun, Hong Kong from May to November 2005 (43 males and 69 females) with a mean age of 17.7 years; median 16.2 years; range 10.3 to 39.7 years. All patients were healthy, non smokers, who had not been on antibiotic or steroid therapy for a period of 6 months prior to study. The orthodontic treatment plan included at least one full arch fixed orthodontic appliance (FOA) therapy with planned treatment duration not less than one year. All patients received oral hygiene instructions and flossing instructions before the start of and during FOA therapy. Baseline data (T0) of oral carriage of *Candida* species was obtained prior to appliance insertion by collecting oral rinse samples as described below. FOAs were then inserted and rinse samples obtained on sequential visits (T1 to T10), over a 12-month period.

A total of 97 subjects (87%) (38 males and 59 females) remained in the study after 12 months and altogether 760 oral rinse samples were obtained since the beginning of FOA therapy. Collection of oral rinse samples was performed approximately 45 days apart.

Consistent candidal carriers, defined as those who generated at least a single colony forming unit (CFU) of growth of *Candida* from oral rinse samples on culture, were identified. These *Candida albicans* isolates were collected for phenotyping, genotyping and dendrogram analysis as described below.

Informed consent was obtained from the patients or their parents/guardians before the experiments and the experimental protocol was approved by the institutional Ethics Committee of the University of Hong Kong.

3.2. Collection of *Candida* isolates and growth conditions

The yeasts were collected using the oral-rinse technique of Samaranayake *et al.* (22). In brief, each

patient was requested to rinse the mouth for 60 seconds with 10ml Phosphate Buffered Saline or PBS (pH 7.2, 0.1M) supplied in a sterile 15ml Universal container. The sample was expectorated into the container and transferred to the laboratory immediately afterwards. Oral rinse was concentrated by spinning at 1700g for 10 minutes in the centrifuge (Beckman Instrument Incorporation. Model: CS-15R Centrifuge. Palo Alto, California, USA) and the pellet resuspended in 1ml sterile PBS (pH 7.2. 0.1M) and vortex-mixed for 30 seconds. The concentrated oral rinse was then dispensed onto a Sabouraud's dextrose agar (SDA; Oxoid) in an Archimedean spiral using a Spiral Plater (Spiral Biotech. Model: Autoplate 4000. Exotech Inc. Gaithersburg, Maryland, USA).

The plates were then incubated aerobically for 48 hours at 37°C. A single yeast colony per sample was randomly chosen by the same investigator and subcultured on SDA and incubated for 18 hours at 37°C. The pure yeast cultures obtained was then harvested, suspended in distilled water, aliquoted in sterile vials and stored at -70°C prior to phenotyping and genotyping.

The organisms were phenotyped using the germ tube test, growth at 45°C, chlamydospore production (23) and, API® ID 32C assimilation tests (BioMérieux, France). API® ID 32C system utilized the ability of *Candida albicans* isolates to assimilate 31 different carbohydrates as sole sources of carbon. After 24 to 48 hours of incubation, growth in each cupule was read using the ATB® Expression™ and identification of biotype was performed. The yeasts were then stored in 2ml microtubes (Sarstedt, Numbrecht, Germany) at -70°C for genotyping. Patients who consistently carried *Candida* species throughout the study period were considered as consistent candidal carriers, and *Candida albicans* isolates from these patients were selected for the genotyping study.

3.3. Preparation of DNA for RAPD Analysis

DNA for RAPD analysis was prepared as described by Samaranayake *et al* (8). Yeast obtained from stock cultures stored at -70°C were subcultured on SDA plates at 37°C for 20 hours. Cells were resuspended thoroughly in 586µl of 50mM EDTA. 15µl of 20mg/ml lyticase (Promega®, Madison, WI, USA) was gently added to cell suspension and incubated at 37°C for 60 minutes to digest the cell walls, then cooled to room temperature.

The sample was centrifuged again at 16,000g for 2 minutes, the supernatant removed, 300µl of Nuclei Lysis Solution (Promega®) was added to the cell pellet and gently mixed, afterwards 100µl of Protein Precipitation Solution (Promega®) was added and vortex mixed for 20 seconds and the sample let to sit on ice for 5 minutes. The sample was then centrifuged at 16,000g for 30 minutes at 4°C. The supernatant containing the DNA was transferred to a clean 1.5ml microcentrifuge tube containing 300µl of isopropanol.

The microcentrifuge tube was gently mixed by inversion until the thread-like strands of DNA form a visible mass, and the sample was centrifuged at 13,000-16,000g for 30 minutes. The supernatant was decanted and

the tube was drained on clean absorbent paper. 300µl of 70% ethanol was added and the tube was gently inverted several times to wash the DNA pellet. The sample was centrifuged again at 16,000g for 15 minutes, and the ethanol was aspirated. The tube was drained with pipette and the pellet allowed to air-dry for 5-10 minutes. 50µl of DNA Rehydration Solution (Promega®) which was a Tris-EDTA buffer was added, together with 1.5µl of RNase Solution (Promega®) to the purified DNA sample. The sample was vortexed for 1 second. The sample was centrifuged briefly in a microcentrifuge for 5 seconds, and the liquid was collected and incubated at 37°C for 15 minutes. The DNA was rehydrated by Tris-EDTA buffer and stored the DNA at 4°C.

3.4. Primer sequences

Optimal primer for genotyping of consistent candidal carriers was found using custom synthesized primers (Gibco BRL, Hong Kong) which has been previously used by us (8). The following primers were used in two randomly chosen consistent candidal carriers to obtain the optimal RAPD profiles results: OBU1 (5' - CAC ATG CTT - 3'), OBU2 (5' - CAC ATG CCT - 3'), OBU3 (5' - CGC ATG CTT - 3'), RSD10 (5' - CCG CAG CCA - 3'), RSD11 (5' - GCA TAT CAA TAA GCG GAG GAA AAG - 3'), RSD12 (5' - GGT CCG TGT TTC AAG ACG - 3'). Since RAPD profiles of these two carriers suggested RSD11 was the most appropriate primer, we used RSD11 for subsequent genotyping of the consistent candidal carriers.

3.5. RAPD analysis

PCR cycles were performed in GeneAmp® PCR system 9700 (Applied Biosystems, USA). A 50µl volume of the PCR master mix contained approximately 50-100 ng of yeast DNA template, 5µl of PCR buffer (10 × PCR buffer is 0.5 M KCL-0.2 M Tris (pH = 8.4), a 200µM concentration of each dNTP, 25mM $MgCl_2$, a 1µM concentration of primer, and 1.5U of Taq polymerase (Life Technologies, Frederick, Maryland, USA.).

The first five cycles included 30 seconds denaturation at 94°C, and 2 minutes of annealing at 39°C with primer RSD11; this was followed by 2 minutes of primer extension and then followed by 45 cycles of 30 seconds denaturation at 94°C, 2 minutes annealing at 45°C and 2 minutes primer extension at 72°C. The reaction was then held at 72°C for 10 minutes. Control tubes without template DNA were included in each run, and reproducibility was checked for each reaction (24, 25). The PCR products were electrophoresed in 1.5% agarose gel for approximately 2 hours in TBE buffer (89 mM Tris/HCl, 89nM boric acid, 2.5mM EDTA, pH 8.0), stained with ethidium bromide and visualized under UV transillumination. The RAPD analysis was repeated on two further separate occasions with strains recovered from the stock kept at -70°C, and was found to yield the identical profile on all three occasions.

3.6. Computer-assisted analysis of data and histogram and dendrogram generation

The different banding positions of RAPD fingerprinting patterns of multiple isolates of *Candida*

Table 1. Demographic data of patients who were identified as consistent Candidal carriers

Patient ID	Sex	Age (Yr)	Visit No	*Candida albicans* isolates identified
P1	M	14	11	11
P2	M	15	7	7
P3	M	17	9	9
P4	F	13	11	10
P5	F	18	11	10
P6	M	22	11	10
P7	F	25	11	10
P8	F	15	10	7
P9	F	16	11	10
P10	F	12	7	7
P11	M	14	11	10
			Total 110	101

M: Male, F: Female

albicans of consistent candidal carriers were analyzed using the Dendron® 3.0 program (Solltech Inc.). For the analysis of relationships among a number of strains, Dendron® constructs histograms and dendrograms by the unweighted pair group method (26). Each lane pattern was compared to every other lane pattern through computations of a similarity coefficient, S_{AB}, which compares the band positions. Two gel patterns in which no bands co-migrate result in an S_{AB} of 0 and two patterns in which all bands co-migrate and exhibit identical band positions result in an S_{AB} of 1.0. The value of S_{AB} of 0.8 was arbitrarily used as the threshold for clustering of similar strains. Thus, in the Dendron® program, the two strains with the highest S_{AB} are grouped, with a branch-point corresponding to the S_{AB}. The program then searches for the strain–strain or strain–unit pair with the next highest S_{AB} and groups them, with a branch point corresponding to that S_{AB}. The process continues to include all strains. A unit can be two or more strains, and a branch-point for a unit–strain or unit–unit is determined by the mean S_{AB} between each member of the unit and another strain or unit. Therefore, branch-points involving a unit are not as accurate as S_{AB} values calculated for two strains.

In dendrogram construction, the data for two Candida albicans isolates or two banding patterns (lanes A and B) can be represented by the binary values 0 and 1, where 0 indicates no band at a position and 1 indicates a band at that position. The similarity coefficient (SAB) for the pair of strains, A and B, was calculated by the formula:

$$SAB = 1 - \left[\sqrt{(b+c)/(a+b+c)} \right]$$

where a is the number of bands common to both lanes A and B (coded as 1,1), b is the number of bands in lane A with no counterpart in lane B (coded as 1,0) and c is the number of bands in lane B with no counterpart in lane A (coded as 0,1). The pair of strains with the highest SAB value is grouped into a unit with a branch-point corresponding to its SAB value.

To develop the dendrogram in the current study, the PCR gel images of all the isolates were captured by a Gel-Doc CCD camera (Bio-Rad®) and then digitized into the Dendron® database, normalized according to invariable band positions and relocated with Dendron® software according to the manufacturer's instructions.

4. RESULTS

4.1. Determination of consistent and inconsistent sequential oral carriage of *Candida* species

Demographic data of patients who were identified as Consistent candidal carriers are shown in Table 1. In total out of 97 individuals in the cohort, 11 (or 11%) yielded *Candida* from oral rinse samples at each visit. Oral candidal carriage before insertion of the appliance, designated as T0, was 32% (Figure 1). The candidal carriage gradually increased and peaked to 50% at T5 (*i.e.,* the fifth visit approximately five months after appliance insertion), then decreased and levelled to around 45%, and finally rose again at T10 (49%). Significant differences in oral candidal carriage rate in the cohort were noted between T0 versus T5, and T0 versus T10 (p-value less than 0.05, chi-square test).

Figure 2 illustrates the profile of the candidal carriers versus non-candidal carriers from T0 to T10. 11% of patients consistently carried *Candida* species in the oral cavity, and were deemed as consistent carriers; 64% of patients were inconsistent carriers, *i.e.,* they carried *Candida* species erratically, and 25% were consistent non-candidal carriers, *i.e.,* they never carried *Candida* species throughout the experimental period of 12 months. In total, 75% at least once carried *Candida* species throughout this period; 15% changed from a non-candidal carrier to a candidal carrier state after FOA insertion and, none of the subjects converted from a carrier to a non-carrier after appliance insertion.

4.2. Non-albicans *candida* carriage

Other non-albicans *Candida* species identified from the consistent candidal carriers were: *C. tropicalis* (4 isolates), *C. parapsilosis* (2 isolates), *S. cerevisiae* (2 isolates), *C. globosa* (1 isolate).

4.3. Biotypes of consistent candidal carriers

A total of 110 *Candida* isolates were collected from all the visits of the 11 consistent candidal carriers. Speciating these isolates using the commercially available API® ID 32C system indicated that 101 strains (92%) belonged to *Candida albicans*. Of these *Candida albicans* isolates, 83% belonged to the same major biotype with an API® ID 32C profile of 7347340015 and the remainder belonged to 9 other minor biotypes as per the typing method of Williamson *et al.* (27) (Table 2). One patient (P11) carried only the most common biotype (7347340015) throughout all the visits, while others carried a mixture of biotypes.

4.4. RAPD genotyping of sequential clinical *Candida albicans* isolates

Clonal variability of 101 sequential *Candida albicans* isolates from 11 patients (patients P1 to P11) was determined by RAPD genotyping. Primer RSD11 showed the best molecular profile resolution and hence was used throughout. Representative RAPD profiles of sequential clinical isolates of one individual (patient P10 with 7 total visits) is shown (Figure 3). In general, the RAPD profiles of these sequentially isolated strains indicated that they

Table 2. Biotyping results of a total of 101 *Candida albicans* isolates from 11 patients using the API® 32C system

Biotype	No.	%
7347340015	83	82.1%
7146340015	7	6.9%
7147340015	3	3.0%
7347340011	2	2.0%
7143340015	1	1.0%
7146340011	1	1.0%
7147340011	1	1.0%
7346340011	1	1.0%
7347340415	1	1.0%
7753347777	1	1.0%
Total	101	100.0%
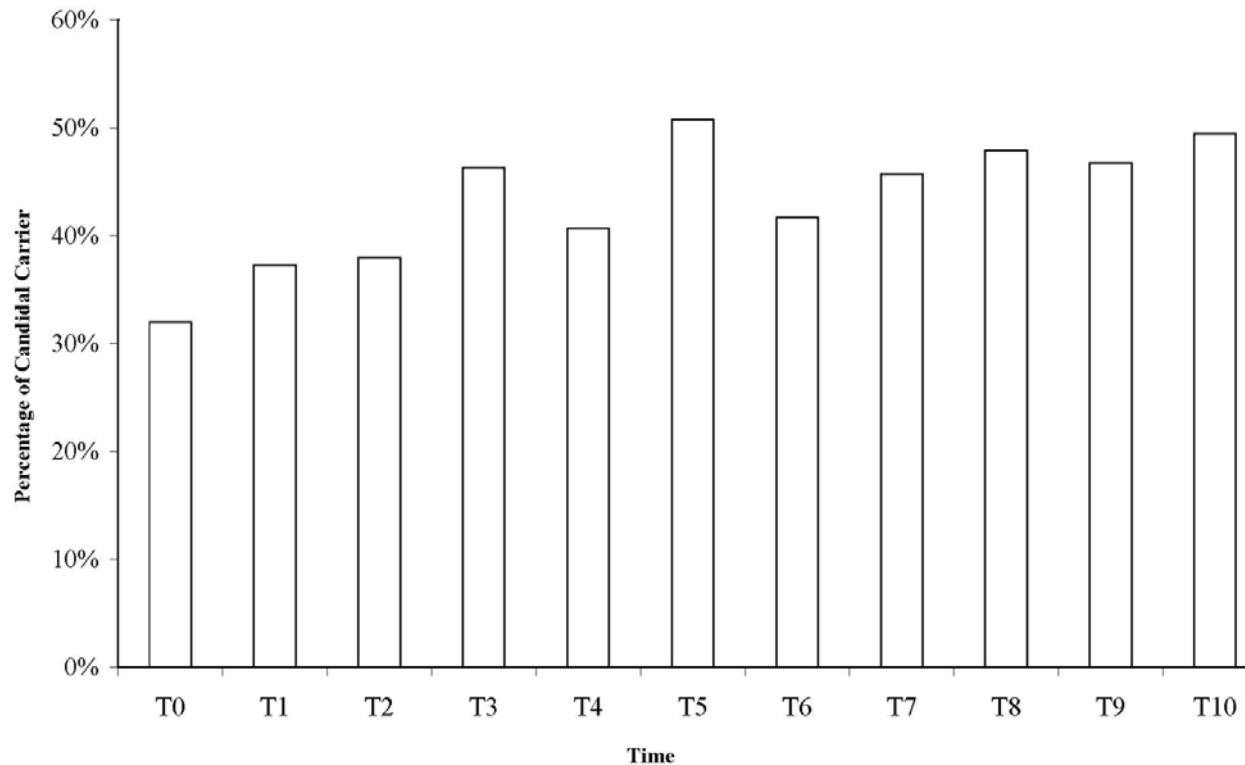
Figure 1. Oral candidal carriage rate of the study cohort over a one year period (sampled on 11 visits from T0 to T10).
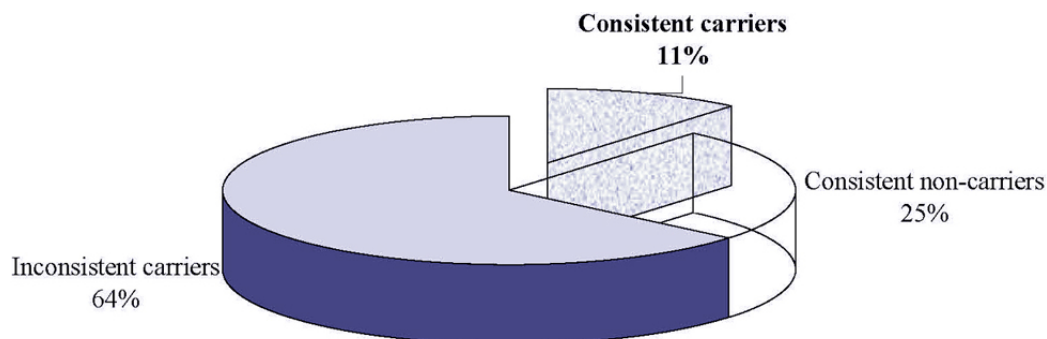

Figure 2. Percentage of consistent and inconsistent candidal carriers, and non-candidal carriers from T0 to T10.

belonged to the identical genotype throughout the study period, from T0 to T10. Another example shows the genotypes of *Candida albicans* isolates from, visits T0 to T10, of patient P3 (Figure 4). As can be seen the genotypes of isolates from visits T7 and T8 in this patient were markedly different from the earlier visits indicating a genetic 'shift' in this particular isolate after a 12 month period. Finally, patient P1 shows persistence of identical genotypes from visits T0 to T10, although some minor bands variations were noted in some isolates (Figure 5).
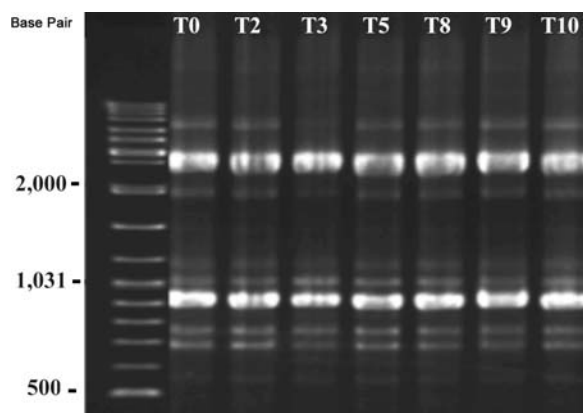
Figure 3. RAPD gel profiles of 7 sequential oral *Candida albicans* isolates from patient P10 (using the primer RSD11). Note the consistent genotypic profiles throughout the study period.
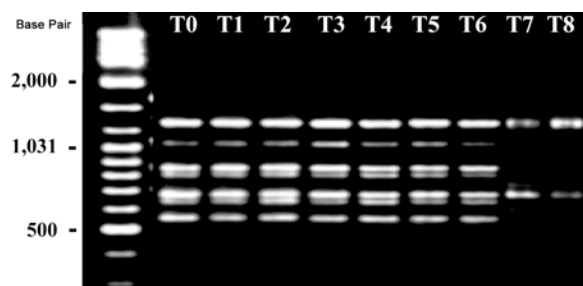

Figure 4. RAPD gel profiles of 9 sequential oral *Candida albicans* isolates from patient P3 (using the primer RSD11). Note the genotypic 'shuffling' in late visits T7 and T8.
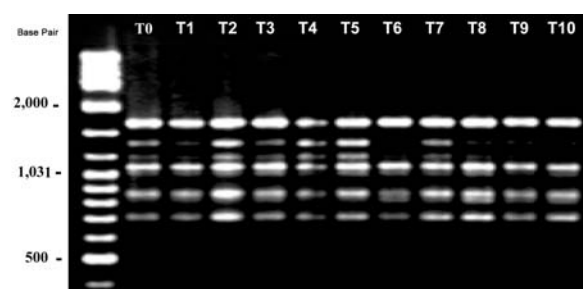

Figure 5. RAPD gel profiles of 11 sequential oral *Candida albicans* isolates from patient P1 (using the primer RSD11). Note the sporadic appearance of new genotypes indicating genetic 'shuffling'.

4.5. Dendrogram analysis

Dendrograms generated by the Dendron® software for sequential *Candida albicans* isolates of the eleven consistent candidal carriers are shown (Figure 6). Further examples of genotype variations in isolates during the study period (from T0 to T10) are given below. Isolates from three patients (P1, P8, P10) showed no changes in genotype profile throughout the observation period as

shown by the single 'tightly connected' cluster with a S_{AB} threshold value of greater than or equal to 0.80. On the other hand we noted two 'tightly connected' clusters for patient P2: Cluster I had four isolates, and Cluster II had three isolates at S_{AB} values of greater than or equal to 0.80 (Figure 6). All except one patient (P8) had at least one set of identical strains (*i.e.,* $S_{AB} = 1.0$).

Interestingly, the genotypes of the late visits seemed to be markedly different in all eleven patients when compared with the genotypes isolated from early visits. For examples, in patient P2, isolates from T8, T9, T10 formed a closely related cluster different from those of the earlier visits (Figure 6). In addition, in patient P3, late visits of T7, T8 formed a cluster which was connected to other cluster of earlier visits at S_{AB} of 0.73 (Figure 6). For patient P9, the first two visits after insertion of FOA exhibited identical strains but these were unrelated to those from other eight visits which were identical (Figure 6). Similar findings were found in P4, P6, P7, P8, P10 and P11. This phenomenon indicates a possible gradual genetic 'drift' that occurs in these *Candida albicans* isolates of FOA wearers over the study period.

The composite dendrogram of all 101 sequentially isolated oral isolates of *Candida albicans* from 11 healthy patients during FOA therapy showed that they were genetically diverse and formed multiple clusters (Figure 7). There were 21 tightly connected clusters at S_{AB} of 0.8, and 18 clusters at a lower S_{AB} of 0.6. We noted extensive variations of genotypes of *Candida albicans* among the subjects, with 65 genotypes among 101 isolates. There were only minor changes amongst the genotypes of *Candida albicans* in six candidal carriers (P1, P5, P8, P9, P10, P11) implying the phenomenon of 'shuffling' within one genetic group, but in the remaining five patients, genotypes significantly differed over the study period, with new genotypes emerging especially after a prolonged period indicating a possible genetic 'shifts' rather than a 'genetic drift or shuffling' (for instance P2 [T8, T9, T10]), P3 [T7, T8], P4 [T10], P6 [T8, T9, T10], P7 [T10]).

5. DISCUSSION

Oral yeast carriage rate in healthy populations ranges from 2.0% to 71% based on numerous studies (28, 29). In our study, oral prevalence of *Candida* before treatment (at T0) was 32% which is higher than the prevalence rate of 24% previously reported in healthy ethnic Chinese adults (30), and 12.5% in healthy Chinese children (31). This figure, however, is comparable to the 34% reported in a healthy cohort of Chinese vegetarians (32). There is a single study conducted south-, east- and west-central China who reported a very high oral yeast carriage rate of 66% in healthy rural adults (33). With the exception of the latter study, our baseline data is comparable to those of others in the literature.

To our knowledge the current study is the longest longitudinal investigation on the genotypes of *Candida albicans* in a group of healthy candidal carriers, reported in the indexed literature. We were able to identify 11% of
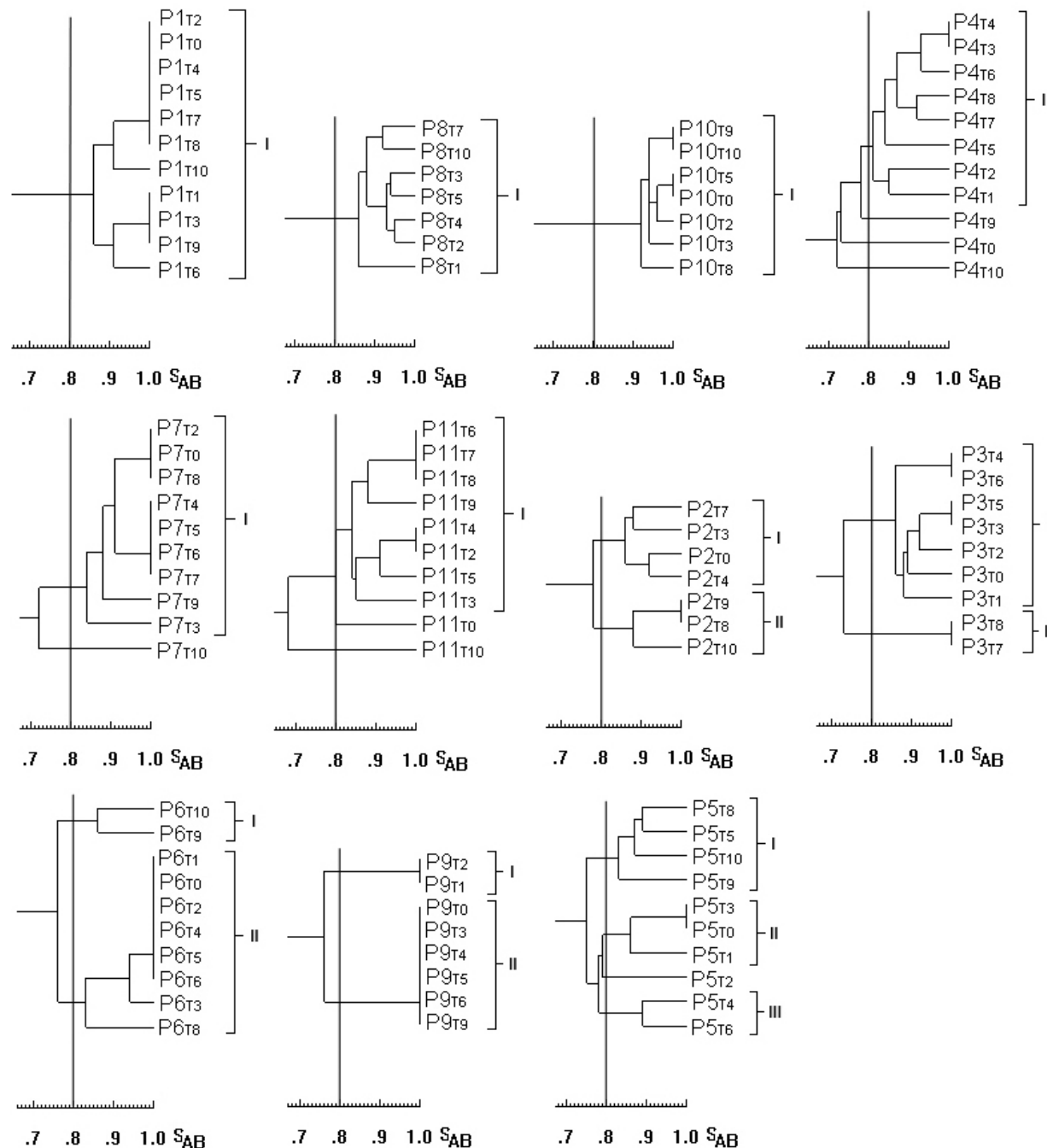
Figure 6. Dendrograms of RAPD profiles of *Candida albicans* from all 11 candidal carriers (P1 to P11), arranged according to the number of clusters, in ascending order.

consistent carriers in the cohort by regularly collection of samples over a 12 month period (from T0 to T10). It is still unknown why some patients, 25% in our study, do not carry *Candida* species throughout the experiment. Further studies on the nature of the buccal cells and saliva and other immune factors of these individuals should shed some light on the reasons why some individuals are prone to oral candidal carriage while others are not.

It is known that poor oral hygiene leading to increased plaque accumulation can increase the oral yeast carriage in patients undergoing FOA therapy (3) while on the contrary more stringent advice and oral hygiene instructions to patients can possibly overcome increased plaque levels in patients wearing FOA. The gradual increase of candidal carriage after insertion of FOA noted in our study (Figure 1) appears to be mainly due to the wearing of appliance, and not due to the deterioration of
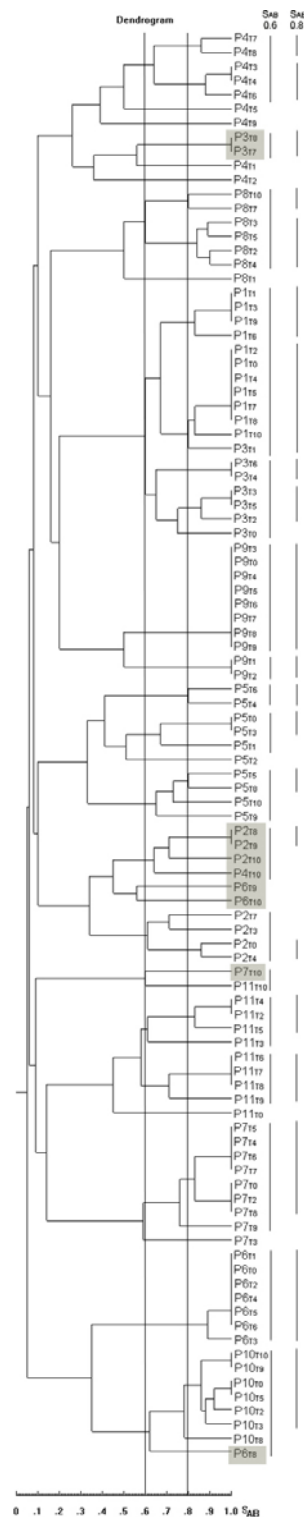
Figure 7. Composite dendrogram generated for 101 sequentially isolated oral isolates of *Candida albicans* during FOA therapy of 11 patients (P1 to P11). 21 clusters were identified at S_{AB} value of 0.80, while 18 clusters were identified at S_{AB} value of 0.60. Shaded areas indicate the genotypes of *Candida albicans* from 5 patients (P2 [T8, T9, T10]), P3 [T7, T8], P4 [T10], P6 [T8, T9, T10], P7 [T10]) which demonstrated 'shuffling' and random distribution of the genotypes.

oral hygiene as our cohort regularly visited hygienists for professional cleaning on average every four months during the study period.

In total 14% of the subjects converted from the state of a non-candidal carrier to candidal carrier during the study period. In a previous 3-month study at our institution we have reported a similar observation of 19% conversion, but no conclusions could be drawn due to the small sample size (3).

Candida albicans was the most common yeast isolated in our subjects which is similar to previous reports (28, 29, 32). In a recent study comparing yeast carriage between North American and mainland Chinese reported that 39% of rural adults in Northern China carried *Candida parapsilosis*, while only 9% carried *Candida albicans* (33). The latter finding is markedly different from previous studies including the current observations. This could possibly be explained by the societal factors which might relate to lifestyle, diet, regular dental care which favour the selection of *Candida albicans* over other species, especially in the Western countries. This contention is further supported by our previous studies, where *Candida albicans* was the predominant oral *Candida* species, in ethnic Chinese in the city of Hong Kong, with perhaps the most westernized lifestyle in China (33).

The predominance of a single biotype of *Candida albicans* in candidal carriers, as noted here, has been reported previously (34-37). Recently, Song *et al.* reported three predominant biotypes of *Candida albicans* obtained from healthy subjects in Sweden, one of which was the same biotype as ours (7347340015) (12). This indicates a wide geographic distribution of this biotype, confirming a previous report of Tsang *et al.* who found the identical biotype in Hong Kong, Germany and England (37).

All except one subject (P8) in our study carried at least one pair of genetically identical isolates during the 12-month study period. This was more common in some patients than others, for examples, most of the *Candida albicans* isolates of patients P1, P6, P7 and P9 were genetically identical throughout this period (Figure 6). This finding implies that genetic shuffling occurs in some strains of colonizing *Candida* but not in others, a phenomenon which warrants further study. Previous workers who conducted similar studies have noted that healthy individuals exhibited more diverse genotypes as well as genetic clusters of *Candida* than patients with HIV infection (8), symptomatic oral candidiasis (12), and marginal periodontitis (12, 20). Similarly, Xu *et al.* found greater genotypic diversity in healthy subjects carrying *Candida albicans* than those derived from clinical specimens, regardless of the HIV status of subjects (38).

A number of workers, including us, have previously demonstrated the phenomenon of genetic 'shuffling' in *Candida albicans* strains during progression of HIV disease (8, 10, 16-18). However, to our knowledge, this is the first long term study in a healthy cohort extending over a period of 12 months. This phenomenon

of genotypic 'shuffling' is likely to be due to the evolutionary changes in the candidal genome due to adverse environmental pressures that always exist in the oral cavity.

Nonetheless, our data indicate that genotypes of 6 of 11 patients did not exhibit major genotypic shifts during the study period. This concurs with data of Whelan *et al.* who noted identical *Candida albicans* strains causing recurrent infection in AIDS patients over a six-month study period (39). Similar findings have been reported by others and they postulated that the same strain could survive over a prolonged period in the oral cavity of HIV-infected individuals due to the defects in host immunity (9, 39, 40). However, our results imply that the same *Candida* strain may persist intra-orally without undergoing major genetic changes even in healthy individuals with a robust immune system. Yet, the reason why some strains persist without genetic changes and others resort to genetic 'shuffling' remains elusive and warrants further study.

It has been suggested that, in studies of this nature, multiple strains of *Candida* species should be selected at each visit for genetic analysis because of the polyclonality of oral *Candida* (8, 12, 13, 41), although there are other studies where collection of a single isolate of *Candida* species per visit has been performed (9). In the current study of 97 patients with 11 consistent candidal carriers, selection of multiple strains at each visit was prohibitively expensive and hence not performed. Yet our data provides a fascinating insight into the behavior of *Candida* species within the oral environment and show us a glimpse of a survival mechanism of this common, human, opportunist pathogen.

6. ACKNOWLEDGEMENTS

7. REFERENCES

1. N. E. Atack, J. R. Sandy and M. Addy: Periodontal and microbiological changes associated with the placement of orthodontic appliances. A review. *J Periodontol*, 67(2), 78-85 (1996)

2. B. U. Zachrisson: Gingival condition associated with orthodontic treatment. II. Histologic findings. *Angle Orthod*, 42(4), 353-357 (1972)

3. U. Hagg, P. Kaveewatcharanont, Y. H. Samaranayake and L. P. Samaranayake: The effect of fixed orthodontic appliances on the oral carriage of *Candida* species and Enterobacteriaceae. *Eur J Orthod*, 26(6), 623-629 (2004)

4. L. P. Samaranayake and T. W. MacFarlane: An in-vitro study of the adherence of *Candida albicans* to acrylic surfaces. *Arch Oral Biol*, 25(8-9), 603-609 (1980)

5. C. M. Sedgley and L. P. Samaranayake: The oral prevalence of aerobic and facultatively anaerobic gram-

negative rods and yeasts in Hong Kong Chinese. *Arch Oral Biol*, 39(6), 459-466 (1994)

6. A. P. Kam and J. Xu: Diversity of commensal yeasts within and among healthy hosts. *Diagn Microbiol Infect Dis*, 43(1), 19-28 (2002)

7. X. Zeng, Q. M. Chen, M. H. Nie and B. Q. Li: The genotypic profiles of *Candida albicans* isolates from patients with oral lichen planus. *Sichuan Da Xue Xue Bao Yi Xue Ban*, 36(2), 193-195 (2005)

8. Y. H. Samaranayake, L. P. Samaranayake, R. S. Dassanayake, J. Y. Yau, W. K. Tsang, B. P. Cheung and K. W. Yeung: 'Genotypic shuffling' of sequential clones of *Candida albicans* in HIV-infected individuals with and without symptomatic oral candidiasis. *J Med Microbiol*, 52(Pt 4), 349-359 (2003)

9. F. Barchiesi, L. F. Di Francesco, P. Compagnucci, D. Arzeni, O. Cirioni and G. Scalise: Genotypic identification of sequential *Candida albicans* isolates from AIDS patients by polymerase chain reaction techniques. *Eur J Clin Microbiol Infect Dis*, 16(8), 601-605 (1997)

10. P. Boerlin, F. Boerlin-Petzold, J. Goudet, C. Durussel, J. L. Pagani, J. P. Chave and J. Bille: Typing *Candida albicans* oral isolates from human immunodeficiency virus-infected patients by multilocus enzyme electrophoresis and DNA fingerprinting. *J Clin Microbiol*, 34(5), 1235-1248 (1996)

11. G. Pizzo, G. M. Giammanco, S. Pecorella, G. Campisi, C. Mammina and M. D'Angelo: Biotypes and randomly amplified polymorphic DNA (RAPD) profiles of subgingival *Candida albicans* isolates in HIV infection. *New Microbiol*, 28(1), 75-82 (2005)

12. X. Song, E. R. Eribe, J. Sun, B. F. Hansen and I. Olsen: Genetic relatedness of oral yeasts within and between patients with marginal periodontitis and subjects with oral health. *J Periodontal Res*, 40(6), 446-452 (2005)

13. X. Song, J. Sun, G. Store, E. R. Eribe, B. F. Hansen and I. Olsen: Genotypic relatedness of yeasts in thrush and denture stomatitis. *Oral Microbiol Immunol*, 21(5), 301-308 (2006)

14. F. C. Odds, A. D. Davidson, M. D. Jacobsen, A. Tavanti, J. A. Whyte, C. C. Kibbler, D. H. Ellis, M. C. Maiden, D. J. Shaw and N. A. Gow: *Candida albicans* strain maintenance, replacement, and microvariation demonstrated by multilocus sequence typing. *J Clin Microbiol*, 44(10), 3647-3658 (2006)

15. K. Vargas, S. A. Messer, M. Pfaller, S. R. Lockhart, J. T. Stapleton, J. Hellstein and D. R. Soll: Elevated phenotypic switching and drug resistance of *Candida albicans* from human immunodeficiency virus-positive individuals prior to first thrush episode. *J Clin Microbiol*, 38(10), 595-607 (2000)

16. M. A. Pfaller, J. Rhine-Chalberg, S. W. Redding, J. Smith, G. Farinacci, A. W. Fothergill and M. G. Rinaldi: Variations in fluconazole susceptibility and electrophoretic karyotype among oral isolates of *Candida albicans* from patients with AIDS and oral candidiasis. *J Clin Microbiol*, 32(1), 59-64 (1994)

17. J. Schmid, F. C. Odds, M. J. Wiselka, K. G. Nicholson and D. R. Soll: Genetic similarity and maintenance of *Candida albicans* strains from a group of AIDS patients, demonstrated by DNA fingerprinting. *J Clin Microbiol*, 30(4), 935-941 (1992)

18. D. Metzgar, A. van Belkum, D. Field, R. Haubrich and C. Wills: Random amplification of polymorphic DNA and microsatellite genotyping of pre- and posttreatment isolates of *Candida* spp. from human immunodeficiency virus-infected patients on different fluconazole regimens. *J Clin Microbiol*, 36(8), 2308-2313 (1998)

19. P. P. Chong, Y. L. Lee, B. C. Tan and K. P. Ng: Genetic relatedness of *Candida* strains isolated from women with vaginal candidiasis in Malaysia. *J Med Microbiol*, 52(Pt 8), 657-666 (2003)

20. J. Xu, T. G. Mitchell and R. Vilgalys: PCR-restriction fragment length polymorphism (RFLP) analyses reveal both extensive clonality and local genetic differences in *Candida albicans*. *Mol Ecol*, 8(1), 59-73 (1999)

21. J. Hannula, M. Saarela, B. Dogan, J. Paatsama, P. Koukila-Kahkola, S. Pirinen, H. L. Alakomi, J. Perheentupa and S. Asikainen: Comparison of virulence factors of oral *Candida dubliniensis* and *Candida albicans* isolates in healthy people and patients with chronic candidosis. *Oral Microbiol Immunol*, 15(4), 238-244 (2000)

22. L. P. Samaranayake, T. W. MacFarlane, P. J. Lamey and M. M. Ferguson: 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*, 15(7), 386-388 (1986)

23. L. P. Samaranayake: Essential microbiology for dentistry. Churchill Livingstone, Edinburgh (2006)

24. P. F. Lehmann, D. Lin and B. A. Lasker: Genotypic identification and characterization of species and strains within the genus *Candida* by using random amplified polymorphic DNA. *J Clin Microbiol*, 30(12), 3249-3254 (1992)

25. P. Steffan, J. A. Vazquez, D. Boikov, C. Xu, J. D. Sobel and R. A. Akins: Identification of *Candida* species by randomly amplified polymorphic DNA fingerprinting of colony lysates. *J Clin Microbiol*, 35(8), 2031-2039 (1997)

26. P. H. A. Sneath and R. R. Sokal: Numerical Taxonomy. The Principles and Practice of Numerical Classification. W.H. Freeman, San Francisco (1973)

27. M. I. Williamson, L. P. Samaranayake, T. W. MacFarlane: Biotypes of *Candida albicans* using the API 20C system. *FEMS Microbiol Lett*, 37(1), 27-29 (1986)

28. L. P. Samaranayake and T. W. MacFarlane: Oral candidosis edited by Lakshman P. Samaranayake, T. Wallace MacFarlane. Wright, London (1990)

29. F. C. Odds: *Candida* and candidosis: a review and bibliography. Bailliere Tindall, London (1988)

30. C. M. Sedgley and L. P. Samaranayake: The oral prevalence of aerobic and facultatively anaerobic gram-negative rods and yeasts in Hong Kong Chinese. *Arch Oral Biol*, 39(6), 459-466 (1994)

31. C. M. Sedgley, L. P. Samaranayake, J. C. Chan and S. H. Wei: A 4-year longitudinal study of the oral prevalence of enteric gram-negative rods and yeasts in Chinese children. *Oral Microbiol Immunol*, 12(3), 183-188 (1997)

32. C. M. Sedgley, C. S. Chu, E. C. Lo and L. P. Samaranayake: The oral prevalence of aerobic and facultatively anaerobic gram-negative rods and yeasts in semi-recluse human vegetarians. *Arch Oral Biol*, 41(3), 307-309 (1996)

33. J. Xu and T. G. Mitchell: Geographical differences in human oral yeast flora. *Clin Infect Dis*, 36(2), 221-224 (2003)

34. Y. Y. Xu and L. P. Samaranayake: Oral *Candida albicans* biotypes in Chinese patients with and without oral candidosis. *Arch Oral Biol*, 40(6), 577-579 (1995)

35. X. Song, J. Sun, B. Frode Hansen, I. Olsen: Oral distribution of genera, species and biotypes of yeasts in patients with marginal periodontitis. *Microb Ecol Health Dis*, 15, 114-119 (2003)

36. M. I. Williamson, L. P. Samaranayake and T. W. MacFarlane: Biotypes of oral *Candida albicans* and Candida tropicalis isolates. *J Med Vet Mycol*, 24(1), 81-84 (1986)

37. P. C. Tsang, L. P. Samaranayake, H. P. Philipsen, M. McCullough, P. A. Reichart, A. Schmidt-Westhausen, C. Scully and S. R. Porter: Biotypes of oral *Candida albicans* isolates in human immunodeficiency virus-infected patients from diverse geographic locations. *J Oral Pathol Med*, 24(1), 32-36 (1995)

38. J. Xu, C. M. Boyd, E. Livingston, W. Meyer, J. F. Madden and T. G. Mitchell: Species and genotypic diversities and similarities of pathogenic yeasts colonizing women. *J Clin Microbiol*, 37(12), 3835-3843 (1999)

39. W. L. Whelan, D. R. Kirsch, K. J. Kwon-Chung, S. M. Wahl and P. D. Smith: *Candida albicans* in patients with the acquired immunodeficiency syndrome: absence of a novel of hypervirulent strain. *J Infect Dis*, 162(2), 513-518 (1990)

40. S. Redding, J. Smith, G. Farinacci, M. Rinaldi, A. Fothergill, J. Rhine-Chalberg and M. Pfaller: Resistance of *Candida albicans* to fluconazole during treatment of oropharyngeal candidiasis in a patient with AIDS: documentation by in vitro susceptibility testing and DNA subtype analysis. *Clin Infect Dis*, 18(2), 240-242 (1994)

41. W. G. Merz: *Candida albicans* strain delineation. *Clin Microbiol Rev*, 3(4), 321-334 (1990)

Abbreviations: FOA: Fixed Orthodontic Appliance, PBS: Phosphate Buffered Saline, SDA: Sabouraud's Dextrose Agar, RAPD: Random Amplification of Polymorphic DNA, PCR: Polymerase Chain Reaction

Key Words: *Candida albicans*, Genotypes, Phenotypes, Fixed Orthodontic Appliance

Send correspondence to: Professor L.P. Samaranayake, Oral Bio-Sciences, Faculty of Dentistry, Prince Philip Dental Hospital, 34 Hospital Road, Sai Ying Pun, Hong Kong SAR, China. Tel: 852-2859-0342, Fax: 852-2547-6133, E-mail: lakshman@hkucc.hku.hk

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