'Genotypic shuffling' of sequential clones of *Candida albicans* in HIV-infected individuals with and without symptomatic oral candidiasis

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Although HIV-infected individuals harbour multiple strains of oral *Candida albicans*, little is known of their micro-evolution over time. Therefore, a prospective study was conducted with 16 HIV-infected ethnic Chinese individuals with and without symptoms of oropharyngeal candidiasis to evaluate the genotype distribution of oral *C. albicans* isolates during HIV disease progression. Oral-rinse samples were obtained from all individuals and up to five *C. albicans* colonies were selected for each visit, over a 12 month period of multiple visits. After identification of isolates using standard mycological criteria, the genetic similarities of yeast isolates within and between sequential clones of *C. albicans* were assessed by DNA fingerprinting through random amplification of polymorphic DNA (RAPD). The results of RAPD gel profiles and the lineage of each isolate were further analysed using commercially available software. RAPD studies revealed the prevalence of up to 14 different genotypes per individual during the study period, with multiple genotypes isolated simultaneously from a single oral rinse. Computer analysis of RAPD profiles revealed that yeasts isolated over sequential visits from symptomatic individuals demonstrated a striking level of relatedness compared with isolates from asymptomatic individuals. Genetically identical *C. albicans* strains also formed 'loosely' connected subclusters that overlapped multiple visits, implying genetic 'shuffling' in these isolates during disease progression. These data point to varying evolutionary genetic trends in *C. albicans* associated with symptomatic oral candidiasis and asymptomatic carriage in HIV disease.

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INTRODUCTION

Candida albicans is recognized as the most important agent of mucosal candidiasis in individuals with HIV infection (Scully, 1992; Pfaller *et al.*, 1994), and up to 90 % of infected individuals suffer from at least one episode of oropharyngeal candidiasis during the course of their disease (Samaranayake & Holmstrup, 1989; Barchiesi *et al.*, 1997). The frequency of isolation of *C. albicans* and the clinical recurrence of oral candidiasis increase with advancing HIV infection (Torssander *et al.*, 1987; Korting *et al.*, 1988), possibly due to the underlying immune deficiency and the commonly administered short- or long-term antifungals, which favour the selection of resistant strains (Sangeorzan *et al.*, 1994; Rex *et al.*, 1995; Barchiesi *et al.*, 1997; Redding *et al.*, 1997). This, in turn, implies that subclones of *C. albicans* with variable genotypes may colonize the oral cavity simultaneously. In this event, it is difficult to determine with certainty the contribution, if any, of individual clones to infection (Soll *et*

al., 1988; Johnson *et al.*, 1995) and, further, the presence of more than one clonal type may have important therapeutic implications, as some may have different susceptibilities to antifungal agents (Korting *et al.*, 1988; Pfaller *et al.*, 1994; Samaranayake *et al.*, 2001). However, little is known of clonal variations in *C. albicans* during either recurrent episodes of oral candidiasis or asymptomatic carriage over prolonged periods in HIV disease.

Recent advances in molecular techniques have generated several typing methods for genetic assessment of strain relatedness in *C. albicans* which, in turn, have facilitated detailed studies of the molecular epidemiology of this ubiquitous yeast. These molecular techniques include pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and the use of specific probes. These techniques have helped to delineate subtypes of colonizing and/or infecting *C. albicans* strains over multiple infective episodes in patients with HIV infection (Pfaller *et al.*, 1994; Sangeorzan *et al.*, 1994; Barchiesi *et al.*, 1995, 1997; Boerlin *et al.*, 1996; Dromer *et al.*, 1997; White *et al.*, 1997),

Abbreviations: RAPD, random amplification of polymorphic DNA; SDA, Sabouraud's dextrose agar.

the transmission of these strains from partners in healthy individuals (Schroppel *et al.*, 1994) and the role of different strains in recrudescence infection (Bart-Delabesse *et al.*, 1993; Pfaller *et al.*, 1994; Challacombe *et al.*, 1995; Le Guennec *et al.*, 1995; Lischewski *et al.*, 1995; Metzgar *et al.*, 1998).

Of the currently available *Candida* genotyping techniques, RAPD is relatively cost-effective (for large numbers of isolates) and matches the resolving power of electrophoretic karyotyping. This, together with the availability of computer-assisted software systems that generate dendrograms of genetic relatedness among *C. albicans* isolates, has significantly advanced lineage studies over progressive infective episodes or during asymptomatic carriage (Soll, 2000).

The main aim of this investigation was to characterize the subtypes of 443 oral *C. albicans* isolates during HIV disease progression in a cohort of 16 HIV-infected individuals in southern China, during sequential patient visits. For this purpose, we utilized the RAPD genotyping method and the resulting gel profiles were further analysed using the program Dendron 3.0 (Solltech Inc.). Special emphasis was given to the tracing of the lineages of particular strains or the emergence of new strains of *C. albicans* over sequential visits during an observation period of 12 months. To our knowledge, this is the first molecular epidemiological study from the Asian region that comprehensively characterizes the lineage of a large number of sequential, oral *C. albicans* isolates in HIV infection.

METHODS

Patients. A cohort of 16 ambulatory ethnic Chinese patients with symptomatic HIV infection (14 males and 2 females; mean age 35.5 years; median 34 years; range 26–47 years; belonging to CDC stage IV, A to C) who attended the AIDS Unit, Department of Health, Hong Kong, SAR, China, was followed up for a period of 1 year to monitor oral carriage of *C. albicans*. Of the 16, ten were heterosexuals, four were homosexuals and two were haemophiliacs. Ten of the 16 individuals presented with symptomatic oral candidiasis on one or more visits during the 1 year study period, while the remainder were asymptomatic with respect to this condition. The patients with candidiasis were on intermittent antifungals for sporadic episodes of the infection and were treated with nystatin, fluconazole, ketoconazole, clotrimazole and itraconazole, as appropriate. On each patient visit, samples for culture were taken irrespective of clinical signs and symptoms of oral candidiasis. In total, 101 oral-rinse samples were obtained during the observation period as described below. Each of the 16 patients attended the clinic between four and eight times during this period.

Ethics Committee approval from the Department of Health, Hong Kong, and informed patient consent were obtained as a routine for the longitudinal study.

Collection of *C. albicans* isolates and growth conditions. A total of 443 *C. albicans* isolates were obtained from the cohort of 16 HIV-infected individuals. The organisms were recovered using the oral-rinse technique of Samaranayake *et al.* (1986). In brief, the patients were requested to rinse the mouth for 60 s with 10 ml PBS (pH 7.3, 0.1 M) supplied in a sterile Universal container. The sample was expectorated into the container and immediately transferred to the laboratory, where the oral rinse was concentrated by spinning at 1700 g for 10 min and resuspended in 2 ml sterile PBS and vortex-mixed for 30 s. The

concentrated oral rinse was then dispensed onto a Sabouraud's dextrose agar (SDA; Gibco) plate in an Archimedean spiral using a Spiral Plater (model DU, Spiral Systems Inc.). The plates were incubated for 48 h at 37 °C and up to five yeast colonies per sample were randomly chosen by a single investigator and subcultured onto SDA plates. The pure yeast cultures were then harvested, suspended in water in sterile vials and stored at –20 °C. The organisms were identified by germ-tube test, growth at 45 °C, chlamydospore production and API 20C AUX assimilation tests (bioMérieux) and the phenotype was defined using CHROMagar *Candida* plates (Odds & Bermaerts, 1994). Their identity were also reconfirmed using the new improved APILAB Plus (bioMérieux) to exclude *Candida dubliniensis*. The yeasts were then stored in vials with multiple glass beads (Microbank, Pro-Lab Diagnostics) at –70 °C, subcultured monthly on SDA and maintained at 4 °C during the experimental period. Purity of cultures was confirmed periodically by visualization of Gram-stained organisms and germ-tube test.

Genotyping of *C. albicans* isolates

Preparation of DNA for RAPD. Yeasts obtained from stock cultures stored at –70 °C were subcultured on YPD broth (1 % peptone, 1 % yeast extract, 2 % glucose), solidified with 1.5 % agar, at 37 °C for 24 h and single colonies were transferred to 20 ml YPD broth and incubated at 30 °C under aerobic conditions to the stationary phase (as assessed by the measurement of OD₆₀₀). Following incubation, yeasts were harvested by centrifugation at 4000 g for 5 min and washed in 1 M sorbitol (dissolved in deionized water). The yeast pellet was resuspended in 1.5 ml SE buffer [1.2 M sorbitol, 0.1 M EDTA, pH 8.0, containing 3 µl β-mercaptoethanol (Sigma) and 0.5 mg yeast lytic enzyme (Lyticase, Sigma)], incubated at 37 °C for at least 1 h until formation of spheroplasts and harvested by centrifugation at 2500 g for 5 min. These spheroplasts were washed twice in SE buffer and resuspended in 1.5 ml 0.15 M NaCl, 0.1 M EDTA (pH 8.0) and lysed by addition of proteinase K (final concentration 500 µg ml^{–1}) and SDS (1 %, w/v, final concentration) followed by the addition of RNase (500 µg ml^{–1}) and incubated at 55 °C for 1 h. The supernatant obtained after centrifugation at 13 000 g was extracted twice with phenol and once with phenol/chloroform prior to precipitation of DNA by addition of an equal volume of 2-propanol. The DNA precipitated was dissolved in 100 µl TE buffer (10 mM Tris/HCl, 0.1 mM EDTA, pH 8.0) (Bostock *et al.*, 1993).

RAPD analysis. Thermocycling was performed in a model PTC-150 minicycler machine (MJ Research). PCRs contained approximately 200 ng yeast DNA as template, 5 µl 10× PCR buffer (200 mM Tris/HCl, pH 8.4, 500 mM KCl), 200 µM dNTPs, 25 mM MgCl₂, 1 µM primer and 1.5 U *Taq* polymerase (Life Technologies). The first five cycles included 30 s denaturation at 94 °C, 2 min annealing at 52 °C [primer RSD10 (5'-CCGACGCCA-3') or RSD12 (5'-GGTCCGTGTTCAAGACG-3'); Life Technologies] and 2 min primer-extension at 72 °C, followed by 45 cycles of 30 s denaturation at 94 °C, 2 min annealing at 57 °C (primer RSD10 or RSD12) and 2 min primer-extension at 72 °C. The reaction was then maintained at 72 °C for 15 min. Control tubes without template DNA were included in each run and reproducibility was checked for each reaction (Lehmann *et al.*, 1992; Steffan *et al.*, 1997). The PCR products were electrophoresed in agarose gels (1.2 %) for approximately 2 h at room temperature in TBE buffer (89 mM Tris/HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.0), stained with ethidium bromide and visualized with UV light.

Computer-assisted analysis of data and histogram and dendrogram generation. The different banding positions of RAPD fingerprinting patterns of multiple isolates of *C. albicans* were analysed using the program Dendron 3.0 (Solltech Inc.). For the analysis of relationships among a number of strains, Dendron constructs histograms and dendrograms by the unweighted pair group method (Sneath & Sokal, 1973). 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. In the current study, a value of S_{AB} of 0.80 was arbitrarily used as the threshold for clustering of similar strains, since it is roughly halfway between the mean value for dissimilarity and identity (Soll, 2000).

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 *C. 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 (S_{AB}) for the pair of strains, A and B, was calculated by the formula:

$$S_{AB} = 1 - [\sqrt{(b+c)/(a+b+c)}]$$

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 S_{AB} value is grouped into a unit with a branch-point corresponding to its S_{AB} value.

To develop the dendrogram in the current study, the gel images of all the isolates were captured by a digital camera (Kodak DC290) and then digitized into the Dendron database, normalized according to invariable band positions and relocated with Dendron software according to the manufacturer's instructions.

RESULTS

RAPD genotyping of sequential clinical *C. albicans* isolates

RAPD analysis was performed to determine the clonal variability of 443 colonizing oropharyngeal *C. albicans* strains obtained from 16 HIV-infected individuals. Based on our earlier experiments (Dassanayake *et al.*, 2000; Waltimo *et al.*, 2001), two primers, RSD10 and RSD12, were used to distinguish the different yeast genotypes. The molecular profile resolution was greatest with primer RSD12 for all isolates except for those from individuals P1 and P8, where resolution with primer RSD10 was superior

(not shown). Therefore, when genotypic data derived from dendrogram profiles were pooled (see below), strains from patients P1 and P8 were excluded.

Because of the large number of strains studied, only the RAPD profiles of sequential isolates generated with RSD12, from an individual without symptomatic oral candidiasis (28 oral isolates of individual P46 from seven sequential visits), is provided as an example (Fig. 1).

In general, gross examination of the fingerprint profiles of sequential isolates clearly demonstrated the persistence of the identical genotype over several visits as well as the emergence of new genotypes within the oral niche of each individual during the study period.

Dendrogram analysis

The RAPD gel profiles analysed using the dendrogram program helped determine (i) the recurrence of identical strains, (ii) infection/infestation with new strains or (iii) the selection of specific strains during HIV disease progression of the study cohort (Soll, 2000). As stated above, an S_{AB} of 0.80 was taken as an arbitrary threshold of genetic relatedness (Schmid *et al.*, 1992). In general, sequentially isolated *C. albicans* isolates formed clusters comprising two to three or more strains at S_{AB} values of ≥ 0.80 . The remaining, distantly related strains of *C. albicans* were connected at S_{AB} values < 0.80 .

An example of a dendrogram based on S_{AB} values, generated for 28 sequential *C. albicans* isolates from patient P46, is presented in Fig. 2(a). The S_{AB} ranged between 0.58 and 1.0 and the mean S_{AB} was 0.68, with a standard deviation of 0.09. The Dendron database formed four clusters, containing two (cluster I), five (cluster II), seven (cluster III) and three (cluster IV) isolates, at S_{AB} values of ≥ 0.80 . There were two identical strains in cluster I (3b, 3c; $S_{AB} = 1.0$). Cluster II comprised two identical strains (6c, 7c) and three other strains (6b, 7a, 7b) connecting at an S_{AB} of 0.81. Three identical strains (4d, 4e, 5a) were seen in cluster III, together with four others connecting at an S_{AB} of 0.83. The seven strains belonging to cluster III and the three strains belonging to cluster IV were connected to each other at a low S_{AB} of 0.76, while the 15 strains of clusters II, III and IV and a further four strains (6a, 6d, 6e, 7d) were connected at an S_{AB} of 0.69. All these were connected to two more isolates (1a, 2c)

Fig. 1. RAPD fingerprints of 28 sequential oral *C. albicans* isolates from HIV-infected patient P46 after RAPD analysis with primer RSD12. Various fingerprints observed are indicated by letters A–F. Lanes M, PCR markers (Sigma).

Fig. 2. Dendrograms generated for sequential oral isolates of *C. albicans* from HIV-infected patients P46 (a, $n = 28$) and P5 (b, $n = 35$) with asymptomatic *Candida* carriage. Vertical dashed lines mark the positions of S_{AB} values of 0.70 and 0.80, the arbitrary thresholds for 'tight' and 'loose' clustering. Heavy vertical lines to the right of each dendrogram mark the position of clusters.

at a very low S_{AB} of 0.68. The two identical strains in cluster I (3b, 3c; $S_{AB} = 1.0$) and five other strains (2a, 2b, 4a, 4b, 5d) were all connected to each other at an S_{AB} of 0.60. To conclude, in patient P46, only 17 of 28 *C. albicans* isolates (61 %) grouped into clusters at an S_{AB} value of > 0.80 , and these included four sets of identical isolates, i.e. isotypes (3c and 3b; 6c and 7c; 4d, 4e and 5a; 2d and 2e). The remainder of the isolates (39 %) from this patient were distantly related.

Similar individual dendrograms based on the S_{AB} values of genetically related sequential strains were generated for the other 15 HIV-infected individuals. Another such dendrogram of sequential isolates from individual P5 is shown in Fig. 2(b).

Genetic relatedness of clones of sequential *C. albicans* isolates

In general, the dendrogram profiles generated from a total 443 *C. albicans* strains from 16 HIV-infected individuals exhibited varying and disparate patterns of relatedness in *C. albicans* isolated during sequential visits either within the

same individual or between individuals. Genetically identical ($S_{AB} = 1.0$) and genetically related ($S_{AB} \geq 0.80$) pairs/multiples of yeast isolates are outlined in Table 1.

As we selected up to five isolates each of *C. albicans* per visit, it was not surprising that, in some patients, up to four isolates from a single visit were DNA isotypes. For example, in patient P2, two isolates each from visits 2 (2a, 2b) and 3 (3d, 3e) and four isolates from visit 4 (4b–4e) were genetically identical (Table 1).

On longitudinal analyses for genetic isotypes of sequential isolates from the same individual, we found this to be a common occurrence. For instance, patient P3 yielded *C. albicans* isotypes on three visits (visits 2, 4 and 6) and patient P36 on four visits (visits 5 and 6; 6 and 7; and 6, 7 and 8) (Table 1). Table 2 summarizes the genotyping data in terms of the number of sequential visits when isotypes were identified for each patient. Thus, 11 of the 16 patients had genetically identical isolates during two to four sequential visits (Table 2). The results also show that the isotypes colonized these individuals generally over one to two visits, although, exceptionally, this was seen for up to four sequential visits, which lasted up to 7 months (patient P9).

Table 1. Clusters of *C. albicans* strains from sequential visits of the same individual that show S_{AB} values of ≥ 0.80

Clusters are numbered according to the cluster-generation profile, from top to bottom (see Fig. 2). S_{AB} values are given in parentheses after the strains that make up the cluster.

Patient	I	II	III	IV	V	VI	VII	VIII	IX	X
Symptomatic candidiasis group										
P2	1b, 1c (0.85)	2a, 2b (1)	3d, 3e (1)	3b, 4b, 4c, 4d, 4e, 5a (1)						
P3	2a, 4c, 6b (1)	3a, 3b, 3c (0.81)	1a, 1b (0.8)							
P6	1b, 2a (1)	4a, 5a (0.8)	(1e, 2b)*, 3b (0.82)	5b, 5c, 5d (0.83)						
P9	2a, 5e, 6a, 6b, 7d (1)	6d, 6e (1)	2b, 3b, 3c (1)	3e, 5a, 5c, 5d (1)	6c, 7c, 7e (1)					
P10	4e, 5a (0.86)	2e, 5c (0.86)	2b, 4d (0.8)	3b, 4a (0.86)	(3c, 4b), 4c (0.82)	5e, 6a (1)	6b, 6c (0.8)			
P30	5b, 5c, 5d (0.8)	4e, 5a, 6c (0.81)	4b, 4d (1)	(3c, 3e), 4a (0.82)	(2c, 3b), (1d, 3a) (0.8)					
P36	6d, 6e, 7e, 8a, 8c (1)	5b, 6a (1)	5a, 5e (0.82)	6c, 7c (1)	2e, 3a, 3c (0.8)	(2a, 2c, 2d), 2b, 1c, 3d (0.82)	(1a, 1b, 4a), 3e (0.8)	1e, 1d, 3b (0.8)		
P38	2d, 3d, 3a, 2e (0.8)	2a, 2b, 2c (0.8)	5b, 6a, 5c, 6e, 5d (0.8)	6b, 6c (0.8)	4a, 4c, 4e, 4d, 5e (0.8)					
P39	5d, 6c, 6e (1)	6d, 7b (1)	2d, 3a, 3c, 7a, 7d (1)	3d, 7c (1)	(1a, 3b), 1b (0.86)	2b, 2e (0.8)	5a, 5b (1)	5c, 5e, 6a (1)		
P44	3e, 4a (0.8)	6c, 6e (0.81)	1c, 3c (0.81)	3b, 5b (0.82)	1e, 2e (0.8)	4c, 4d (0.8)	1a, 1d, 2b (0.8)	4b, 4e (0.81)	5e, 6d (0.8)	
Asymptomatic <i>Candida</i> carriage group										
P1	3b, 3c (0.82)	5d, 6b, 6c, 6e (0.8)	4e, 5b, 5c (0.81)	6a, 6d (0.85)	3d, 3e, 4a, 4c (0.81)	4b, 4d (0.83)	1b, 2c (0.83)	1a, 2b (0.82)	1c, 2a (0.83)	2d, 2e (0.84)
P4	1a, 5a (0.81)	3e, (4a, 5c), 4d, 5b (0.84)	4b, 4c (0.85)	3c, 4e (0.8)	2a, 2e, 3a, 3b (0.81)	1e, 5d (0.81)	2d, 7d (0.8)			
P5	7d, 7e (0.85)	1e, (2d, 2e) (0.8)	1a, 3d (0.82)	1d, 3a (0.81)	5c, 6c (0.85)	5a, 5e (0.85)				
P8	1b, 1e (0.82)	2e, 3e (0.8)	4b, (4c, 4d), 5a, 5b (0.82)	3b, 3d (0.85)	2d, (4a, 5c) (0.8)	1a, 2a (0.8)	2b, 3c (0.85)	5d, 5e (1)		
P37	5e, 6b (0.85)	7b, 7c (0.8)	1a, 2a (0.82)	1b, 1d (0.8)	3b, 3c (0.8)	4d, 4e (0.82)				
P46	3b, 3c (1)	6b, (6c, 7c), 7a, 7b (0.81)	(4d, 4e, 5a), 5b, 5c, 4c, 5e (0.82)	(2d, 2e), 3a (0.85)						

*Isolates within parentheses have an S_{AB} value of 1.0.

Table 2. Numbers of visits where *C. albicans* strains with S_{AB} values of 1.0 or ≥ 0.80 were isolated

Groups of visits in parentheses indicate that strains with the S_{AB} value shown were isolated at those visits; e.g. (3, 4, 5) indicates that isolates from visits 3, 4 and 5 formed a cluster with the S_{AB} shown. The isolation of different groups of strains in the same S_{AB} class is represented by e.g. $\times 2$, for the isolation of two different pairs/triplets of strains at the same visits.

Patient	$S_{AB} = 1.0$		$S_{AB} \geq 0.80 \text{ and } < 1.0$	
	Number	Visits	Number	Visits
Symptomatic candidiasis group				
P2	3	(3, 4, 5)	0	—
P3	3	(2, 4, 6)	0	—
P6	2	(1, 2) $\times 2$	3	(1, 2, 3)
P9	4	(2, 5, 6, 7)	0	—
	2	(2, 3); (3, 5)		
P10	2	(5, 6); (3, 4)	2	(4, 5); (2, 5); (2, 4); (3, 4) $\times 2$
P30	2	(1, 3); (2, 3)	2	(3, 4)
			3	(4, 5, 6); (1, 2, 3)
P36	3	(6, 7, 8)	2	(2, 3); (1, 3)
	2	(5, 6); (6, 7)	3	(1, 2, 3)
P38	0	—	2	(2, 3) $\times 2$; (5, 6) $\times 2$; (4, 5)
P39	2	(5, 6); (6, 7); (3, 7); (1, 3); (5, 6)	2	(1, 3)
	3	(2, 3, 7)		
P44	0	—	2	(3, 4); (1, 3); (3, 5); (1, 2) $\times 2$; (5, 6)
Asymptomatic <i>Candida</i> carrier group				
P1	0	—	2	(5, 6); (4, 5); (3, 4) $\times 2$; (1, 2) $\times 3$
P4	2	(4, 5)	2	(1, 5) $\times 2$; (3, 4); (2, 3) $\times 2$; (2, 7)
			3	(3, 4, 5)
P5	0	—	2	(1, 2); (1, 3) $\times 2$; (5, 6)
P8	2	(4, 5)	2	(2, 3); (4, 5) $\times 2$; (2, 3)
			3	(2, 4, 5)
P37	0	—	2	(5, 6); (1, 2)
P46	2	(6, 7); (4, 5)	2	(6, 7) $\times 2$; (4, 5); (2, 3)

'Tight' and 'loose' clusters of genetically related *C. albicans* strains

The dendrogram data of multiple isolates obtained sequentially from individual patients were further evaluated to determine strain relatedness in terms of 'tight' and 'loose' clusters (Whelan *et al.*, 1990). This method of evaluation helps to determine the degree of 'genetic shuffling' that occurs in *C. albicans* populations. Demarcation lines were drawn on the dendrogram at S_{AB} values of 0.72 and 0.80 to denote arbitrary thresholds for 'loose' and 'tight' clustering, respectively. Fig. 2(b) shows a dendrogram constructed from the S_{AB} values computed for 35 strains from patient P5. We could distinguish six small clusters and three larger clusters of 'loosely connected' *C. albicans* isolates within this yeast population. In cluster II, two identical isolates from visit 2 were closely related to a single isolate from visit 1. Clusters II, III and IV formed a 'loosely' connected subcluster that contained closely related strains from visits 1, 2 and 3 (as well as a single strain from visit 6). A similar 'loosely' connected subcluster was seen with a majority of isolates from visits 5, 6 and 7 [as well as single isolates from each of

visits 2 and 4 (2a, 4e)]. This indicates gradual genetic shuffling or genetic drift that occurred in these *C. albicans* isolates during the study period of 12 months. Similar relationships amongst 'loose' clusters were seen in almost all patients (e.g. Fig. 2a) at varying demarcation values, confirming the aforementioned observation.

Genetic relatedness of isolates from symptomatic/asymptomatic patients

To determine the genetic relatedness amongst sequential *C. albicans* isolates from both the symptomatic and asymptomatic groups, composite dendrograms were separately generated for 93 isolates from three individuals with a history of symptomatic oral candidiasis (P10, P30 and P36) and asymptomatic individuals (P5, P8 and P46). The yeasts in these two groups were first assessed for intra-group and then for inter-group genetic relatedness.

The composite dendrogram for symptomatic patients displayed a striking degree of variation amongst yeasts from different patients, as closely delineated clusters were seen for

each individual (Fig. 3). For instance, all yeast isolates from patient P36 grouped into three loose clusters, I, IV and V. Within cluster I, isolates 6c and 7c and isolates 6d, 6e, 7e, 8a and 8c were identical, while strain 6b was 'loosely' connected to the seven other strains at a low S_{AB} of 0.68 (Fig. 3). Within cluster IV, there were four smaller clusters with S_{AB} values of 1.0 or ≥ 0.80 . These smaller clusters were joined by four other *C. albicans* strains, resulting in a low S_{AB} of 0.75. A similar pattern of clustering was seen for patients P10 and P30. A total of 26 sequential isolates from patient P10 grouped into two clusters (II and VII), while 27 sequential isolates from patient P30 grouped into two further clusters (III and VI). However, three yeast isolates, 30-5e, 36-7d and 36-8e, were highly dissimilar to all other isolates and were loosely connected to the rest of the group at an S_{AB} of 0.40. To conclude, sequential isolates from all three symptomatic

cases grouped into three disparate 'loosely' connected clusters, implying differences in clonality in spite of their origin from the same geographical location.

The composite dendrogram for 87 sequential *C. albicans* strains from three individuals without a history of symptomatic oral candidiasis (P5, P8 and P46) was, however, genetically more diverse (i.e. formed many loosely connected clusters) (Fig. 4) than the strains from the symptomatic group described above (Fig. 3). The vast majority of isolates (except for ten strains) grouped into 11 separate clusters. The ten isolates that did not belong to any of the above clusters were two, three and five strains from patients P5, P8 and P46, respectively. For example, a total of 28 strains belonging to P46 were grouped in clusters V and VI, while 46-2c was seen in cluster IV with two strains from patient P5 and 46-1a was seen in cluster VIII amongst six isolates from patient P8. Thirty-five isolates from P5

Fig. 3. Composite dendrogram generated for sequential oral isolates of *C. albicans* from HIV-infected patients P10, P30 and P36 with symptomatic oral candidiasis. See Fig. 2 for further details.

Fig. 4. Composite dendrogram generated for sequential oral isolates of *C. albicans* from HIV-infected patients P5, P8 and P46 with asymptomatic oral *Candida* carriage. See Fig. 2 for further details.

grouped into clusters II, III, IV, VII, X and XI, while a single isolate, 5-2a, was an exceptional outlier at a lower S_{AB} of 0.65. Another isolate, 5-2c, was identical to two strains from P8 (8-4a and 8-5c). The majority of isolates from P8 fell into clusters VIII and IX.

Thus, in general, the composite dendrogram of three asymptomatic patients (Fig. 4) demonstrated a greater degree of random distribution of yeast isolates compared with the yeasts from their symptomatic counterparts (Fig. 3). Strains 5-6e, 5-3b, 8-5e, 8-5d, 8-3a, 46-2a, 46-2b, 46-5d, 46-3b, 46-3c remained unclustered, distantly related to all other isolates in the composite dendrogram, with S_{AB} values between 0.62 and 0.54.

Genetic diversity of colonizing strains in symptomatic and asymptomatic groups

To determine the genetic relatedness, if any, of colonizing strains of *C. albicans* in symptomatic and asymptomatic individuals, dendrograms of isolates from these two groups (with three patients in each group) were joined to produce a larger composite dendrogram of 180 yeasts (data not shown). This composite dendrogram revealed random distribution of isolates and random cluster formation, implying that infectious strains in HIV-infected individuals do not represent a genetically distinct clone, distinct from commensal strains. For instance, the clonal delineation of yeast isolates from symptomatic individuals P10, P30 and P36 that is evident in Fig. 3 was not seen when the dendrograms obtained from six patients, P10, P30, P36, P5, P8 and P46, belonging to the symptomatic as well as asymptomatic groups were pooled together to create a single dendrogram.

On further analysis, we noted a higher degree of dissimilar clonality in sequential *C. albicans* isolates from asymptomatic *Candida* carriers compared with the symptomatic group. In order to study this phenomenon further, we analysed the maximum numbers of sequential visits where identical ($S_{AB} = 1$) or closely related ($S_{AB} \geq 0.80$) strains were isolated (Table 2). The results indicated that only three of six (50%) asymptomatic individuals carried identical strains on sequential visits in comparison with eight of ten (80%) of the symptomatic group of patients, giving further credence to the results described above ($P < 0.05$).

DISCUSSION

Longitudinal studies on the oral colonization and infection patterns of the human fungal pathogen *C. albicans* have mostly been performed with a single isolate from each patient per visit (Barchiesi *et al.*, 1997), and there have been calls for studies using multiple strains from a single visit due to the polyclonality of *C. albicans* in the oral niche (Merz, 1990). Hence, we employed a widely used genotyping method to characterize up to five randomly selected isolates per visit to screen for the presence of multiple clones of *C. albicans* among a select group of HIV-infected individuals over a period of 1 year.

Among the many molecular approaches for genotyping yeasts, e.g. RFLP analysis (Vazquez *et al.*, 1991; Millon *et al.*, 1994), Southern blot hybridization (Lasker *et al.*, 1992; Lockhart *et al.*, 1995), multilocus enzyme electrophoresis (Caugant & Sandven, 1993; Boerlin *et al.*, 1996) and RAPD (Bart-Delabesse *et al.*, 1995; Holmberg & Feroze, 1996), the latter technique is the least labour-intensive, requiring only small amounts of DNA, and is the technique of choice for large-scale clinical epidemiological studies (Dassanayake & Samaranayake, 2003). Furthermore, RAPD analysis is highly analogous to other genome-scanning protocols such as multilocus enzyme electrophoresis and Southern blot hybridization with the moderately repetitive DNA probe Ca3 (Pujol *et al.*, 1997). Although RAPD is a very effective method for evaluating and comparing the genetic profiles of clones of *C. albicans*, the subjective interpretation of band profiles becomes unwieldy when a large number of isolates are compared. As we compared RAPD profiles of up to 40 isolates from a single individual in this study, an automated computer program, Dendron, was utilized (Soll, 2000). Dendron compares every newly scanned strain with all previously scanned and recorded *C. albicans* strains in the dendrogram and generates a measure of relatedness that is considered closely related if the S_{AB} value is ≥ 0.80 (Soll *et al.*, 1989, 1991; Schmid *et al.*, 1992, 1993; Lockhart *et al.*, 1996).

Our results indicate that some patients maintained the identical *C. albicans* isotype on contiguous sequential visits, while, in others, this was found irregularly, yet periodically (Fig. 2). It is difficult to provide an explanation for the periodic emergence of genetic isotypes in the same individual. One possibility is that we failed to isolate the genetically identical strain during successive visits due to the random selection of only five yeast colonies from the primary culture. Ideally, evaluation of a larger number of isolates per patient visit may provide an answer to the variable frequency of genetic isotypes seen in the current study. Yet, such an approach in many patients over a lengthy period is practically demanding and highly resource-intensive. Another reason for the former observation could be endogenous reinfection/infestation with the identical strain from a distant body site (e.g. skin, vagina) or exogenously from a spouse or sexual partner. Periodic endogenous reinfection with identical *C. albicans* strains has been reported previously in patients with AIDS (Schmid *et al.*, 1992) and those with recurrent vulvovaginitis (Soll *et al.*, 1989).

The large number of strains that are 'loosely' connected (i.e. S_{AB} between 0.80 and 0.70) observed in this study suggests that genetic shuffling has taken place during HIV disease progression. Similar genetic shuffling in *C. albicans*, leading to evolution of subtypes during HIV disease progression, has been reported by several other researchers (Schmid *et al.*, 1992; Pfaller *et al.*, 1994; Boerlin *et al.*, 1996; Metzgar *et al.*, 1998). There are also reports in the literature where techniques such as RFLP have been used to demonstrate genetically diverse *C. albicans* in HIV infection (McCullough *et al.*, 1995; Barchiesi *et al.*, 1997). The large number of *C. albicans* genotypes in HIV infection has been shown to have increased

potential for the development of antimicrobial resistance and increased avidity to buccal epithelial cells (Redding *et al.*, 1994). Whether the process of genetic shuffling contributes to the latter phenomena remains to be determined.

Our data also indicate that oral *C. albicans* populations in HIV disease exhibit not only genetic reshuffling but also longevity in genetic terms by propagating the identical genotype over a considerable period. Whelan *et al.* (1990) reported that patients with AIDS were frequently infected with the same strain, and repeat isolates from individual patients were generally the same. Redding *et al.* (1994) also reported the persistence of the same *C. albicans* subtype over a 2-year period in a single patient with recurrent episodes of oropharyngeal candidiasis. Also, Barchiesi *et al.* (1997) reported that four of five AIDS patients were infected with the same DNA subtypes of *C. albicans* throughout each episode of infection during a 5-year observation period. Another group also reported the persistence of the same genotype during a longitudinal study with 30 HIV-infected patients carrying oral *C. albicans* in their oropharynx treated with fluconazole (Millon *et al.*, 1994). To conclude, our data, with the largest battery of *Candida* isolates genotyped to date, reaffirm the existence of two parallel phenomena, genetic reshuffling and propagation of genetic isotypes of *C. albicans* in HIV disease progression.

The current data also suggest that oral *C. albicans* strains of HIV-infected individuals with symptomatic candidiasis are relatively closely related, as these strains exhibited a significantly lower degree of genetic shuffling during the 1-year study period compared with the asymptomatic cohort. It is possible that more resistant and hence stable strains acquired during recurrent infections during multiple courses of antimicrobial treatment persist over a prolonged period. In contrast, *C. albicans* strains from asymptomatic individuals were randomly distributed within the dendrogram, sometimes sharing identical profiles with yeasts from other HIV-infected individuals. Similar findings have been reported by Schmid *et al.* (1992), where *C. albicans* strains were genetically less diverse in a group of AIDS patients than oral commensals from patients with no signs of oral thrush at the time of sampling. It is interesting in this context that Vargas *et al.* (2000) reported that *C. albicans* isolates from HIV-positive individuals prior to thrush episodes demonstrated increased switching frequency or the emergence of new strains with increased switching frequencies.

Clinically, the simultaneous presence of multiple oral strains of *C. albicans* has important implications for treatment of candidiasis, as they may exhibit variable susceptibilities to antifungal agents. Epidemiological surveying with molecular identification tools will be one of many requirements for the proper management of candidal infections in the future. Our results strongly indicate that HIV-infected and other compromised individuals with candidiasis must be strictly monitored for the presence of multiple *C. albicans* strains during disease evolution.

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