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Frequency, species and molecular characterization of oral *Candida* in hosts of different age in China

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INTRODUCTION: Research indicates that host age is a determining factor in yeast carriage. From the neonatal period, humans go through several dentition periods, and the emergence and substitution of teeth and changes in living habits greatly change the environment of the oral cavity, and therefore influence colonization by oral commensal organisms, certainly including *Candida* spp. No previous study of *Candida* carriage by different age groups divided by dentition has been reported. This study supplies data on the geographical specificity of *C. albicans* genotypic subgroup distribution.

METHODS: All test individuals came from a single geographical locale over a short period. Following mucosal swab sampling, CHROMagar *Candida*-yeast differential media were used to determine the frequency of carriage and species. All *C. albicans* strains were confirmed by PCR and PCR using primers reported to span a transposable intron region in the 25S rRNA gene was used to determine genotypic subgroups.

RESULTS: The results demonstrate that for the tested population, the frequency of *Candida* species and the distribution of *C. albicans* genotypic subgroups varied with age group. With increasing age, the frequency of *C. albicans* decreases, non-*C. albicans* yeasts increases; Genotypic subgroup A is the dominating strain in the oral cavities of healthy young individuals.

CONCLUSIONS: The influence of dentition substitution on oral yeast carriage was minor.

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Introduction

Over 400 microbial species or types inhabit the mouth (1), and colonization of the mouth by yeasts have been known since Hippocrates reported the presence of oral lesions caused by *Candida* (2). Among them, *C. albicans* and several related *Candida* species are opportunistic pathogens, which live as benign commensal organisms in the oral cavities of healthy individuals (3). As *C. albicans* is a very important opportunistic pathogen and one of the major agents of hospital-acquired infection (4), research regarding the colonization of *C. albicans* in the oral cavities in a variety of pathological conditions are frequently found. However, there are few studies about *Candida* spp. as commensal organisms, and their colonization and physiological succession in healthy individuals. *Candida albicans* and other *Candida* species are the important components of the oral-resident microflora, and can bind to a variety of host cell receptors through both lectin-like and protein–protein-type interactions (5), and can also co-aggregate with bacterial cells of a variety of genera (6), in particular a range of oral *Streptococci* such as *Streptococcus gordonii* (7). The tight interrelationship between yeast and host cells, and yeast and other micro-organisms, indicates that yeast cells contribute to the development, stabilization and maintenance of oral mixed microbial populations, and play an important role in maintaining the balance of the oral cavity (8).

Oral cavity is a very complex environment, including many kinds of tissues, soft tissue and hard tissue, i.e., teeth. During postnatal development, great changes take place in the environment of the oral cavity: from the neonate with no teeth; the emergence of the primary dentition; and replacement of the primary dentition with secondary dentition. Concurrently, living habits change, and therefore, oral commensal organisms change in a physiological succession. Compared with the many studies on the succession of bacterium, there are few about the succession of yeast. A study by Russell and Lay (9) demonstrated that the frequency of oral yeast carriage at birth was low, doubled by the time that infants were discharged from the hospital about seven-day old, and increased sharply after one month at home. Research by Kleinegger (3) indicated that the frequency of oral yeast carriage was 44% of the tested individuals in a group aged from 0.5 to 1.5 years old, 24% in a

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5–7-year-old group, 40% in a 15- to 18-year-old group. Starr (10) investigated the prevalence of oral *C. albicans* at baseline (before dental treatment, aged 8–11 years), post-treatment, and 12, 24, 36 months post-baseline, respectively, 47, 32, 21, 27, 28% of children were positive. These results indicate that the frequency of oral yeast is different in different-aged children; however, the distribution of oral yeast has geographical specificity, and no corresponding information about China exists.

Among the *Candida* species, *C. albicans* is still considered the most frequently encountered fungal pathogen and commensal organism, and strains have been subdivided into biological groups, including genetic subtypes. Recent advances in molecular biology-based technology have enabled detailed analysis of the genetic diversity of *C. albicans*, and some groups of *C. albicans* strains have been genetically characterized and reported. The usefulness of ribosomal sequences for genetic types has been applied to the identification of several fungal pathogens. McCullough (2) reports that PCR primers designed to span the 25S rRNA gene (rDNA) region can be used to classify *C. albicans* strains into four genotypes on the basis of the PCR amplification product; genotype A (450 bp); genotype B (840 bp); genotype C (450 and 840 bp); genotype D (1080 bp; *C. dubliniensis*), and Tamura found an additional genotype, genotype E (1400 bp) (11). These results (2) also indicated that there is a correlation between the *C. albicans* genotype and antifungal susceptibility, and showed diversity in the genotype groups of *C. albicans* isolated from different geographic areas (12).

Therefore, in this article, we describe our analysis of the yeast carriage of different age groups using CHROMagar Candida-yeast differential medium to determine the frequency of carriage and species present, and, by the PCR method of McCullough (2), the subgroups of the *C. albicans* strains. To evaluate the correlation between oral yeast presence, load, species and genotype with the changing oral environment, and accumulate the knowledge of oral yeast carriage.

Material and methods

Collection of isolates

Two hundred and thirty-two volunteers were recruited from the general population of Chengdu City. Subjects were confined to five dentition groups: group A1, neonates, 1–7 days, neonatal dentition; group A2, 3–5 years, primary dentition; group A3, 6–8 years, early mixed dentition; group A4, 12–14 years, late mixed dentition; and group A5, 18–21 years, secondary dentition. The parents of all subjects in group A1 and the subjects in group A2–A5 had resided in Chengdu City for at least one year prior to sampling, and all test individuals were sampled within a two-month period. All volunteers had unremarkable health histories and were free of signs or symptoms of oral candidiasis or other mucosal disease, as determined by oral examination. All individuals in groups A2–A4 were dentate and had no oral devices.

To assess correctly the relationship of carriage and dentition periods, we sampled the oral cavities in the five groups of individuals who exhibited no signs of oral disease, as confirmed by examination by oral medical doctors. Any individuals with even questionable symptoms were eliminated from the study. All the individuals studied were dentate, except for in the neonate group: individuals with orthodontic appliance or dentures were removed from the study.

A sample was collected from the inner cheek mucosa of each subject and the samples were coded according to dentition period groups (A1–A5). Each sample was collected by passing a sterile cotton swab five times across the oral buccal mucosal surface. Immediately after sampling, each swab was immersed in 0.5 ml of sterile PBS in a microcentrifuge tube. The sample tubes were transported from the place of collection to the laboratory within 2 h of sampling. The tubes were rigorously mixed for 30 s using a vortex mixer, and then centrifuged at 6000 ***g*** for 5 min. The supernatant was discarded, the deposit resuspended in 0.1 ml sterile PBS, and 15 μl aliquots spread onto CHROMagar *Candida*^{TM}-yeast differential medium, which were incubated at 37°C for 48 h and read for visual colony color. A single colony of yeast cells was taken from the CHROMagar *Candida*^{TM} medium and spread onto Sabouraud agar medium for pure culture.

Extraction of DNA

Two or three loops of cells from the SDA plates were suspended in 200 μl of TE buffer in a microcentrifuge tube. DNA extraction was carried out using the procedure described by Tamura et al. (13). Briefly, 250 μl of GPT reagent [(6 M guanidine thiocyanate in 50 mM Tris (pH 8.3)] and 450 μl of Tris (pH 8.0)-buffered phenol were added to a suspension of washed yeast cells, and the mixture boiled for 15 min to kill the fungal cells and extract the DNA. Chloroform-isoamyl alcohol (250 μl) was then added and the aqueous phase was separated by centrifugation at 12 000 ***g***, mixed with an equal amount of 100% isopropanol and a 1/10 volume of 3 M sodium acetate, and placed at –20°C for 1 h. Samples were centrifuged at 12,000 ***g*** for 20 min; and the nucleic acid pellet obtained washed with ice-cold 70% ethanol, dried, and resuspended in sterile TE buffer at a concentration of 5 μg/ml.

Candida albicans *identification and genotype determination by PCR*

The primer pairs used for *C. albicans* identification were based on the sequences that confer species specificity of EO3 of *C. albicans* mitochondrion DNA, as described by Miyakawa (14): primer-1 (5′-CACCAACTCGACC AGTAGGG-3′), primer-2 (5′-CGGGTGGTCTATAT TGAGAT-3′). The PCR primer pairs for genotype determination, whose sequences span the site of the transposable intron of the 25S rDNA were those described by McCullough et al. (2). The primer pairs used were CA-INT-L (5′-ATAAGGGAAGTCGGCAA AATAGATCCGTAA-3′) and CA-INT-R (5′-CCTTG GCTGTGGTTTCGCTAGATAGTAGAT-3′). Ampli-

fication reactions were performed in 25 μl of distilled water containing 2.5 μl of each primer (20 pM), 2.5 μl of genomic DNA (5 μg/ml), and PCR kit including TaKaRa Ex Taq (5U/μl) 0.125 μl, 10*Ex Taq Buffer 2.5 μl, $MgCl_2$ (25 mM) 2 μl, dNTP Mixture (2.5 mM each) 2 μl (Ready-to-Go PCR kit; TakaRa Biotechnology (Dalian) Co Ltd., China).

The conditions for *C. albicans* identification PCR were as follows: denaturation by incubation for 5min at 94°C prior to 35 cycles of 94°C for 1 min, 56°C for 2.5 min, and 72°C for 3 min, and a final extension at 72°C for 7 min to ensure complete polymerization of any remaining PCR product. The PCR conditions used for genotype determination were as follows: denaturation for 3 min at 94°C prior to 30 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 2.5 min, and a final extension at 72°C for 10 min in a thermoreactor (Gradient Thermal Cycle, Eppendorf Corp., Germany).

All reaction products were characterized by electrophoresis on 1.5% agarose gels in 1× TBE buffer at 70 V for 100 min and were stained in a solution of 0.5 μg of ethidium bromide per ml and visualized by UV transillumination (BioRad, USA).

The Fisher test was used in statistical analyses and differences among or between groups were assumed to be significant when the probability (P) was less than or equal to 0.05.

Results

Frequency of yeast carriage of different groups

Only 7.5% of the tested individuals in group A1 were positive, while 70% of tested individuals in group A2, 56.4% of group A3, 49.1% of group A4 and 60% of group A5 were positive (Fig. 1a and Table 1). The difference in the frequency of yeast carriage between group A1 and the other groups was significant, as was the difference between groups A4 and A5 ($P < 0.001$).

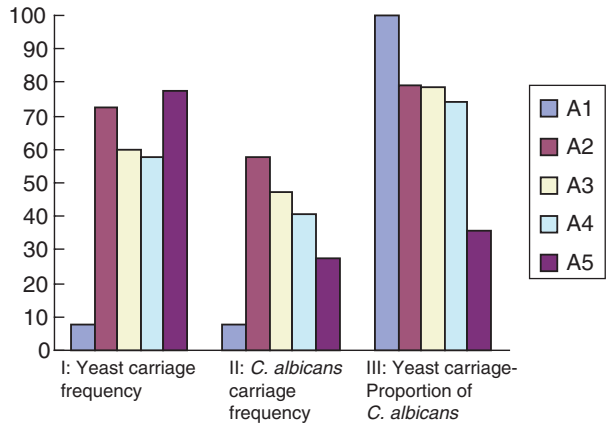

Figure 1 Histograms of the frequency of carriage of all yeast isolates (a), the frequency of carriage of the species *C. albicans* (b), and the proportion of yeast carriage of *C. albicans* (c) of the different dentition period groups. The symbols for the groups are designated on the right-hand side. Group A1: neonate; group A2: primary dentition; group A3: early mixed dentition; group A4: late mixed dentition; group A5: secondary dentition.

The frequency of *C. albicans* carriage in the different groups was 7.5% in group A1, 57.5% in group A2, 47.3% in group A3, 40.1% in group A4, and 20.5% in group A5 (Fig. 1b and Table 1). The difference in the frequency of *C. albicans* carriage between group A1 and the other groups was significant, as was the difference between groups A2 and A5 ($P < 0.001$). Of all of the yeasts detected, the proportion of *C. albicans* was 100% in group A1, 79.3% in group A2, 78.85% in group A3, 76.7% in group A4, and 35.5% in group A5. The difference between A5 and the other groups was significant ($P < 0.001$) (Fig. 1c).

In group A2, one individual had isolates of both *C. albicans* and *C. glabrata*. In group A3, two individuals had isolates of both *C. albicans* and *C. glabrata*. In group A4, two individuals had isolates of both *C. albicans* and *C. glabrata*, and one individual had isolates of both *C. albicans* and *C. tropicalis*. In group A5, five individuals had isolates of both *C. albicans* and *C. glabrata*, and two individuals had isolates of both *C. albicans* and *C. tropicalis*.

Candida albicans *genotypic subgroups*

We found three genotypes of *C. albicans* – genotypes A–C – but did not find other genotypic groups. Genotype A is the dominating component in all dentition groups, with all of the *C. albicans* strains in group A1 being genotype A, and the proportion of genotype A in groups A2 A3 A4 and A5 being 82.6%, 92.3%, 91.3%, and 81.8%, respectively. The differences between the five groups were not significant (Table 2).

Discussion

Yeast is the most important commensal organism in the oral cavity, and the yeast carriage frequency varies as individuals age and according to geographical area. In a review by Odd in 1988 (15), the highest reported frequencies were 71% of schoolchildren in the UK, and 56% of children in Israel. In 1996, Kleinegger (3) studied the frequency, species and strains of oral *Candida* at different host ages, and the results indicated that the frequency, average intensity and genetic relatedness of *C. albicans* strains varied as a function of host age. It is therefore a reasonable possibility that these changes in frequency may be due to physiological changes related to age: changes relating to body fluids and at mucosal surfaces, as natural barriers against yeast colonization, and changes in the living environment and habits of the individual and the ecological environment of the oral cavity. Of the changes in the oral cavity's ecological environment, the substitution of different dentition is the most distinct. These changes would influence the component and species of oral commensal organisms.

Because there is evidence indicating that the distribution of yeast strains has geographical specificity, we collected samples from individuals who had lived in our city for at least one year preceding collection, and performed all collection within a 1-month time window. We chose to test individuals ranging in age from

Table 1 Frequency carriage of yeast of different dentition groups

Group	Total number of individuals	Yeast strains				*C. albicans*	*Yeast*
		C. albicans	*C. tropicalis*	*C. glabrata*	*Other*	*Frequency (%)*	*Frequency (%)*
A1	40	3	0	0	0	7.5	7.5
A2	40	23	4	2	0	57.5	70.0
A3	55	26	2	5	0	47.3	56.4
A4	57	23	3	4	1	40.4	49.1
A5	40	11	5	13	2	27.5	60.0

Table 2 The distribution of *C. albicans* genotypic subgroups in oral cavities of different groups

Groups	*C. albicans* isolates	Genotypic subgroup		
		A	*B*	*C*
A1	3	3/3 (100%)	0	0
A2	23	19/23 (82.6%)	3/23 (13.0%)	1/23 (4.4%)
A3	26	24/26 (92.3%)	1/26 (3.8%)	1/26 (3.8%)
A4	23	21/23 (91.3%)	0	2/23 (8.7%)
A5	11	9/11 (81.8%)	1/11 (9.1%)	1/11 (9.1%)

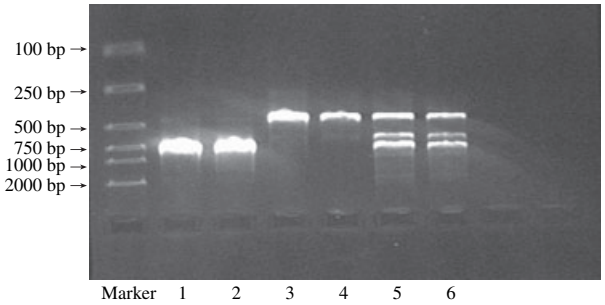

Figure 2 Different genotypic subgroups of *C. albicans*, as shown by PCR. Lanes 1 and 2 (genotypic subgroup B, PCR amplification product is about 840 bp). Lane 3 (ATCC90038 genotypic subgroup A, PCR amplification product is about 450 bp). Lane 4 (genotypic subgroup A, PCR amplification product is about 450 bp). Lanes 5 and 6 (genotypic subgroup C, three PCR amplification product bands: one band is about 450 bp and two bands are of about 840 bp). DNA Marker: DL2000 (TAKARA Dalian).

neonates to about 20 years old. For the neonate group, previous research indicated that there are greatly different carriage frequencies in the different neonatal period: from 5.7% at birth to 82% 4 weeks after birth (3). In this study, we chose a neonate age limit of 7 day in order to examine the initial colonization conditions.

Different sampling and identification methods for *Candida* spp. would certainly influence the results. We studied 55 healthy children of average age 7.4 years, and different positions on the oral mucosa (inner cheek mucosa, dorsal surface of the tongue, and bottom of mouth), different method of sampling (cotton swab and saliva), and different identification methods (CHROMagar *Candida*^{TM} medium and PCR), and compared the different yeast carriage frequencies (data unpublished). Many researchers find that mucosal swabs often produce low frequencies; we found that after swab sampling, use of centrifugal condensing of the sampling liquid can effectively improve the carriage detection frequency. The results indicate that the method of inner cheek mucosal swab in association with centrifugation, and culture and identified with CHROMagar *Candida*^{TM} culture medium is an ideal and convenient way for studying yeast and *C. albicans* in the oral cavity.

Our results show the yeast carriage frequency is very low in the neonatal group, just 7.5%, which accords with the 5.7% in the study by Russell and Lay (2). Because of the low frequency in the neonate group, the difference between the other groups and the neonate group was significant. The highest frequency of carriage of yeast and *C. albicans* was in the primary dentition group. With increasing age, the frequency of *C. albicans* decreases: However, the frequency of yeast carriage does not decrease: because the carriage of non-*C. albicans* yeast increases. There is a large proportion of individuals in group A5 with *C.glabrata* isolated, the possible reason maybe lies in the increased social contact in this age group different from the other age group. And at the same time, *C. glabrata* is the second most common *Candida* pathogen after *C. albicans* (16), causing both bloodstream and mucosal infections (17, 18), the frequently isolated of *C. glabrata* in the group of secondary dentition may indicate both *C. glabrata* and *C. albicans* are all benign commensal organisms in the oral cavities of healthy adults. The non-significant difference between the carriage frequency of oral yeasts and *C. albicans* in groups A2–A4 suggests that substitution of dentition has a minor influence of colonization.

From the tested individuals, we found three genotypic *C. albicans* groups, genotypes A–C, but could not isolate genotypes D and E. Genotype D (*C. dubliniensis*) is frequently found in immunocompromised patients, especially in HIV-infected patients (19), and we could not find it in healthy individuals. Genotype E is very rare, and we could not find it in this research. In all groups, genotypic subgroup A is the dominated subgroup, with genotypes B and C seldom isolated. Therefore, we can draw a conclusion that genotypic subgroup A is the dominating strain in the oral cavities of healthy young individuals. It is worthwhile to note that for the PCR amplification of genotype C in this research, three bands are obvious (Fig. 2), different from the result of two bands in other researches (2, 11), indicating the diversity of this transposable intron, which needs continuous study.

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