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Distribution of *Candida albicans* in the oral cavity of children aged 3-5 years of Uygur and Han nationality and their genotype in caries-active groups

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ABSTRACT. We analyzed the distribution of *Candida albicans* in the oral cavity of 3-5-year-old children of Uygur and Han nationalities as well as their genotypes in caries-active groups in the Urumqi municipality. CHROMagar Candida was separately cultivated, and we identified 359 Uygur and Han children aged 3-5 years. We randomly selected 20 Han children and 20 Uygur children for this study. We chose a bacterial strain for polymerase chain reaction (PCR) 25S rDNA genotyping and random amplified polymorphic DNA (RAPD) genotyping. The rate of caries-active in Han children was higher than that in Uygur children, with values of 39.6 and 24.3%, respectively. The detection rate of *C. albicans* was closely correlated to the caries filling index classification (χ^2 = 31.037, P = 0.000, r = 0.421; χ^2 = 80.454, P = 0.000, r = 0.497). PCR of 25S rDNA from 40 strains of Han and Uygur children revealed 3 genotypes, while RAPD analysis revealed 5 genotypes. The distribution of 25S rDNA genotyping of Han children from PCR differed from that of Uygur children (χ^2 = 7.697, P = 0.021),

both of which were mainly the A type. RAPD genotyping of both Han and Uygur children showed similar results (χ^2 = 1.573, P = 0.814). There were differences in the distributions of *C. albicans* in children of different nationalities. *C. albicans* is a key factor causing caries. The PCR 25S rDNA genotyping method is simple and sensitive, while the RAPD genotyping method is reliable and comprehensive.

Key words: *Candida albicans*; Random amplified polymorphic DNA; Polymerase chain reaction; 25S rDNA-PCR

INTRODUCTION

Candida albicans, also known as *Saccharomyces albicans*, is widely distributed in the natural environment. Infection with *C. albicans* can cause candidiasis, oral lichen planus, and vitiligo, among other conditions. Recently, *C. albicans* has been shown to cause caries (Nikawa et al., 2003). *Streptococcus mutans*, lactic acid bacteria, and actinomycetes have traditionally been recognized as the main factors causing caries bacteria in the oral cavity; however, *C. albicans* is frequently present in caries-active groups, and the rate of detection of *C. albicans* in the oral cavity is much higher than that in those who are caries-free (Ollila and Larmas, 2008; Raja et al., 2010). *C. albicans* has been detected in children's dentine caries at a rate of 71-97% (de Carvalho et al., 2006), indicating the importance of this pathogen in causing caries. There have been no studies examining the relationships between regional and national differences and *C. albicans* infection rates. Therefore, this study was conducted to determine the rate of *C. albicans* in the oral cavity of Han and Uygur children in Urumqi. Our results may provide guidance for treating caries-active disease.

MATERIAL AND METHODS

Main reagents

CHROMagar Candida (CAC) and the Biospin Fungus Genomic DNA Extraction Kit were obtained from BioFlux (South San Francisco, CA, USA). *Taq* polymerase chain reaction (PCR) reagents and nucleic acid dye were obtained from BioMed (Beijing, China), the MarkDL2000, and MarkIV were obtained from Sangon Biotech (Beijing, China).

Research target

This study was approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (No. 20071108-5) and registered at the Chinese Clinical Center (registration No. ChiCTR - PRC - 00000030). Parents of the children involved in the study signed informed consent forms. A stratified cluster sampling method was used to randomly select 389 kindergarten children aged 3-5 years from 9 schools in 3 districts of the Urumqi municipality, including 144 Uygur children and 245 Han children. Inclusion criteria were: all children had not taken antibiotics within 1 month before the inspection, had no systemic or congenital diseases, showed no symptoms of oral mucosal disease, and had not received orthodontic tooth treatments. Two hours before the test, children began to fast and washed their

mouth with phosphate-buffered saline mouthwash.

Cultivation, identification, and separation of *C. albicans*

For clinical sample collection, we used sterile toothpicks to take samples from 1/3 of the mandibular on the first molar tooth buccal cervical and 1/3 of the plaque from caries-free children (Corby et al., 2005). A Sterilizing excavator-spoon was used to sample caries-affected dentin and caries-damaged enamel from children with caries; samples were immediately placed into sterile centrifuge tubes containing 500 μL phosphate-buffered saline transmission fluid, and the sample was mixed for 2 min using a vortex machine. The sample volume was adjusted to 300 μL and centrifuged at 6000 *g* for 10 min. The clear liquid was removed, and 100 μL sterile phosphate-buffered saline was added to resuspend the pellet; 20 μL was used to inoculate CAC medium and the cells were grown at 37°C for 48 h.

Blue-green Gram stain was placed on the CAC medium for bacterial colony identification. There were 25 children with *C. albicans* in the Uyghur nationality and 56 in the Han nationality (based on decayed, missing, and filled teeth [dmft] ≥ 5). Twenty Han children were randomly selected and the typical slab single bacterial colonies on each tablet were inoculated on CAC, grown at 37°C, and purified 3 times. Purified single bacterial colonies were picked on CAC and placed in sterile ShaShi fluid; the culture was shaken for 24 h at 37°C.

PCR identification method of *C. albicans*

DNA was extracted from *C. albicans* based on the instructions in the Biospin Fungus Genomic DNA Extraction Kit. A spectrophotometric method was used to test DNA purity and content, and DNA was preserved at -20°C until use.

PCR amplification and electrophoresis

The general primers ITS1 and ITS2 (ITS1, GGAAGTAAAAGTCGTAACAAGG; ITS2, GCTGCGTTCTTCATCGATGC) were used for the PCR system. Samples were 20 μL, including 10 μL 2X EasyTaq PCR SuperMix, 0.5 L of each upstream and downstream primers, and 2.0 μL plaque DNA template. The PCR was run at 95°C for 5 min, 95°C for 30 s, 50°C for 30 s, and annealing at 72°C for 30 s for 40 cycles, and then a final extension at 72°C for 10 min.

Internal transcribed spacer (ITS) sequencing molecular identification

Purified PCR products of the randomly selected caries-active children of both Han and Uyghur nationalities were tested to determine their DNA sequences. We used ITS1-ITS2 two-way sequencing. Sequencing results are compared with the BLAST data and GenBank database, and strains were determined. Based on BLAST results, we selected homologous sequences with ≥98% reliability for genotyping.

Genotyping

The *C. albicans* clinical strains of Han and Uyghur nationality children based on ITS sequencing results were chosen as research targets.

C. albicans PCR 25S rDNA genotyping

Primer sequences for 25S rDNA were CA - INT L: 5'-ATAAGGGAAGTCGGCAAA ATAGATCCGTAA-3' and CA - INT - R: 5'-CCTTGGCTGTGGTTTCGCTAGATAGTAGA T-3' (McCullough et al., 1999). The PCR system included 15 μL 2X Easy Taq PCR SuperMix, 1 μL 10 μM DNA template primer, and ddH_2O balance to a total volume of 30 μL. Reaction conditions were: 94°C for a 3-min degeneration, followed by 94°C for 1 min, 60°C for 1 min, and 72°C for 30 s for a total of 30 cycles. The reaction product was run on 15 g/L agarose gel electrophoresis at 100 V for 30 min with Genefinder nucleic acid dye. Bands were visualized under ultraviolet (UV) illumination. Different genetic groups were determined according to the size of the amplification products.

Random amplified polymorphic DNA (RAPD) genotyping of *C. albicans*

The primers RSD6: 5'-GCGATCCCCA-3' (Waltimo et al., 2001) and RSD12: 5'-GCATATCAATAAGCGCAGGAAAAG-3' (Leung et al., 2000) were used for the PCR with a total volume of 30 μL that included 15 μL 2X EasyTaq PCR SuperMix and 1 μL 10 mM DNA template primer. Reaction conditions were: 94°C for 30 s, 27°C for 2 min, and 72°C for 2 min for 5 cycles, followed by 94°C for 30 s, 32°C for 2 min, and 72°C for 2 min for 45 cycles, and finally a 15-min elongation step at 72°C. The reaction product was subjected to 12 g/L agarose gel electrophoresis at 50 V for 120 min with Genefinder nucleic acid dye. Bands were visualized under UV illumination. Genotypes were determined according to the size and number of amplification products. The RAPD analysis experiment was repeated in accordance with the above experimental conditions to test the repeatability and stability of RAPD.

Statistical analysis

The SPSS17.0 statistical software was used for data analysis (SPSS, Inc., Chicago, IL, USA). Differences between the Han and Uygur groups regarding genotype distribution were determined using the Pearson χ^2 test. $P < 0.05$ was considered to be significant.

RESULTS

Cultivation and identification of *C. albicans*

After culturing at 37°C for 24 h, *C. albicans* formed white or pale green oblate colonies on the tablet, turning an emerald green or turquoise color after 48 h with sizes ranging 1-3 mm in diameter. Colonies with smooth surfaces and edges were inoculated into medium. After identification of Gram stains, large numbers of positive Gram-stained ovoid spores and pseudohyphae were observed under a microscope (Figure 1).

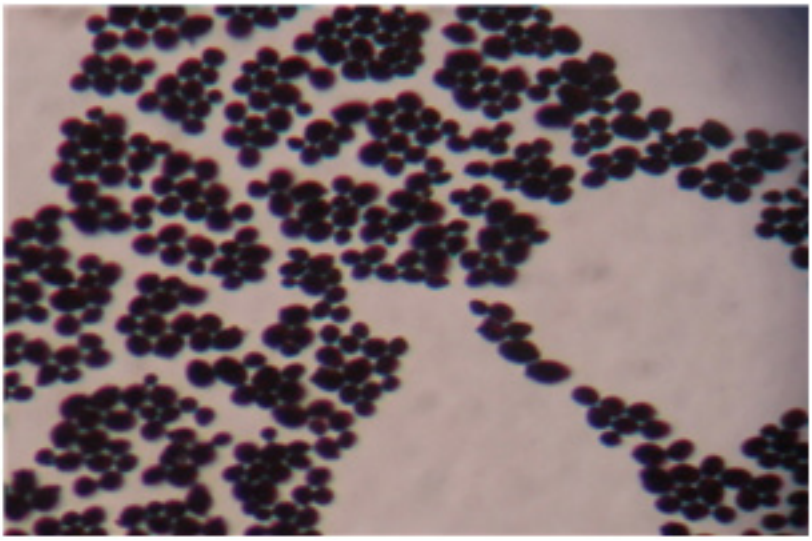

Figure 1. *Candida albicans* Gram staining.

PCR products

C. albicans PCR identification results are shown in Figure 2. CAC identification medium showed green colonies that were bright and clear.

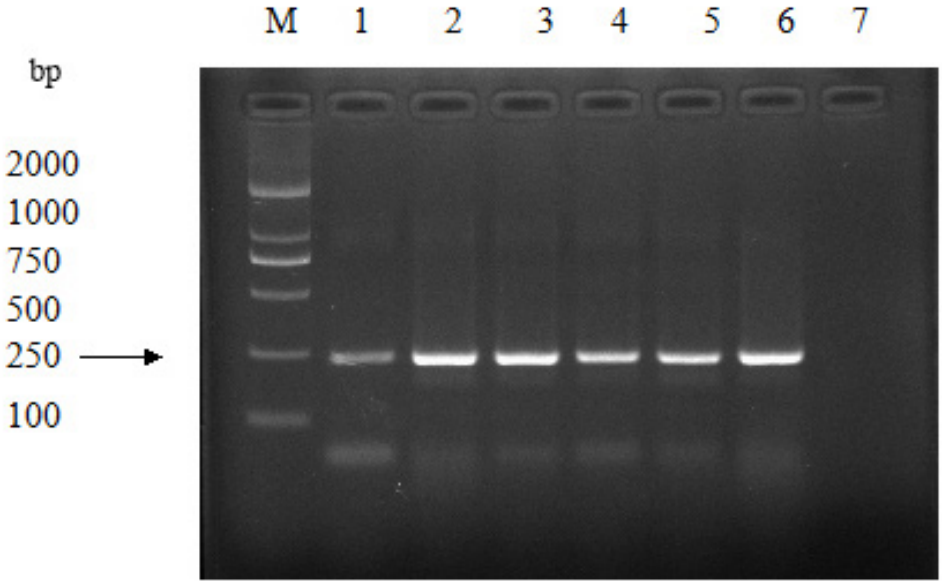

Figure 2. *Candida albicans* PCR identification. Amplification products of approximately 200 bp. *Lane M*: DNA marker: DL2000; *lane 7*: negative control; *lanes 1-6*: *C. albicans* identification products.

Cavity prevalence

The distribution of oral cavities in Uygur and Han nationality children are shown in Table 1. The detection rate of *C. albicans* of the Han and Uygur nationalities were 39.6 and 24.3%, respectively, and their distribution difference was statistically significant ($\chi^2 = 9.453$, $P < 0.05$); the dmft value was divided into 4 levels (Gábris et al., 1999): 0, $0 < dmft \leq 3$, $3 < dmft \leq 6$, and $dmft > 6$. The detection rate of *C. albicans* without caries of children in both nationalities was low, and there was no statistically significant difference ($\chi^2 = 1.102$, $P > 0.05$). In groups of $0 < dmft \leq 3$, there were differences in *C. albicans* distribution ($\chi^2 = 6.403$, $P < 0.05$); in the $3 < dmft \leq 6$ groups, there was no difference in *C. albicans* distribution ($\chi^2 = 0.694$, $P > 0.05$); in groups of $dmft > 6$, there was a significant difference in *C. albicans* distribution ($\chi^2 = 8.795$, $P < 0.05$). The *C. albicans* detection rate and dmft grading were correlated in Uygur children ($\chi^2 = 31.037$, $P < 0.001$, $r = 0.421$); the Han nationality *C. albicans* detection rate and dmft grading were also correlated ($\chi^2 = 80.454$, $P < 0.001$, $r = 0.497$). The *C. albicans* detection rate of classification was positively correlated with dmft.

Table 1. Distribution of oral *Candida albicans* based on decayed, missing, and filled teeth (dmft) classification in Han and Uygur ethnicity children (%).

dmft	Uygur ethnicity		Han ethnicity		χ^2	P
	Detected	Non-detected	Detected	Non-detected		
0	2 (3.8)	51 (96.2)	8 (8.25)	89 (91.75)	1.102	0.294
$0 < dmft \leq 3$	4 (13.8)	25 (86.2)	18 (41.9)	25 (58.10)	6.403	0.011
$3 < dmft \leq 6$	11 (44.0)	14 (56.0)	25 (43.5)	21 (56.50)	0.694	0.405
$6 < dmft$	18 (48.6)	19 (51.4)	46 (78.0)	13 (22.0)	8.795	0.003
Total	35 (24.3)	109 (75.7)	97 (39.6)	148 (60.0)	9.453	0.002
χ^2	31.037		80.454			
P	0.000		0.000			
r	0.421		0.497			

ITS sequencing and molecular identification

The ITS sequencing results confirmed the identity of *C. albicans*, and the reliability was ≥98%.

C. albicans genotyping

As shown in Figure 3, based on main band number and location of PCR products on the agarose gel, *C. albicans* was divided into 3 genotypes (Millar et al. 2005). Based on the results of the Pearson χ^2 test, the high-caries oral *C. albicans* PCR 25S rDNA genotyping distribution was different between the Han and Uygur children ($\chi^2 = 7.697$, $P < 0.05$). The A genotype in the Han and Uygur groups accounted for 45.0 and 75% of the identified types, respectively. The distribution of genotype A in both groups showed no significant difference ($\chi^2 = 3.750$, $P > 0.05$). The structure rate of genotype B in both groups showed no significant difference ($\chi^2 = 0.173$, $P > 0.05$). The structure rate of genotype C in both groups showed a significant difference ($\chi^2 = 7.025$, $P < 0.05$); thus, the structure rate of genotype C of oral *C. albicans* PCR 25S rDNA genotyping was significantly different between high-caries children of the two nationalities, while there was no difference in genotypes A and B (Table 2).

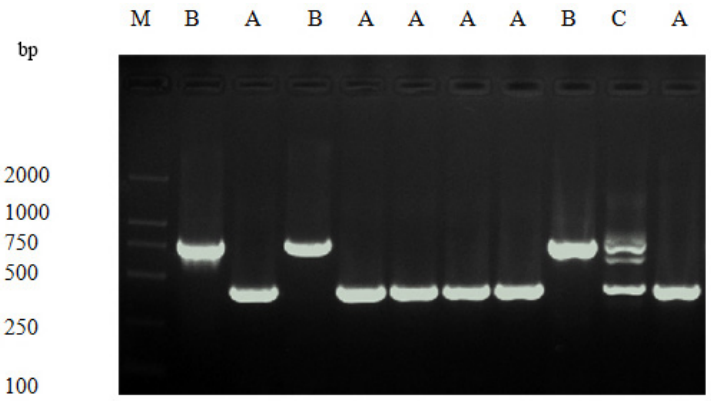

Figure 3. *Candida albicans* PCR 25S rDNA genotyping. *Lane M*: DNA marker DL2000.

Table 2. Formation of genotype oral *Candida albicans* of Han and Uighur children.

Ethnic group	A type	B type	C type	Totality
Han	10 (50.0)	3 (15.0)	7 (35.0)	20
Uighur	15 (75.0)	4 (20.0)	1 (5.00)	20

C. albicans RAPD genotyping

As shown in Table 3, between the 2 RAPDs (RSD6, 12), primer RSD6 produced the largest number of bands with more clear patterns and consistent results. Thus, RSD6 was selected for DNA amplification of 40 *C. albicans* isolates. The RAPD method was used to detect 5 stable genotypes (Figure 4). After the Pearson χ^2 test, a high rate of dental caries and *C. albicans* genotyping distribution by RAPD in Han and Uygur children showed no significant differences ($\chi^2 = 1.573$, $P = 0.814$), and constituent ratios between groups showed no significant differences.

Table 3. Uygur and Han RSD6-RAPD genotyping.

Ethnic group	1 type	2 type	3 type	4 type	5 type	Totality
Han	7	3	4	3	3	20
Uighu	10	2	2	4	2	20

DISCUSSION

It has been reported that regardless of the presence or absence of a smear layer on teeth, *C. albicans* can easily adhere to the enamel, dentin, and cementum surfaces (Klinke et al., 2009). The acid produced can dissolve hydroxyapatite at a rate 20 times higher than that of *S. mutans* and *Lactobacillus* species. *C. albicans* can also use carbohydrates that *S. mutans* cannot, such as sorbitol and xylitol. Thus, *C. albicans* may have strong cariogenic potential. The relationship between *C. albicans* and caries has received increasing attention (Kędzia et al., 2008; Marchant et al., 2011). Our results showed that Han children have a wider distribu-

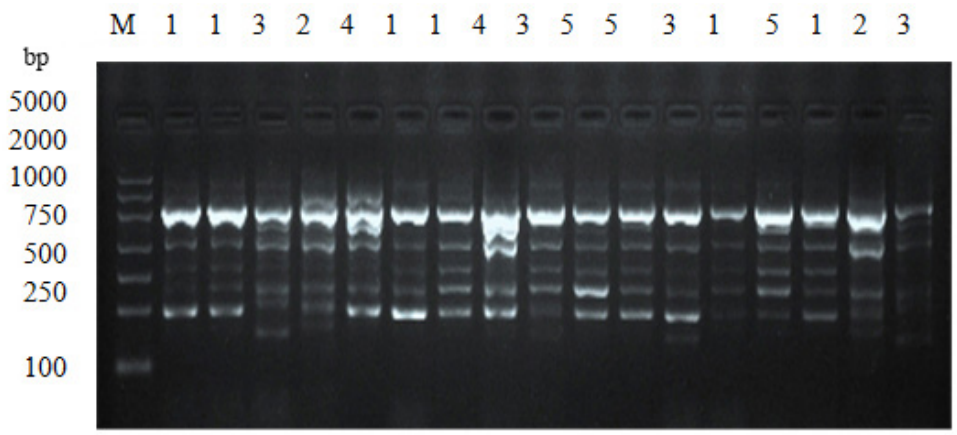

Figure 4. *Candida albicans* RAPD genotyping. *Lane M*: DNA marker MarkIV. *Lanes 1-5*: stable genotypes.

tion of oral *C. albicans* genotypes than Uygur children, which has not been previously documented in China or other countries. In addition, the detection rate of oral *C. albicans* showed a positive relationship with increasing dmft, which agrees with the results of Marchant et al. (2011) and Radford et al. (2000), suggesting that *C. albicans* may be a risk factor for the development of dental caries in children.

Genotyping studies are beneficial for understanding epidemic strain ranges, predisposing factors, and biological flora polymorphisms, which can be used to develop effective prevention measures. *C. albicans* genotyping technologies include restriction enzyme polymorphism analysis, microsatellite polymorphism analysis, PCR-25S rDNA analysis, and RAPD analysis. Among these methods, PCR-25S rDNA technology and the RAPD technique are the most commonly used. PCR-25S rDNA refers to the PCR analysis of *C. albicans*' ITS, which is located between the 5.8S and 28S rDNA regions (ITS2). Because of the lack of mature ribosomes, the ITS shows low selection pressure, a fast evolutionary rate, and is more likely to exhibit an extremely wide range of genetic polymorphisms. In addition, ITS sequences are of moderate length and are widely used in systematic studies of species within a genus or intraspecific groups. Using PCR, RAPD technology is a molecular technique that can be used for polymorphism analysis of an unknown sequence. Using genomic DNA as a template, single synthetic random polymorphic nucleotide sequences as primers, and a thermostable DNA polymerase, PCR can be used to identify genetic polymorphisms.

In this study, we examined 20 randomly selected samples of oral *C. albicans* from high-caries children of each of the Han and Uygur nationalities. PCR 25S rDNA technology was used to analyze genotypes. To enhance the reliability of genotyping results, we confirmed the 40 clinical isolates as *C. albicans* using an ITS test, which showed a Gene homology ≥98%; this method also demonstrated the effectiveness and credibility of CAC oral plaque detection.

C. albicans PCR 25S rDNA genotyping technology divided the isolates into 5 types, including an A-type PCR product at 480 bp, B-type at 840 bp, and C-type with 3 clear amplification products, with 2 bands at 840 bp and 1 at 450 bp. All showed strong specificities, which slightly differed from the results of McCullough (1999). The C type may have been generated through sexual reproduction of A and B types, or may be a transitional type of the 2 groups. The introns contain a specific polymorphism; however, we were unable to identify the poly-

morphism in this study. We did not observe the presence of the D or E types. The dominant *C. albicans* type in Han children was the A type, while the C type was the second most prevalent, and the B type was observed least frequently, which agrees with the results of Liu and Lu (2012). Using the RSD6 primers, we determined 5 types of stable genotypes and there was no difference in high-caries distribution RAPD typing between nationalities.

Comparison of the results showed that the PCR 25S rDNA and RAPD genotyping methods were not completely consistent but could still be used for *C. albicans* genotyping. The PCR 25S rDNA method is simple and showed high repeatability and specificity compared to RAPD analysis, and that the gene sequences determined using RAPD were more polymorphic than those obtained using PCR 25S rDNA. However, the amplification conditions can be optimized and standardized to obtain stable typing. There are advantages and disadvantages to both methods, and thus employing the methods together increases the overall reliability. McCullough et al. (1999) genotyped *C. albicans* resistance and found that *C. albicans* resistant and sensitive strains of different genetic makeup have become the basis for developing new and effective antifungal treatments. A *C. albicans* PCR 25S rDNA study showed that high oral caries involving *C. albicans* are most frequently of the A-type genotype, indicating that those carrying *C. albicans* genotype A are more susceptible to caries. The distribution of C-type genetic showed no differences, which may be related to the pathogenicity of *C. albicans* and its mechanisms of action. This also suggests that different environments may lead to the selective growth of different host families of *C. albicans*, reflecting differences in the oral microenvironment between the 2 nationalities. However, in the present study of the *C. albicans* gene using RAPD, type 1 was the most dominant, suggesting that carriers of type 1 may be more susceptible to caries.

In summary, children of the Uygur and Han nationalities 3-5 years in age show differences in the distribution of high-caries genotyping, which may be related to ethnic, geographic, and dietary differences.

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