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Study on the Distribution at Species Level of Genus *Candida* in Human Oral Cavities, Using Culture and Multiplex PCR Methods

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Abstract

Purpose: Although the genus *Candida* is frequently isolated from human oral cavities, the distribution at the species level of these organisms has been little reported. The purpose of the present study was to assess the distribution at the species level of the genus *Candida* in human oral cavities. **Methods:** This study was performed using culture and Multiplex PCR methods. Moreover, the genotyping classification of *C. albicans* was analyzed with a PCR. **Results:** Of all subjects (n = 90), detection frequency of genus *Candida* was 42.2%. Genus *Candida* was not detected in the subjects between 0 to 9 years old, and there was no difference in the detection frequencies of this organism among each generation from 10s to 80s. *C. albicans* was the most dominant species, followed by *C. parapsilosis*, *C. glabrata*, and *C. dubliniensis*. Plural *Candida* species tended not to be detected in the individual sample. Genotype A was dominant in the *C. albicans* isolates. **Conclusion:** These results indicated that *C. albicans* of genotype A was dominant and that the genus *Candida* rarely coexists with other *Candida* species, in each individual oral cavity.

Keywords

Candida, *Candida albicans*, Oral Cavity, Multiplex PCR

1. Introduction

The increasing population of immunocompromised patients due to infection

with human immunodeficiency virus (HIV), chemotherapy, organ transplantation and the common use of indwelling intravascular devices have significantly increased the incidence of candidiasis [1] [2]. Systemic candidiasis in hospitalized patients is a significant cause of morbidity and mortality among severely ill individuals and candidemia has been ranked the fourth most prevalent cause of bloodstream infections with its attributable mortality (40%) exceeding that of bacteremia [3] [4] [5] [6]. *Candida albicans* is generally accepted as being the most pathogenic member of the genus and the dominant causative agent of candidiasis and a major nosocomial pathogen [7] [8] [9]. However, there has been a significant upward trend in the emergence of non-*albicans Candida*, especially *Candida glabrata*, *Candida parapsilosis*, and *Candida tropicalis* [10] [11] [12]. In addition, because several non-*C. albicans Candida* species are frequently resistant to common antifungal agents, accurate identification methods are essential for the establishment of appropriate antifungal therapy.

We previously developed a one-step multiplex PCR method with the ability to identify and differentiate eight medically important *Candida* species (*i.e.*, *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. dubliniensis*, *C. guilliermondii*, *C. krusei*, and *C. lusitaniae*) using only one PCR tube per sample [13]. Our multiplex PCR method is easy because the use of MightyAmp DNA Polymerase Ver.3 (Takara) means that DNA extraction is not necessary, and species identification and detection using this method only takes approximately 2 hours. Thus, our method is useful to allow the prevalence of the eight medically important *Candida* species to be fully clarified.

Several *Candida* species are components of the commensal oral flora that are often isolated from the oral cavity of healthy humans [14] [15] [16]. The most common *Candida* species that harbors the oral cavity is *C. albicans*. Although the genus *Candida* is frequently isolated from human oral cavities, the distribution at the species level of these organisms in this organ has been little reported.

The strain delineation within *C. albicans* and its distribution among patients are important for the identification of the dominant types of *C. albicans* responsible for candidiasis and to determine the relationships of their subtypes to human disease. Genotyping is highly sensitive and offers greater discrimination compared to biotyping, thus allowing more detailed studies on the epidemiology and pathogenesis of microorganisms [17]-[22]. McCullough *et al.* [21] reported the use of polymerase chain reaction (PCR) for differentiation of *C. albicans* using primers designed to span the 25 S rRNA gene (rDNA). The advantage of using the technique is that it can detect *Candida dubliniensis* (genotype D) as well as determine the genotypes of *C. albicans* [21] [22]. McCullough *et al.* [21] confirmed that genotype B belongs to the same taxon as type I *Candida stellatoidea* and genotype D belongs to the same taxon as *C. dubliniensis*. The use of this genotype analysis method is simple and reproducible when reference *C. albicans* strains are used [22]. In addition, a new genotype of *C. albicans* with Group I intron, genotype E, was reported using the similar approach [23].

The purpose of the present study was to assess the distribution at the species level of the genus *Candida* in human oral cavities, using culture and multiplex PCR methods. Moreover, the genotyping classification of *C. albicans* was analyzed with a PCR.

2. Materials and Methods

2.1. Subjects

Ninety volunteers (43 men, 47 women; range 4 - 88 years) who visited Nihon University Hospital, School of Dentistry at Matudo, during 2021, participated in the present study. They had no systemic disease and received no antibiotic therapy for at least 3 months, and also none wore a denture. All participants were asked not to brush, rinse, or smoke immediately prior to the assessment and not to eat or drink for at least 2 h beforehand. Subjects were divided into nine groups, *i.e.*, 0 to 9 years old (under 10; average age: 6.6), 10 to 19 years old (the 10s; average age: 13.1), 20 to 29 years old (the 20s; average age: 24.4), 30 to 39 years old (the 30s; average age: 35.2), 40 to 49 years old (the 40s; average age: 45.6), 50 to 49 years old (the 50s; average age: 55.4), 60 to 69 years old (the 60s; average age: 63.3), 70 to 79 years old (the 70s; average age: 72.6), and 80 to 89 years old (the 80s; average age: 84.0). The present study was approved by the Ethics Committee of Nihon University School of Dentistry at Matsudo, Japan (EC 20-022). Informed consent was obtained from all subjects.

2.2. Clinical Samples

Paraffin-stimulated whole saliva samples were collected in a sterile microcentrifuge tube. All samples were dispersed by sonication for 30 s in an ice bath (50 W, 20 kHz, Astrason® System model XL 2020, NY, USA). Portions (100 µl) of appropriate dilutions of these samples were inoculated on CHROMagar™ *Candida* (CHROMagar, Paris), a commercial selective medium for the genus *Candida*.

Selective medium plates were cultured at 30˚C for 2 days under aerobic conditions. After cultivation, the number of CFU/ml on a selective medium was calculated and compared.

2.3. Identification of *Candida* Species Isolated from Clinical Samples

Twenty-four of the approximately 50 colonies that grew on the selective medium plate per subject were randomly isolated and subcultured, and their species identifications were then confirmed by a multiplex PCR analysis. Subcultured isolates were suspended in 1.0 McFarland standard in 100 µl of distilled water, and 5.6 µl of the suspension was used as a template for PCR. The multiplex PCR condition and PCR primers used in this study were performed as described previously [13]. Briefly, the multiplex PCR mixture contained 0.2 µM of each primer, 10 µl of 2 × MightyAmp Buffer Ver.3 (Takara Bio Inc., Shiga, Japan), 0.4 µl of MightyAmp DNA Polymerase (Takara), and 5 µl of the template in a final

volume of 20 μl. PCR reactions were performed in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, CA, USA). PCR conditions included an initial denaturation step at 98˚C for 2 min, followed by 30 cycles consisting of 98˚C for 10 s and 68˚C for 1 min. PCR products were analyzed by 2.0% agarose gel electrophoresis before being visualized by electrophoresis in 1 × Tris-borate-EDTA on a 2% agarose gel stained with ethidium bromide. A 100-bp DNA ladder (Takara Biomed, Shiga, Japan) was used as a molecular size marker.

2.4. Genotyping Classification of *C. albicans* Isolates

The genotyping classification of *C. albicans* isolates was analyzed with a PCR as follows. Subcultured *C. albicans* isolates were suspended in 1.0 McFarland standard in 100 μl of distilled water, and 5.6 μl of the suspension was used as a template for PCR. PCR was performed as described previously [21]. Briefly, the PCR mixture contained 0.2 μM of CA-INT-L primer (5'-ATAAGGGAAGTCGGCAAAATAGSTCCGTAA-3') and CA-INT-R (5'-CCTTGGCTGTGGTTTCGCTAGATAGTAGAT-3') primer, 10 μl of 2 × MightyAmp Buffer Ver.3 (Takara Bio Inc., Shiga, Japan), 0.4 μl of MightyAmp DNA Polymerase (Takara), and 5.6 μl of the template in a final volume of 20 μl. PCR was carried out in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler). PCR conditions included an initial denaturation step at 98˚C for 2 min, followed by 30 cycles consisting of 98˚C for 10 s and 68˚C for 1 min. PCR products were analyzed by 2.0% agarose gel electrophoresis and visualized by gel staining with ethidium bromide. A 100-bp DNA ladder was used as a molecular size marker (Takara Biomed).

3. Results

3.1. Detection Frequency of Genus *Candida* in Saliva Samples

The detection frequencies of genus *Candida* in saliva samples from ninety healthy subjects are shown in Table 1. The detection frequencies of genus *Candida* in saliva samples from the under 10, 10s, 20s, 30s, 40s, 50s, 60s, 70s, and 80s groups were 0%, 40%, 40%, 20%, 70%, 40%, 60%, 60%, and 50%, respectively. The mean numbers of *Candida* species in each subject were 0 CFU/ml, and 5 CFU/ml, 590 CFU/ml, 46 CFU/ml, 659 CFU/ml, 375 CFU/ml, 139 CFU/ml, 96 CFU/ml, and 17 CFU/ml, respectively.

3.2. Detection Pattern of *Candida* Species in *Candida* Positive Samples

The detection patterns of *Candida* species in *Candida positive* samples are shown in Table 2. Of 38 *Candida* positive samples, the sample numbers of *C. albicans* alone, *C. dubliniensis* alone, *C. parapsilosis* alone, *C. albicans* and C. *glabrata*, *C. albicans* and unidentifiable *Candida* spp., and *C. albicans* and *C. dubliniensis* and *C. glabrata* were 32 (84.2%), 1 (2.6%), 1 (2.6%), 1 (2.6%), 2 (5.3%), and 1 (2.6%), respectively.

Table 1. Detection frequency of Genus *Candida* in each generation.

Age (Average)	No. of subjects	No. *of Candida* positive samples (Frequency, %)	Average CFU/ml
0 - 9 (6.6)	10	0 (0)	0
10 - 19 (13.1)	10	4 (40)	5
20 - 29 (24.4)	10	4 (40)	590
30 - 39 (35.2)	10	2 (20)	46
40 - 49 (45.6)	10	7 (70)	659
50 - 59 (55.4)	10	4 (40)	375
60 - 69 (63.3)	10	6 (60)	139
70 - 79 (72.6)	10	6 (60)	96
80 - 89 (84.0)	10	5 (50)	17

Table 2. Detection pattern of *Candida* species in *Candida* positive samples.

Detected *Candida* species	No. of samples n = 38 (Frequency, %)
C. albicans alone	32 (84.2)
C. dubliniensis alone	1 (2.6)
C. parapsilosis alone	1 (2.6)
C. albicans + *C. glabrata*	1 (2.6)
C. albicans + unidentifiable *Candida* spp.	2 (5.3)
C. albicans + *C. dubliniensis* + *C. glabrata*	1 (2.6)

3.3. Distribution of *C. albicans* Genotypic Subgroups

The distribution of *C. albicans* genotypic subgroups is shown in **Table 3**. Of 36 *C. albicans* positive samples, the number of *C. albicans* classified in genotypes A, B, C, D, and E was 23 (63.9%), 8 (22.2%), 5 (13.9%), 0 (0%), and 0 (0%) respectively. **Figure 1** shows the genotyping classification of *C. albicans* isolates by PCR. Amplicon sizes of genotypes A, B, and C were 450 bp, 840 bp, and 450 and 840 bp, respectively.

4. Discussion

The genus *Candida* belongs to the Fungi kingdom, class of deuteromycetes, and comprises between 150 and 200 species. In recent years, distinct shifts in the distribution of *Candida* species isolated from nosocomial infections have been reported. Although *Candida albicans* remains the most frequent cause of candidemia and haematogenously disseminated candidiasis, an increasing number of hospital-acquired infections due to other *Candida* species, so-called non-*albicans* *Candida* species, is being observed [24] [25]. Candidemia is often associated with human immunodeficiency virus (HIV) or advanced medical and surgical interventions that compromise patient immunity, e.g., bone-marrow or solid-organ

Table 3. Distribution of *C. albicans* genotypic subgroups.

Genotype	No. of samples (Frequency, %) n = 36
Genotype A	23 (63.9)
Genotype B	8 (22.2)
Genotype C	5 (13.9)
Genotype D	0 (0)
Genotype E	0 (0)

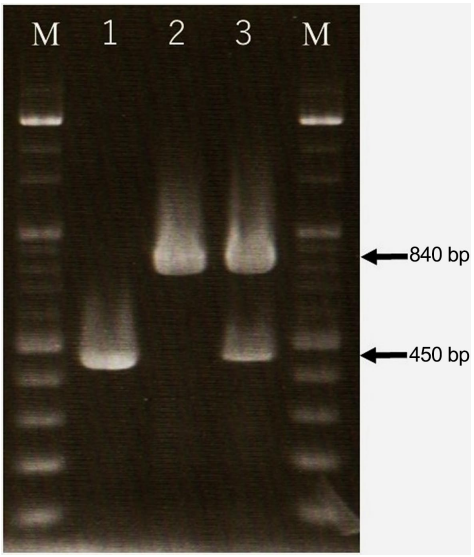

Figure 1. Genotyping classification of *C. albicans* isolates by a PCR. Lanes: 1, *C. albicans* isolate of genotype A; 2, *C. albicans* isolate of genotype B 3, *C. albicans* isolate of genotype C; M, molecular size marker (100-bp DNA ladder).

transplants, aggressive chemotherapy and broad application of antifungal agents [26]. In fact, nosocomial fungal blood-stream infections are an increasingly significant cause of morbidity, with an estimated mortality of 25% - 38% [27]. *C. albicans* is the most common and clinically relevant pathogen of the genus. However, there has been a significant upward trend in the emergence of non-*albicans Candida*, especially *Candida glabrata*, *Candida parapsilosis*, and *Candida tropicalis* [10] [11] [12]. In addition, because several non-*C. albicans Candida* species are frequently resistant to common antifungal agents, accurate identification methods are essential for the establishment of appropriate antifungal therapy [28].

Several *Candida* species are components of the commensal oral flora that are often isolated from the oral cavity of healthy humans [14] [15] [16]. The most common *Candida* species that harbors the oral cavity is *C. albicans*. Although oral yeasts remain dormant under physiologic conditions; however, under opportunistic conditions, they may transform into contagious pathogens and in-

duce oral diseases such as oral candidiasis or thrush [29] [30]. Results from a recent clinical study reported that the subgingival oral biofilm is a reservoir for increased *Candida* colonization [31]; and in susceptible patient groups (such as individuals with poor oral hygiene status), oral *Candida* growth can contribute to the progression of periodontal diseases such as chronic periodontitis [32].

Although the genus *Candida* is frequently isolated from human oral cavities, the distribution at the species level of this organism in this organ has been little reported. Saliva is an excellent sample that reflected the intraoral conditions, and collecting samples is easy and rapid [33]. Therefore, paraffin-stimulated whole saliva was used as the clinical specimen in the present study. Of all subjects (n = 90), the average detection frequency of the genus *Candida* was 42.2%. Some studies have reported that the carrier rate of oral *Candida* species in healthy subjects ranges between 17% and 75% [34] [35]. The result in this study was the almost middle level of those in the previous studies. In this study, the genus *Candida* was not detected in the subjects between 0 to 9 years old, and there was no significant difference in the detection frequencies of this organism among each generation from 10s to 80s. Previous studies reported that risk factors associated with an increase in oral *Candida* colonization included immunosuppression, advanced age, steroid therapy, habitual tobacco smoking, edentulism, denture-wearing, and poor oral hygiene status [16] [36] [37]. In this study, all subjects had no systemic disease and received no antibiotic therapy for at least 3 months, and also none wore a denture. The oral cavities of dentate healthy elderly people might not be particularly proper for the reservoir of the genus *Candida*, compared with those of young people. Moreover, interestingly, it was indicated that the oral cavities of children under 10 years old might not be suitable for *Candida* colonization. Further studies might be needed to confirm this.

In this study, *C. albicans* was the most dominant species, followed by *C. dubliniensis*, *C. glabrata*, and *C. parapsilosis*. Our results support previous studies showing that *C. albicans* is an integral component of the normal oral flora [29] [38]. In this study, plural *Candida* species tended not to be detected in the individual sample. This finding indicated that the genus *Candida* rarely coexists with other *Candida* species in each individual oral cavity.

In this study, three genotypes of *C. albicans*, namely, genotypes A, B, and C, were found. However, genotypes D and E were not detected. Genotype A was dominant in the *C. albicans* isolates, followed by genotypes B and C. Qi *et al.* also reported that three genotypic *C. albicans* groups (A, B, and C) were detected, and genotypic subgroup A was dominant in healthy oral mucosa of all age groups [39]. When there is micro-ecological disequilibrium in the oral environment, *C. albicans* of genotype A may be capable of colonization on dental surfaces.

5. Conclusion

In this study, the distribution at the species level of the genus *Candida* in human oral cavities was analyzed. Our findings indicated that *C. albicans* of genotype A

was dominant and the genus *Candida* rarely coexists with other *Candida* species in each individual oral cavity. Because the pathogenicity of *C. albicans* belonging to genotype A remains unclear in human oral cavities, it was considered that further exploration would be needed in the future.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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