Identification of Putatively Virulent Candida Species in Saliva Obtained from Elderly Individuals in Gwangju

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Candida albicans and their associated Candida species are opportunistic pathogens which exists as normal flora in the oral cavities of healthy individuals. In response to physiological changes in the host, these yeasts can become pathogenic, resulting in oral candidiasis. The rapid detection and identification of Candida species in clinical laboratories are extremely important for the management of patients with hematogenous candidiasis. The presently available culture and biochemical methods for detection and species identification of Candida are time-consuming and lack the required sensitivity and specificity. In this study, we have established a seminested PCR (snPCR) using universal and species-specific primers for detection of Candida species in saliva. The universal outer primers amplified the 3′end of 5.8S ribosomal DNA (rDNA) and the 5′end of 28S rDNA, including the internally transcribed spacer 2 (ITS2), generating 350–410-bp fragments from the four commonly encountered Candida spp., viz., C. albicans, C. tropicalis, C. glabrata, and C. parapsilosis. The saliva from 331 healthy and, over 50 years of aged people lived in Dong-gu, Gwangju city, was collected. Total DNA were extracted by Hoffman–Winston yeast total DNA prep. method and performed the snPCR. Results appeared to be negative on 292 people (88.2%), however, 26 people (7.9%) were positive Candida albicans, 6 people (1.8%) were positive Candida glabrata, 5 people (1.5%) were positive Candida tropicalis, and only 2 person (0.6%) were positive Candida parapsilosis. These result showed that detection and identification of Candida species could be established by saliva analysis, so that snPCR on saliva is useful method of diagnosis of clinical fields.

Key words: C. albicans, Candida, Candidiasis, C. glabrata, C. parapsilosis, C. tropicalis, saliva, sn-PCR

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I. INTRODUCTION

Nosocomial candidiasis is a major fungal infection occurring mostly in patients undergoing prolonged...
hospitalization due to a variety of underlying conditions\(^1\). Bloodstream infections due to Candida are now regarded as the fourth most frequent cause of septicemia, with a mortality rate of about 50%\(^2\). Diagnosis of candidemia or hematogenous candidiasis has been problematic due to the low positivity of blood cultures. Even in patients with autopsy-proven systemic candidiasis, the rate of recovery from blood cultures ranged between 40 and 60%\(^3\). Although various laboratory tests based on detection of Candida–specific antibodies, antigens, or metabolites have been developed, they all suffer from lack of specificity and/or sensitivity, besides being time-consuming\(^4\).

In order to overcome the limitations of conventional diagnostic tests, DNA–based methods have been developed for the detection of Candida species and offer a potentially more sensitive means of diagnosing systemic candidiasis\(^5,6\). The use of PCR–based tests to detect Candida DNA in body fluids has produced encouraging results\(^7\textsuperscript{–}\textsuperscript{11}\). However, detection of Candida species by PCR lacks sensitivity when the test is performed with blood or serum specimens\(^12\). DNA amplification with universal fungal primers followed by detection using species–specific probes greatly improved the sensitivity of Candida detection\(^13\textsuperscript{–}\textsuperscript{16}\), but probing methods involved the use of radioactivity and/or laborious and time–consuming additional steps.

Saliva is a complex endocrine secretion important in oral cavity homeostatic maintenance\(^17,18\). It is well known that the functions of saliva with regard to flow and molecular composition (proteins, glucoproteins, and phosphoproteins) are to protect oral tissues against desiccation and environmental aggressions, modulate demineralisation–remineralisation, lubricate the occlusal surfaces, and to maintain the ecological balance\(^19\).

Antifungal drugs are widely prescribed in both the community and hospital settings, but despite recent developments, there are still only a limited number of licensed antifungal agents available in comparison to the number of antibacterial agents\(^20\). Candida albicans and non–albicans species have various degrees of sensitivity to the common antifungal agents; consequently, information on this sensitivity would be helpful in predicting the likely efficacy of subsequent treatment\(^21\textsuperscript{–}\textsuperscript{23}\).

The purpose of present study is to identify the Candida species using by snPCR in saliva. And to prescribe specific antifungal drugs to Candida species, antifungal drug sensitive test was performed.

### II. MATERIALS and METHODS

#### 1. Reference Organisms

The reference strains used in the study were *C. albicans* KCTC 7965, *C. parapsilosis* KCTC 7214, *C. tropicalis* KCTC 7212 and *C. glabrata* KCTC 7219.

#### 2. Saliva

A total of 331 institutionalized over 50 age elderly individuals resident in Dong–gu, Gwangju, Korea, were studied.

Saliva specimens were collected between 9:00 and 11:30 a.m. All subjects were confirmed that they had not consumed food or drink nor taken medicine in the preceding hour. In addition, none of the patients had smoked tobacco, brushed their teeth, or taken an antimicrobial mouth rinse 1 hr before saliva collection.

Whole saliva was collected during patient were asked to chew a gum base (3 g, Lotte, Korea) and to expectorate whole saliva into 50 mL tube for 3 min.
Then, 1 ml saliva was separated by centrifugation at 13,000 rpm for 10 min in a 1.5 ml tube. After centrifugation of each specimen to collect pellet any insoluble particles, the volume of the saliva supernatant was eliminated.

3. DNA Preparation

For DNA extraction of Candida spp., 3 ml culture of the yeast in YM broth was grown overnight to stationary phase and yeasts were harvested by centrifugation at 1,200 g. For DNA extract from saliva, 1 ml of saliva were harvested by centrifugation at 1,600 g. The each of pellets were resuspended in 200 μl lysis buffer (10 mM Tris, pH 8.0 containing 2% v/v Triton-X-100, 1% w/v SDS, 100 mM NaCl, and 1 mM EDTA) along with three glass beads (200 microns). The suspension was vortexed at maximum speed for 3 min. The lysates were deproteinized with a mixture containing phenol, chloroform, and isoamyl alcohol (25:24:1). The DNA from the aqueous phase was precipitated with 2.5 volumes of chilled ethanol, washed with 70% (v/v) ethanol, dried, and resuspended in 50 μl of TE buffer.

4. PCR Primers.

A 22-bp forward primer, CTSF (5′-TCGCATCGATGAAGAACGCAGC-3′), and a 25-bp reverse primer, CTSR (5′-TCTTTTCCTCCGCTTATTGATATGC-3′), capable of amplifying the 3′ end of 5.8S rDNA and the 5′ end of 28S rDNA, including the intervening spacer region, were synthesized by Bioneer, Inc., Daejeon Korea. Species-specific oligonucleotide primers for snPCR were derived from the ITS2 regions of C. albicans (CADET, 5′-ATTGCTTGCGGCGGTAACGTCC-3′), C. parapsilosis (CPDET, 5′-ACAAACTCCAAAACTTCTTCCA-3′), C. tropicalis (CTDET, 5′-AACGCTTATTTTGCTTAGGCT-3′), and C. glabrata (CGDET, 5′-TAGGTTTTACAACAATCGGTGT-3′) (24,25).

5. DNA Amplification and Detection

Amplification of target DNA was carried out in thin-walled 0.2 ml PCR tubes in a total volume of 20 μl containing AccuPower™ PCR PreMix, 10 pmol each of CTSF and CTSR primers, 1 μl of DNA extracted from culture or 5 μl of DNA extracted from saliva, and 17 μl of SigmaWater. After amplification in the first step, 1 μl of the product was further amplified using the initial reverse primer (CTSR) and a species-specific forward primer in four separate tubes corresponding to each of the Candida species to be detected. For snPCR, the reaction mixture consisted of AccuPower™ PCR PreMix; 5 pmol of CTSR together with 5 pmol of CADET, CPDET, CGDET, or CTDET; 1 μl of the first PCR product; and 17 μl of SigmaWater. All reagents except primers were obtained from BIONEER Corp. PCR cycling was carried out in a MyGenie 96 Thermal Block (Bioneer, Korea) under the following conditions: denaturation at 94°C for 1 min, annealing at 46°C for 30 s, and extension at 72°C for 1 min. An initial denaturation step at 94°C for 5 min and a final extension step at 72°C for 10 min were also included. Optimum amplification was determined to be obtained with 40 cycles of the first PCR, followed by 45 cycles of the snPCR for DNA extracted from saliva.

Appropriate positive controls were included in each test run, including the DNA template by each of Candida spp, total DNA during PCR assays.

To detect amplified DNA fragments, agarose gel electrophoresis was performed as described previously. The gels were exposed to UV light and photographed. The sizes of amplified DNA fragments were identified by comparison with molecular size marker DNA (100 bp DNA ladder).
6. Antifungal Drug Sensitivity Testing

*In vitro* antifungal activities of amphotericin B (Sigma, USA), fluconazole (Sigma, USA) and ketokonazole (Sigma, USA) were assessed by Kirby–Bauer test on YM agar plates.

Information regarding previous dental and medical histories, including receipt of any antifungal agent in the preceding 6 months was obtained by interview and/or from the patient records. The antifungal susceptibility of isolates from patients who had not received antifungals,

### III. RESULTS

#### 1. Standardization of snPCR

The PCR amplification of rDNAs from the four *Candida* species, viz., *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata*, using universal fungal primers (CTSF and CTSR) resulted in amplification of a single DNA fragment of the expected size (Data not shown).

Reamplification of the product of the first PCR with CTSR and the species–specific primers corresponding to the ITS2 sequences from *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata* resulted in specific amplification of single DNA products of the expected sizes. For example, CTSR and CGDET amplified a 140–bp product in snPCR only when the first PCR was performed with template DNA from *C. glabrata* and not when it was performed with DNAs from *C. albicans*, *C. parapsilosis*, and *C. tropicalis*. Similar results were obtained with other primer combinations for specific detection of *C. albicans*, *C. parapsilosis*, and *C. tropicalis*. The results of these experiments established the species specificity of the snPCR (Fig. 1A).

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**Fig. 1.** A: Lanes 1 to 4, snPCR amplification using primers CTSR and CPDET, CTDET, CADET, and CGDET and DNAs from *C. parapsilosis*, *C. tropicalis*, *C. glabrata*, and *C. glabrata*, respectively. Lane M: 100–bp molecular size marker, B: Identification of *Candida albicans*, *Candida glabrata*, *Candida parapsilosis* and *Candida tropicalis* by snPCR of saliva DNA, C: Bar diagram showing the *Candida* spp, infection rate by the diagnostic tests using snPCR to detect various *Candida* spp, in clinically suspected patients, Y axis represents the Number of isolates,
2. Species Identification of Candida isolates on Saliva

Semi–nested PCR (snPCR) was established using universal and species–specific primers for detection of Candida spp, in saliva. The universal outer primers were amplified the 3’ end of 5.8S ribosomal DNA (rDNA) and the 5’ end of 28S rDNA, including the internally transcribed spacer 2 (ITS2), generating 350–410 bp fragments from the four commonly encountered Candida spp., viz., C. albicans, C. tropicalis, C. glabrata, and C. parapsilosis (Fig. 1B).

snPCR Results appeared to be negative on 292 people (88.2%), however, 26 people (7.9%) were positive of Candida albicans, 6 people (1.8%) of Candida glabrata, 5 people (1.5%) of Candida tropicalis and only 2 people (0.6%) were positive of Candida parapsilosis (Fig. 1C). In Candida albicans isolation group, 11 persons were male and 15 persons female (Fig. 2A). In age range of fifties to eighties, 6, 10, 9
Fig. 3. In vitro susceptibility of Candida spp. to three antifungal agents and that of mixture. (A) amphotericin B (50 μg), K: ketoconazole (50 μg), F: fluconazole (50 μg), AK: amphotericin B (25 μg) + ketoconazole (25 μg), AF: amphotericin B (25 μg) + fluconazole (25 μg), KF: ketoconazole (25 μg) + fluconazole (25 μg), AKF: amphotericin B (16.6 μg) + ketoconazole (16.6 μg) + fluconazole (16.6 μg). (A) Candida albicans, (B) Candida glabrata, (C) Candida parapsilosis, and (D) Candida tropicalis.

3. Antifungal Drug Sensitivity Testing

Ketoconazole and fluconazole exhibited the lowest drug concentration to C. parapsilosis. Except for C. tropicalis, the majority of strains were fully susceptible to ketoconazole. In present study, C. tropicalis were more resistant to amphotericin B, ketoconazole, and fluconazole than other Candida strain (Fig 3A–D), Table 1 showed the antifungal drug sensitivity against Candida species.

Table 1. Antifungal drug sensitivity of C. albicans, C. tropicalis, C. parapsilosis, and C. tropicalis strains. + indicates the sensitivity of each antifungal drug against candida species.

<table>
<thead>
<tr>
<th>Anti-fungal drug</th>
<th>C. albicans</th>
<th>C. glabrata</th>
<th>C. parapsilosis</th>
<th>C. tropicalis</th>
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<tbody>
<tr>
<td>Amphotericin B</td>
<td>+</td>
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<td>Ketoconazole</td>
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<td>Fluconazole</td>
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and 1 persons were infected, respectively (Fig. 2B).

Of the Candida glabrata infection group, only 6 females were isolated (Fig. 2C). In age range, 3 persons for fifties, 2 for sixties, and 1 for seventies were isolated (Fig. 2D). For Candida parapsilosis, only 2 males were detected; 1 for sixties and 1 for seventies (Fig. 2E–F), 4 persons for male and 1 for female was isolated by Candida tropicalis, comprising of 1 person in fifties, 2 in sixties and 2 in seventies, respectively (Fig 2G–H).

IV. DISCUSSION

In the present study, snPCR assays targeting species-specific sequences in the rDNA have been established for the specific detection of four clinically important Candida species by using reference strains.

In a review of Odds of 41 studies in which the frequency of carriage was measured in the oral cavities of healthy individuals, the four highest reported frequencies were 71% of schoolchildren in the United Kingdom, 69% of a nursing staff in Germany, 56% of children in Israel, and 54% of infants in the United Kingdom. In a study of 52 healthy woman in Iowa City, the frequency was 56%. For the study of age range, Wilkeson et al. found that 88% of
eldery individuals in a long-term-care facility either carried or were infected with a *Candida* spp., while 11.8% of eldery healthy individuals, in present study, were carried with a *Candida* spp. This fact implies that carried or infected with a candida spp. is more vulnerable to immunocompromised persons.

These snPCRs were evaluated for *Candida* species identification with clinical isolates and for direct detection as well as specific identification of *Candida* species in saliva samples from patients. The target for snPCR amplification was rDNA. Although PCR assays with several other target sequences have been reported in the literature, the use of rDNA for sensitive detection of *Candida* was considered to be most suitable because it is present in multiple copies (50 to 100 copies) per *Candida* genome, and PCR assays with multiple-copy targets are usually more sensitive than those with single-copy targets. Moreover, between the highly conserved rDNA subunits are the internally transcribed spacers, which contain sequences unique to each *Candida* species, and thus the use of primers corresponding to these regions facilitates species identification. Although several PCR assays based on amplification of the rDNA have been recently reported, species identification usually involved further manipulation of the amplified products, i.e., restriction enzyme digestion and analysis, use of radioactive and enzyme-labeled probes, and DNA sequencing. Many of these procedures required prolonged hybridization times and the use of hazardous radioactive materials. Moreover, species identification by the use of a biotinylated probe and detection by enzyme immunoassay showed lower specificity for *C. glabrata*. In contrast, the snPCR established in this study has an average processing time of 9 to 10 hr, does not require the use of hybridization probes and radioactive substances, and is specific for the detection of all four *Candida* species tested.

Nevertheless, other researchers have reported that non-*albicans* *Candida* species are able to produce extracellular PL. This was not observed in other study, but the absence of PL activity in non-*albicans* *Candida* species may be related to the egg yolk-based plate method used, which is a traditional method for screening PL activity but is not specific as egg yolk contains substrates for both PLs and lipases. Phospholipase activity is an important determinant of pathogenicity in *C. albicans* infections, and it is assumed that blood isolates of *C. albicans* possess greater PL activity than commensal isolates. The majority of the strongly positive isolates were isolated from the patients, but the difference between the two patients' groups was not significant.

In vitro resistance to antifungals was verified in both species, but *C. tropicalis* seemed to be more resistant to tested antifungals than *C. albicans*. Additionally, in present study, where *C. tropicalis* was isolated (12.8% of cases) and was isolated *C. albicans* (66.6% of cases), an appropriate antifungal therapy had to be employed.

Finally, detection and identification of *Candida* spp. could be established by saliva analysis, so that snPCR on saliva is useful method of diagnosis for clinical fields. And antifungal drug sensitive test give information, which can facilitate the selection of appropriate antifungal agents.

V. CONCLUSIONS

Results were appeared to be negative on 292 people (88.2%), however, 26 people (7.9%) were positive of *Candida albicans*, 6 people (1.8%) of *Candida glabrata*, 5 people (1.5%) of *Candida tropicalis*, and only 2 persons (0.6%) of *Candida parapsilosis*.
These results show that detection and identification of Candida spp., could be established by saliva analysis, so that snPCR on saliva is useful method of diagnosis for clinical fields.

In conclusion, the snPCR that was established and evaluated in this study is a specific and sensitive method for the diagnosis of candidemia or hematogetic candidiasis caused by the four most commonly encountered Candida species, i.e., C. albicans, C. parapsilosis, C. tropicalis, and C. glabrata. Besides being rapid, the snPCR has the added advantage of identifying patients infected with more than one Candida species. This information can facilitate the selection of appropriate antifungal agents.

VI. REFERENCES


