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ORIGINAL ARTICLE

# Antifungal Susceptibility Testing with Etest for *Candida* Species Isolated from Patients with Oral Candidiasis

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**Background:** The necessity of performing antifungal susceptibility tests is recently increasing because of frequent cases of oral candidiasis caused by antifungal-resistant *Candida* species. The Etest (BioMerieux, Marcy l'Etoile, France) is a rapid and easy-to-perform *in vitro* antifungal susceptibility test. **Objective:** The purpose of this study was to determine the minimal inhibitory concentrations (MICs) of antifungal agents by using the Etest for *Candida* species isolated from patients with oral candidiasis. **Methods:** Forty-seven clinical isolates of *Candida* species (39 isolates of *Candida albicans*, 5 isolates of *C. glabrata*, and 3 isolates of *C. tropicalis*) were tested along with a reference strain (*C. albicans* ATCC 90028). The MIC end points of the Etest for fluconazole, itraconazole, voriconazole, and amphotericin B susceptibility were read after the 24-hour incubation of each isolate on RPMI 1640 agar. **Results:** All *Candida* isolates were found susceptible to voriconazole and amphotericin B. However, all five isolates of *C. glabrata* were resistant to itraconazole, among which two isolates were also resistant to fluconazole. **Conclusion:** This study revealed that the Etest represented a simple and efficacious method for antifungal susceptibility testing of *Candida* species isolated from oral candidiasis patients. Therefore, voriconazole and amphotericin B should be recommended as effective alternatives for the treatment of oral candidiasis.

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-Keywords-
*Candida*, Etest, Oral candidiasis

## INTRODUCTION

*Candida* species, which are part of the normal flora in the oral cavity, may cause opportunistic infections under various circumstances that compromise host immunity. Oral candidiasis is recently increasing due to old age, denture use, diabetes, systemic steroid and antibiotic use, pernicious anemia, malignancy, radiation therapy on the head and neck, and cell-mediated immunodeficiency[1,2]. With the increasing use of antifungal agents, several reports on antifungal resistance have been published[3-15]. Therefore, antifungal susceptibility testing becomes necessary for the effective treatment of oral candidiasis.

The Etest (BioMerieux, Marcy l'Etoile, France) is a recently commercialized antimicrobial susceptibility test. A long plastic strip consisting of a predefined concentration gradient of an antifungal is placed on an inoculated agar plate. After incubation for the diffusion of the antifungal, the oval-shaped inhibition zone of candidal growth may indicate the minimal inhibitory concentrations (MICs). Thus, the Etest is simple, rapid, and easy to use. Moreover, the results of the Etest are consistent with those of a reference method, the broth microdilution procedure[16-18]. As a basis for the treatment of infection caused by oral *Candida* species, the MICs of fluconazole (FCZ), itraconazole (ICZ), voriconazole (VCZ), and amphotericin B (AMB) for *Candida* isolates from patients with oral candidiasis were determined by performing Etest on RPMI 1640 agar.

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## MATERIALS AND METHODS

### Materials

#### 1) *Candida* isolates

Among the patients who visited the Department of Dermatology of Dongguk University Gyeongju Hospital for a year (September 2013 to August 2014), those suspected of having oral candidiasis were subjected to a potassium hydroxide test of skin lesions in which hyphae were observed along with conidia. Oral candidiasis was finally diagnosed with fungal culture in 45 patients. Forty-seven isolates of *Candida* species (39 of *C. albicans*, 5 of *C. glabrata*, and 3 of *C. tropicalis*) were obtained, and two cases of *C. albicans* co-infection with either *C. glabrata* or *C. tropicalis* were observed (Table 1). These isolates were tested for susceptibility against FCZ, ICZ, VCZ, and AMB. Oral *Candida* species were identified according to colony morphology, microscopic features, germ tube test, and API 20C kit test (BioMerieux). *Candida* albicans ATCC 90028 was used as the reference strain.

#### 2) Culture media

Sabouraud's dextrose agar (SDA) was used for the subculture of fungal isolates. For the antifungal susceptibility testing with Etest, RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 8.4 g L-glutamine, 34.5 g morpholinepropanesulfonic acid, 20 g glucose, and 17 g Bacto agar (Becton Dickinson and Company, Sparks, MD, USA) were dissolved in 1 L distilled water, adjusted to pH 7.0, autoclaved at 121°C for 15 minutes, and poured into 150 mm Petri dishes with 4.0±0.5 mm depth.

### Methods

#### 1) Inoculation, Etest condition, and culture

*Candida* isolates from oral candidiasis patients were inoculated on SDA along with the reference strain, and incubated at 35°C in moist condition for 3 to 4 days. Yeast colonies (>1 mm diameter) on SDA were suspended in 0.85% sterile saline solution to adjust to a turbidity of a 0.5 McFarland standard. A sterile cotton swab was used to spread 500 μl fungal suspension evenly on a 150-mm RPMI plate in three directions. Etest strips were placed on plates that had been dried for 15 minutes at room temperature. The strip end with a lower concentration of the antifungal was positioned first. Etest strips of FCZ (0.016～256 μg/ml), ICZ (0.002～32 μg/ml), VCZ (0.002～32 μg/ml), and AMB (0.002～32 μg/ml) were placed perpendicular to each other on an RPMI plate. The MIC of each drug was determined after incubation for 24 and 48 hours at 35°C in moist condition.

#### 2) Determination of minimal inhibitory concentrations with Etest and of susceptibility

The MICs of the antifungals were read after incubation for 24 and 48 hours, from the scale where the growth inhibition ellipse edge intersected the strip. If the end point of growth inhibition is clear, the scale was read. The MICs of azoles, however, were difficult to read because of trailing, which is a reduced but persistent growth of *Candida* sp. even at high concentration of azoles, and thus determined at the concentration where the colony size was apparently reduced (Fig. 1). As the trailing effects of azoles become aggravated and the MIC of AMB markedly increases after 48 hours of incubation, Etest reading was done to determine the MICs after 24 hours. The final MIC values were based on the consensus between two readers. For quality control, the reference strain was tested simultaneously with the *Candida* isolates. Guidelines for the *in vitro* susceptibility of *Candida* species were adapted from the M27-A3 document from the Clinical and Laboratory Standards Institute (CLSI). For FCZ, the MIC for susceptibility was ≤8 μg/ml, that for susceptible-dose dependence was 16 to 32 μg/ml, and that for resistance was ≥

**Table 1.** Candida species isolated from 45 patients with oral candidiasis

| *Candida* species | No. of patient |
|---|---|
| *C. albicans* | 37 |
| *C. glabrata* | 4 |
| *C. tropicalis* | 2 |
| *C. albicans*+*C. glabrata** | 1 |
| *C. albicans*+*C. tropicalis** | 1 |

*Mixed cultures of *Candida* species.

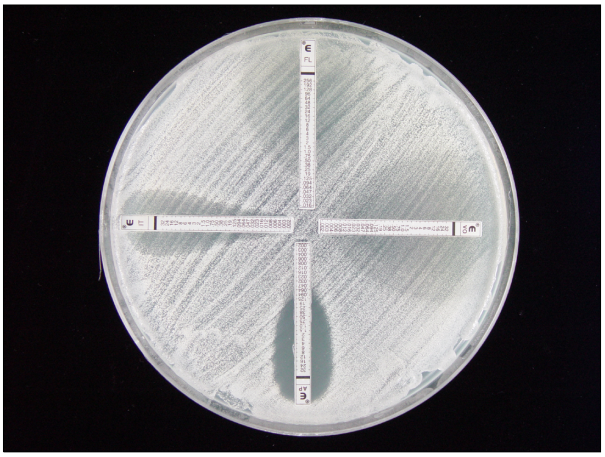

**Fig. 1.** The Etest result of a *Candida albicans* isolate tested against fluconazole, itraconazole, voriconazole, and amphotericin B.

**Table 2.** CLSI (M27-A3) and literature guidelines for *in vitro* susceptibility testing of *Candida* species

| Antifungal agent | Interpretative criteria (μg/ml) | | |
|---|---|---|---|
| | Susceptible | Susceptible-dose dependent | Resistant |
| Fluconazole | ≤8 | 16～32 | ≥64 |
| Itraconazole | ≤0.125 | 0.25～0.5 | ≥1 |
| Voriconazole | ≤1 | 2 | ≥4 |
| Amphotericin B* | ≤1 | - | >1 |

CLSI: Clinical and Laboratory Standards Institute. *Literature guidelines for amphotericin B were adapted from references[19,20].

**Table 3.** Quality control ranges of *Candida albicans* ATCC 90028, a reference strain for antifungal susceptibility test by CLSI guideline

| Antifungal agent | MIC range (μg/ml) | |
|---|---|---|
| | CLSI guideline | Our results* |
| Fluconazole | 0.125～0.5 | 0.25 |
| Itraconazole | 0.064～0.25 | 0.064～0.094 |
| Voriconazole | 0.004～0.016 | 0.008～0.012 |
| Amphotericin B | 0.125～0.5 | 0.125～0.19 |

CLSI: Clinical and Laboratory Standards Institute, MIC: minimal inhibitory concentration. *Our results were determined after incubation for 24 hours.

64 μg/ml. For ICZ, the MIC for susceptibility was ≤0.125 μg/ml, that for susceptible-dose dependence was 0.25 to 0.5 μg/ml, and that for resistance was ≥1 μg/ml. For VCZ, the MIC for susceptibility was ≤1 μg/ml, that for susceptible-dose dependence was 2 μg/ml, and that for resistance was ≥4 μg/ml. As interpretative criteria for AMB have not been defined in the CLSI guidelines, the MIC for susceptibility to AMB was ≤1 μg/ml, whereas the MIC for resistance to AMB was >1 μg/ml, according to Sanitá et al.[19] and Negri et al.[20] (Table 2).

## RESULTS

### Quality control with a reference strain

The MICs for the reference strain, *C. albicans* ATCC 90028, determined after 24-hour incubation were 0.25 μg/ml against FCZ, 0.064 to 0.094 μg/ml against ICZ, 0.008 to 0.012 μg/ml against VCZ, and 0.125 to 0.19 μg/ml against AMB. From the repeated tests, identical results were obtained within a ±1 grade range, which are acceptable according to the quality control guidelines (Table 3).

### Minimal inhibitory concentrations of antifungals for *Candida* isolates

The MICs for 39 isolates of *C. albicans* were 0.064 to 0.75 μg/ml against FCZ, 0.002 to 0.094 μg/ml against ICZ, 0.002 to 0.016 μg/ml against VCZ, and 0.012 to 0.19 μg/ml against AMB. The MICs for the five isolates of *C. glabrata* were 16 to 64 μg/ml against FCZ, >32 μg/ml against ICZ, 0.38 to 1.0 μg/ml against VCZ, and 0.064 to 0.19 μg/ml against AMB. The MICs for the three isolates of *C. tropicalis* were 0.25 to 0.75 μg/ml against FCZ, 0.012 to 0.25 μg/ml against ICZ, 0.008 to 0.094 μg/ml against VCZ, and 0.094 to 0.25 μg/ml against AMB (Table 4).

**Table 4.** MICs (μg/ml) of antifungal agents for 47 clinical isolates determined by Etest after 24 hours

| *Candida* species (No. of strain) | Antifungal agent | MICs (μg/ml) by Etest |
|---|---|---|
| *C. albicans* (39) | Fluconazole | 0.064～0.75 |
| | Itraconazole | 0.002～0.094 |
| | Voriconazole | 0.002～0.016 |
| | Amphotericin B | 0.012～0.19 |
| *C. glabrata* (5) | Fluconazole | 16～64*,† |
| | Itraconazole | >32† |
| | Voriconazole | 0.38～1.0 |
| | Amphotericin B | 0.064～0.19 |
| *C. tropicalis* (3) | Fluconazole | 0.25～0.75 |
| | Itraconazole | 0.012～0.25* |
| | Voriconazole | 0.008～0.094 |
| | Amphotericin B | 0.094～0.25 |

MIC: minimal inhibitory concentration. *Susceptible-dose dependent, †resistant.

### Antifungal susceptibility of the isolates from oral candidiasis patients

According to CLSI M27-A3 and the guidelines by Sanitá et al.[19] and Negri et al.[20] (Table 2), the MICs of AMB and VCZ were <1.0 μg/ml (0.012～0.25 and 0.002～1.0 μg/ml, respectively), indicating that all isolates of *Candida* (39 of *C. albicans*, 5 of *C. glabrata*, and 3 of *C. tropicalis*) were susceptible. The MICs of FCZ were 0.064 to 64 μg/ml, indicating that the 39 isolates of *C. albicans* and the 3 isolates of *C. tropicalis* were susceptible, 3 of the 5 isolates of *C. glabrata* were susceptible-dose dependent, and the re-

**Table 5.** Susceptibility patterns of *Candida* species isolated from oral candidiasis patients

| *Candida* species (No. of strain) | Antifungal agent | S | SDD | R |
|---|---|---|---|---|
| *C. albicans* (39) | Fluconazole | 39 (100.0) | - | - |
| | Itraconazole | 39 (100.0) | - | - |
| | Voriconazole | 39 (100.0) | - | - |
| | Amphotericin B | 39 (100.0) | - | - |
| *C. glabrata* (5) | Fluconazole | - | 3 (60.0) | 2 (40.0) |
| | Itraconazole | - | - | 5 (100.0) |
| | Voriconazole | 5 (100.0) | - | - |
| | Amphotericin B | 5 (100.0) | - | - |
| *C. tropicalis* (3) | Fluconazole | 3 (100.0) | - | - |
| | Itraconazole | 2 (66.7) | 1 (33.3) | - |
| | Voriconazole | 3 (100.0) | - | - |
| | Amphotericin B | 3 (100.0) | - | - |

Values are presented as number (%). S: susceptible, SDD: susceptible-dose dependent, R: resistant.

maining 2 isolates of *C. glabrata* were resistant. The MICs of ICZ were 0.002 to 2 μg/ml, revealing that the 39 isolates of *C. albicans* and 2 of the 3 isolates of *C. tropicalis* were susceptible, 1 isolate of *C. tropicalis* was susceptible-dose dependent, and the 5 isolates of *C. glabrata* were all resistant (Table 5).

## DISCUSSION

Oral candidiasis is an infection caused by oral *Candida* species; *C. albicans* has been commonly detected, whereas *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. guilliermondii*, and *C. krusei* have been isolated less frequently[21,22]. Recently, however, as in our study, *C. albicans*, *C. glabrata*, and *C. tropicalis* are reported to cause oral infection, as a single species or as mixed causative agents[3,19,23,24]. The treatment of oral candidiasis usually involves local nystatin emulsion or clotrimazole troche in cases without any complication; however, oral administration of azoles such as FCZ or ICZ is necessary for recurrent infections or chronic cases[1,2]. As several studies have been recently reported on antifungal resistance against azoles, susceptibility tests on antifungals need to be performed[3-15].

Among several antifungal susceptibility tests, including the broth microdilution, disk diffusion, and flow cytometry methods, the reference method adapted by the CLSI is the broth microdilution method, which is rather difficult to perform regularly in a laboratory because it is time consuming and tedious to perform[17,18]. The Etest, which is a simple disk diffusion method, has been used recently because its results agree well with those from the broth microdilution method[6,12,14,25-28].

Although several reports have been published worldwide on antifungal susceptibility testing through the Etest of oral *Candida* species[3,4,6,8,11-14,19], there has been only a single report[25] on using the Etest on superficial *Candida* isolates in the Korean dermatological literature.

Therefore, we tested *Candida* species isolated from oral candidiasis patients by using a new Etest method, to determine their susceptibility to azoles including FCZ, ICZ, VCZ, and a polyene, AMB. For quality control, the *C. albicans* ATCC 90028 reference strain was used according to Carvalhinho et al.[4] and Sanitá et al.[19] RPMI 1640 medium was used for the Etest[3,6,16,19]. Incubation was done at 35°C for 24 and 48 hours, as described in Marcos-Arias et al.[3] However, a trailing effect induced by azoles occurred and the MICs of AMB increased obviously after 48 hours. Therefore, the MICs from the Etest were read after 24-hour incubation.

The oral *Candida* species that were isolated, identified, and tested in this study exhibited various results to the tested antifungals. The range of AMB MICs was 0.012 to 0.25 μg/ml and $<1.0$ μg/ml, an interpretable criterion, indicating that all 39 isolates of *C. albicans*, 5 isolates of *C. glabrata*, and 3 isolates of *C. tropicalis* were susceptible to AMB as reported by others[4,8,12,19]. Similarly, the range of VCZ MICs was 0.002 to 1.0 μg/ml and $\leq 1.0$ μg/ml, indicating that all *Candida* isolates were susceptible to VCZ as reported by Mareş et al.[8] and Belazi et al.[11]. However, the range of FCZ MICs was 0.064 to 64 μg/ml, indicating that all 39 *C. albicans* isolates and the 3 *C. tropicalis* isolates were susceptible, but 3 of the 5 isolates of *C. glabrata* were susceptible-dose dependent and the remaining 2 isolates were resistant to FCZ, as observed similarly by others[8,11-14]. Finally, the range of ICZ MICs was from 0.002 to $>32$ μg/ml, indicating that all isolates of *C. albicans* were susceptible and an isolate of *C. tropicalis* was susceptible-dose dependent, but all isolates of *C. glabrata* were resistant to ICZ as described similarly by other reports[3,8,13,14,19].

In summary, all *Candida* isolates were susceptible to AMB and VCZ, which were proven to be more effective than either FCZ or ICZ. When a patient with oral candidiasis does not respond well to other antifungals, VCZ may be recommended as AMB may cause adverse reactions such as nephrotoxicity and hypokalemia. VCZ, a new second-generation triazole derived from FCZ, has a wide spectrum and antifungal activities against various mycoses caused by not only *Candida* species but also *Aspergillus*, *Scedosporium*, and *Fusarium* species[29,30]. In this study, the MICs of antifungals for oral *Candida* species were successfully determined by using a simple method, the Etest, with RPMI 1640 medium in the clinical laboratory. Further study with more clinical isolates needs to be done to investigate the trend of antifungal resistance among oral *Candida* species in Korea.

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