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The frequency, antifungal susceptibility and enzymatic profiles of *Candida* species in cases of onychomycosis infection

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ABSTRACT

Although the frequency of candidal onychomycosis is increasing daily, there is little information in literature about the epidemiology, pathogenesis, and antifungal susceptibility of this dermatological disease. This study aimed to provide information about the epidemiology, pathogenesis, and azole susceptibility of *Candida* species isolated from patients living in a region with continental climate. After identification of the isolated strains using conventional methods, proteinase and phospholipase activities were determined by a plate method and biofilm-forming ability was determined using the microplate method. Susceptibility of the same species to fluconazole (FLU), voriconazole (VRC), miconazole (MNZ), itraconazole (ITZ), and ketoconazole (KTZ) were determined by microdilution method.

The 50 *Candida* isolates included 23 *C. parapsilosis* (46%), 13 *C. albicans* (26%), 4 *C. guilliermondii*(8%), 4 *C.tropicalis* (8%), 2 *C.krusei*(2%), 1 *C.lusitaniae* (2%), 1 *C. sake* (2%), and 1 *C. kefyr* (2%) isolates. The geometric mean (GM) of the minimum inhibitory concentration (MIC) for FLU, KTZ, VRC, MNZ, and ITZ was 0.4 μg/mL, 0.08 μg/mL, 0.08 μg/mL, 0.2 μg/mL, and 0.6 μg/mL, respectively. Proteinase, phospholipase, and biofilm-forming ability were detected in 18%(9/50), 20%(10/50), and 6%(3/50) of the *Candida* isolates, respectively.

We found that the most frequently isolated species is *C.parapsilosis*. On the basis of the GM values, the most effective azoles are ketoconazole and voriconazole. The isolated *Candida* species exhibited low phospholipase, proteinase, and biofilm formation activities.

1. Introduction

Onychomycosis constitutes 30% of superficial fungal infections and 50% of nail infections [1]. The most frequently isolated species are dermatophytes, yeasts, and non-dermatophyte moulds. Previously, only dermatophytes were considered as the most common causative agents; however, *Candida* species have recently been reported among the important pathogens causing onychomycosis infections [2,3].

To understand the pathogenesis of onychomycosis caused by *Candida* species, the host factors and characteristics of *Candida* species need to be examined. Certain factors, such as the age and occupation of patients and the localization of infection are important host factors. In addition to host factors, the virulence factors that microorganism uses to defend itself against the host are also important. These include biofilm formation by the microorganism and production of hydrolytic enzymes (proteinase, phospholipase, and haemolysin). Fungal cells in the extracellular matrix form a thick fungal biomass, leading to difficulties in eradication of the fungal nail infection [4].Phospholipases, a class of hydrolytic enzymes, hydrolyse one or more ester linkages in glycerophospholipids, whereas proteinases break down proteins such as albumin, haemoglobin, and keratin [5]. Due to these enzymes, *Candida* species adhere to the host cell and form an infection by forming lysis.

High-cost and long-term treatments, ineffective management of infection, uncooperative patients, and hepatotoxic and gastrointestinal side effects of drugs result in adverse outcomes in treatment. Therefore, treatment protocols are being developed by international committees [6].Topical and systemic antifungals are used for onychomycosis infection treatment.Fluconazole,itraconazole, imidazoles (clotrimazole, ketoconazole, and miconazole), and allylamines (terbinafine) are agents that are effective against onychomycosis and have a safe profile for human application. When dermatophytes are isolated as agents, terbinafine shows good results whereas azoles, especially itraconazoles,

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are effective in yeast isolates [7,8].

In this study, proteinase, phospholipase, and biofilm formation characteristics of isolated *Candida* species were investigated to understand the pathogenesis of onychomycosis caused by *Candida* spp. In addition, the sensitivity of these species to various azoles was determined by a reference microdilution method to guide clinicians regarding the treatment protocols that can be used.

2. Materials and methods

2.1. Material intake and identification of isolated Candida species

Nail samples were obtained from 1810 patients suspected of onychomycosis in the mycology laboratory of the Kayseri Educational and Research Hospital. First, the nail area was cleaned with alcohol to remove contaminants. Sampling was performed particularly from the proximal and lateral corners of the nail in patients with possible onychomycosis. Nail specimens were then treated with 30% KOH and examined directly under a microscope.The fingernail and toenail specimens were incubated with Sabouraud dextrose agar (SDA, Acumedia, Baltimore, Maryland, USA) supplemented with 50 μg/ml gentamycin (Sigma,Steinheim, Germany) and 100 μg/mL cycloheximide (Sigma, Steinheim,Germany). After inoculation, one of the tubes was placed at 25 °C and the other at 37 °C, and the tubes were assessed weekly. Germ tube test, morphological appearance on corn meal agar with Tween 80, and the API ID 32C (Bio Merieux, France) kit were used to identify the growing *Candida* species.

2.2. Antifungal susceptibility test

Fluconazole [Pfizer Pharmaceuticals Group, New York, NY, USA,] voriconazole, itraconazole, miconazole, and ketoconazole [Sigma-Aldrich, St. Louis, Louis, Missouri, U.S.A.] stock solutions were prepared and administered according to the Clinical and Laboratory Standards Institute (CLSI) M27-A3 reference method [9]. The minimum inhibitory concentration (MIC) values of the antifungals were evaluated according to the CLSIM27-S4 reference method [10].RPMI 1640 (L glutamine, without bicarbonate) (Sigma Chemical Co., St Louis, Mo., USA) was used as the medium for the microdilution method. The antifungal concentration at which growth was significantly reduced (about 50%) for the azoles was considered as the antifungal MIC value. *C.krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 isolates were used as control isolates.

2.3. Proteinase activity

For detection of proteinase activity, bovine serum albumin-agar (SSAA) was used, as described previously [11].For this test, yeast colonies were inoculated into yeast peptone dextrose (YPD) medium following 24–48 h of incubation at 37 °C in SDA, and then incubated for 4 h at 30 °C.The cultures were adjusted to 0.5 McFarland turbidity standard after incubation. The suspension (10 μL) was then mixed with SSAA medium and incubated for 6 days at 30 °C. Strains without the melting zone were evaluated as (-) in terms of acid proteinase activity, strains that spread in a maximum area of 1–2 mm in transparent zone were considered as moderate (+), whereas strains that spread in a 3–5 mm area of the transparent zone were considered to be strong (2+) [12].*C.albicans* ATCC 10231 strains was used as positive control (Fig. 1).

Fig. 1. Proteinase activity.

2.4. Phospholipase activity

An egg yolk agar (YSA) plaque method was used to detect the phospholipase activity of the isolated strains [13].In this test, 10 μLof egg-wrapped agar was inoculated witha yeast suspension of 0.5 McFarland turbidity and incubated at 37 °C for 4days. The phospholipase activity (Pz value) of the strains was evaluated by measuring the apparent ring-shaped precipitation zone around the colony and the Pz value was obtained by calculating the ratio of the colony diameter (a) to the precipitation diameter. The results were evaluated as follows:Pz = 1, negative (-); Pz values between 0.9 and 1.00, (+), those between 0.8 and 0.89, (2+), those between 0.7 and 0.79, (3+), and those < 0.69, (4+) [14].*C. albicans* SC 5314 strain was used as a positive control (Fig. 2).

2.5. Biofilm formation

A modified microplate method (using a plate prepared with brain heart infusion broth (BHIB) containing 0.25% glucose; MMP-BHIB) was used in the experiment [15].The strains were incubated for 24 h at 35 °C in BHIB containing 0.25% glucose and diluted 120 with prewarmed 0.25% glucose containing BHIB. From these suspensions, 200 μL was transferred to sterile 96-well polystyrene microplates (3 wells were used for each strain). Following 48 h of incubation at 37 °C, the wells were washed with PBS, inverted, and stained with 1% crystal violet for 15 min. After washing, 200 μLof ethanol-acetone (80:20, v/v) was added to each of the tablets.The plates were read at 590 nm to determine the optical densities (OD), and the strains were considered negative for biofilm formation in case the OD < 1, weakly positive (+) if 1 < OD < 2; moderate positive (2+) if 2 < OD < 3, and strong positive (3+) if OD > 3. All experiments were repeated twice. *Enterococcus faecalis* strain ATCC 29212 was used as the control.

Fig. 2. Phospholipase activity.

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