**PROTEASE ACTIVITIES OF CANDIDA SPP. ISOLATED FROM IMMUNOCOMPETENT PATIENTS WITH OTOMYCOSIS**

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**Summary:** Otomycosis is a fungal infection of the ear dominantly caused by Candida and Aspergillus spp. The possible virulence factors of Candida spp. are enzymes, such as proteases, phospholipases, phosphatases and esterase. Protease production in Candida strains isolated from patients with otomycosis is, according to our knowledge, not investigated. The present study was aimed at determining in vitro protease activity in 28 strains of Candida spp. (C. parapsilosis, C. famata, C. guilliermondii, C. albicans and C. kefyr) isolated from patients with otomycosis. The majority of isolated strains 25/28 (89.28%) were protease positive. The protease $P_z$ ranged from 0.691 to 0.851. The further investigation is necessary to clarify contribution of protease production to Candida virulence associated with otomycosis.

**Key words:** otomycosis, Candida spp., protease production, virulence

**Introduction**

Otomycosis is a common fungal infection of the ear dominantly caused by Candida spp. and Aspergillus spp. (1–6). Candida spp. are important pathogens in immunocompetent and immunocompromised patients causing infections involving skin, mucosa and deep organs. Due to the increasing incidence in Candida infections there is a great interest on Candida virulence factors which are important in establishing strategies for control and prevention of candidosis (7). The majority of Candida spp. have the ability to produce a variety of enzymes, such as proteases, phospholipases, phosphatases and esterase. Extracellular protease production is considered to enhance the organism’s ability to colonize the skin and penetrate host tissues, and to evade the host’s immune system by degrading a number of proteins important in host defense such as immunoglobulins, complement and cytokines (8), and have the ability to cause damage to host cell membranes in vivo (9). Aspartyl acid protease is the most thoroughly studied proteinase enzyme in Candida spp. (10). There are a number of publications investigating protease production in Candida spp., but, according to our knowledge, the studies on protease activity in strains causing ear infection are lacking.

The aim of this study was to determine in vitro protease activity of 28 strains of Candida spp. isolated from bony portion of the external ear in patients presented with otomycosis.

**Materials and methods**

From April 2001 to March 2003, a total of 67 adults and 23 children presenting with suspected otomycosis were examined at the outpatient otology department. The specimens were taken by cotton swab from bony portion of external ear. All clinical specimens were inoculated onto Sabouraud Dextrose Agar (SDA) slants (Torlak, Belgrade, Serbia and Montenegro) and incubated for seven days at 26 °C and 37 °C and examined macroscopically every day.
Suspected cultures were examined microscopically in order to confirm finding of Candida spp. All Candida isolates were identified by germ tube serum formation test, chlamydospore formation on cornmeal agar and by the battery of fermentation and assimilation tests (API 20C AUX, bioMérieux, France). The isolated strains were preserved at –70°C. Before the protease testing, strains were inoculated on new SDA medium, and incubated for 48h at 37°C.

The protease production was determined according to Aokiel (11) using the test medium consisted of agar plates containing bovine serum albumin (BSA). 60 mL of a solution containing 0.04 g MgSO4 × 7H2O, 0.5 g K2HPO4, 1 g NaCl, 0.2 g dried yeast extract, 4 g glucose and 0.5 g BSA (Fraction V, Sigma Chem Co., St. Louis, Mo., USA). The pH was adjusted to 3.5 with 1 mol/L HCl. The solution was sterilized by filtration, mixed with 140 mL of melted agar and poured into Petri dishes. The yeast inoculum of 10⁶ blastospores/ml was prepared in normal sterile saline and 10 μL of suspension of each strain was inoculated on the plates in triplicate. After the incubation at 37°C for 7 days the diameter of the clear zones around the colonies was considered as a measure of protease production. The protease activity (Pz) was measured and calculated according to the method described by Price (12) in terms of the ratio of the diameter of the colony plus the clear zones. Low Pz signified a high production of the enzyme, i.e. high virulence, while high Pz indicated low production of the enzyme, i.e. low virulence. The average Pz value was obtained with three separate samples of each strain. The C. albicans ATCC 24433, C. parapsilosis DSMZ 5784 and C. kruzei DSMZ 6128 were used as a positive and negative control strains (ATCC-American Type Culture Collection, DSMZ-Deutsche Sammlung von Mikroorganismen and Zellkulturen).

Statistical differences in protease production between Candida strains isolated from children and adults were determined according to Student's t test. A p-value of < 0.05 was considered significant. Statistics could not be performed for the difference between species due to the small number of isolates tested in each species.

Results

In patients suffering from otomycosis 28 Candida strains were isolated from bony portion of external ear, 20 from adults and 8 from children. Five different species were determined: C. parapsilosis 10/28, C. famata 7/28, C. guilliermondii 5/28, C. albicans 5/28 and C. kefyr 1/28. The protease activity of Candida spp. strains was observed three days after inoculation on SDA medium by area of brightness around the colony. The majority of tested strains showed protease activity: seven out of eight isolates in children (87.5%), and eighteen out of twenty Candida isolates in adult patients (90%). The all tested C. albicans, C. famata, C. kefir and C. parapsilosis strains showed in vitro protease production, while only two out of five C. guilliermondii isolates were protease positive (Table I). The Pz protease activity values of clinical isolates ranged between 0.691 and 0.851 (Pz average 0.65). There was no difference between protease production in Candida strains isolated in children and in adults (p < 0.05).

### Discussion

Candida possess constitutive hydrolytic enzymes to aid invasion of host tissues and in this investigation the majority of tested Candida spp. have protease activity. These findings suggest that protease production may play an important role in the pathogenesis of otomycosis caused by Candida spp. It is possible that protease enzymes enhance the ability of Candida spp. to colonize the skin and penetrate host cells which could be important in establishing the infection in the ear. Staib was first to report that C. albicans could use serum proteins as a source of nitrogen and the proteolytic activity related to strain pathogenicity (13). Macdonald and Odds (14) demonstrated that the proteases are produced in vivo. It is reported that secreted proteases are important virulence factor in Candida spp. in skin, mucosal and deep organs infections (15). The secreted acid protease may help the yeast invasion through the keratin protective layer and facilitate initiation of the infection in the ear canal (16). The average pH of skin varies depending of specific site, but ranges within the acid pH optimum of purified protease enzyme. Local lesions create conditions favoring fungal growth and development of mycosis. Most infections are present in cases who has previously underwent medical treatment of the external canal and in patients who underwent surgical procedures, where local lesions such as congestion, increased vascular permeability, raised temperature and acid pH create favorable conditions for the growth of fungi.

<table>
<thead>
<tr>
<th>Candida spp.</th>
<th>Tested strains</th>
<th>Protease positive strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. parapsilosis</td>
<td>10/28</td>
<td>10/10 0.698 +/- 0.260</td>
</tr>
<tr>
<td>C. famata</td>
<td>7/28</td>
<td>7/7 0.851 +/- 0.215</td>
</tr>
<tr>
<td>C. guilliermondii</td>
<td>5/28</td>
<td>5/5 0.691 +/- 0.118</td>
</tr>
<tr>
<td>C. albicans</td>
<td>5/28</td>
<td>5/5 0.713 +/- 0.132</td>
</tr>
<tr>
<td>C. kefyr</td>
<td>1/28</td>
<td>1/1 0.785 +/- 0.112</td>
</tr>
<tr>
<td>*C. albicans ATCC 24433</td>
<td>1/28</td>
<td>1/1 0.612 +/- 0.098</td>
</tr>
<tr>
<td>*C. parapsilosis DSMZ 5784</td>
<td>1/28</td>
<td>1/0 –</td>
</tr>
<tr>
<td>*C. kruzei DSMZ 6128</td>
<td>1/28</td>
<td>1/1 0.720 +/- 0.121</td>
</tr>
</tbody>
</table>

* control strains
It is possible that ability of yeast to adhere to the skin cells is proportional to acid protease production. In experiments on mice adherence to skin was less when *Candida* spp. negative for acid protease secretion were used. The protease probably modifies cell membranes of the host to accept attachment of the fungus or modifies the surface of the yeast cell in a way to promote attachment (17). Protease activity may also be important as a virulence factor in selected body sites and may not necessarily function enzymatically (9).

There are publications investigating protease production in *Candida* spp. isolates from various body sites and the enzyme activity seems to be related to *Candida* virulence in the pathogenesis in invasive candidosis. The strains with higher proteolytic activity are considered more virulent (18). De Bernardis reported high protease activity *in vitro* in all *C. parapsilosis* strains isolated in patients with vaginitis (10). Yamaomoto found that the majority of *C. tropicalis* and *C. parapsilosis* isolates had proteolytic activity while none of *C. glabrata* strains tested secreted the enzyme (19). Kantarcioğlu and Yucel (20) reported in vitro protease production in the most of *C. albicans*, *C. kefyr*, *C. lipolytica*, *C. parapsilosis* and *C. tropicalis* clinical isolates, while none or few *C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. lusitaniae* and *C. rugosa* were protease positive. In the present study the extracellular protease production in *Candida* spp. isolated from patients with otomybos was detected in all of *C. parapsilosis*, *C. famata*, *C. albicans* and *C. kefyr* isolates, while only few *C. guilliermondii* strains produced the enzyme.

Kantarcioğlu and Yucel observed that 78.9% of *Candida* isolates from various body sites were protease positive (20). In our study we detected protease activity in 89.28% of tested *Candida* strains isolated in patients with ear infection.

We conclude that further investigation should continue on protease and other enzymes activity in *Candida* species isolated from different anatomic sites of the ear and these experiments are in progress in our laboratory. The further investigation is necessary to clarify their contribution to *Candida* virulence associated with otomybos and also to determine a possible target for developing novel therapeutic interventions.

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**PROTEAZNA AKTIVNOST Candida SPP. IZOLOVANIH KOD IMUNOKOMPETENTNIH OSOBA SA OTOMIKOZOM**

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**Kratak sadržaj:** Otomikoze su gljivične infekcije uva uzrokovane pre svega *Candida* i *Aspergillus*. Mogući faktori virulencije gljiva roda *Candida* su enzimi kao što su proteaze, fosfolipaze, fosfataze, esterase i dr. Koliko je nama poznato do sada nisu vršena ispitivanja produkcija proteaze kod gljiva roda *Candida*, uzročnika otomikoze. Zbog toga je cilj istraživanja bio da *in vitro* ispita produkcija proteaza kod 28 sojeva gljiva roda *Candida* (*C. parapsilosis*, *C. famata*, *C. guilliermondii*, *C. albicans* i *C. kefyr*) izolovanih kod pacijenata sa otomikozom. Većina ispitanih sojeva, 25/28 (89,28%) pokazala je pozitivnu proteaznu aktivnost sa vrednostima Pz koje su se kretele u opsegu od 0,691 do 0,851. Dalja ispitivanja su neophodna u cilju ispitivanja korelacije uticaja produkcije proteaza gljiva roda *Candida* i njihove virulencije u nastanku otomikoze.

**Ključne reči:** otomikoze, *Candida* spp., produkcija proteaza, virulencija
References


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