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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 Pz ranged from 0.691 to 0.851. The futher 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.

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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, bioMerieux, 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 $MgSO4 \times 7H_2O$, 0.5 g K_2HPO_4, 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 10^6 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 Colection, DSMZ-Deutsche Sammlung von Mikroorganismen and Zellkulturen).

Statistical differences in protease production between *Candida* strains isolated from children and adults were determined according to Studentžs 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. guillermondii* 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 BSA 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. guillermondii* 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 ($p < 0.05$).

Table I Protease activity in 28 isolates of different *Candida* species isolated in patient with otomycosis.

Candida spp.	Tested strains	Protease positive strains	
	n	n	Pz +/– SD
C. parapsilosis	10	10	0.698 +/– 0.260
C. famata	7	7	0.851 +/– 0.215
C. guilliermondii	5	2	0.691 +/– 0.118
C. albicans	5	5	0.713 +/– 0.132
C. kefyr	1	1	0.785 +/– 0.112
**C. albicans* ATCC 24433	1	1	0.612 +/– 0.098
**C. parapsilosis* DSMZ 5784	1	0	–
**C. kruzei* DSMZ 6128	1	1	0.720 +/– 0.121

* control strains

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 patogenicity (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.

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). Yamoamoto found that the majority of *C. tropicalis* and *C. parapsilosis* isolates had proteolitic activity while none C. glabrata strains tested secreted the enzyme (19). Kanatrcioglu 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. guillermondii, 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 otomycosis was detected in all of C. parapsilosis, *C. famata, C. albicans* and *C. kefyr* isolates, while only few *C. guillermondii* strains produced the enzyme.

Kantarcioglu 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 otomycoses 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 gljivama roda *Candida* i *Aspergillus*. Mogući faktori virulencije gljiva roda *Candida* su enzimi kao što su proteaze, fosfolipaze, fosfataze, esteraze i dr. Koliko je nama poznato do sada nisu vršena ispitivanja produkcija proteaze kod gljiva roda *Candida*, uzročnika otomikoza. 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 kretale 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 otomikoza.

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

References

1. Kurnatowski P, Filipiac A. Otomycosis: prevalence, clinical symptoms, therapeutic procedure. Mycoses 2001; 44: 472–9.
2. Ologe FE, Nwabuisi C. Treatment outcome of otomycosis in Ilorin, Nigeria. West Afr J Med 2002; 21: 34–6.
3. Mgbor N, Gugnani HC. Otomycosis in Nigeria: treatment with mercurochrome. Mycoses 2001; 44: 395–7.
4. Nwabuisi C, Ologe FE. The fungal profile of otomycosis patients in Ilorin, Nigeria. Niger J Med 2001; 10: 124–6.
5. Kaur R, Mittal N, Kakkar M, Aggarwal AK, Mathur MD. Otomycosis: a clinicomycologic study. Ear Nose Throat J 2000; 79: 606–9.
6. Amigot SL, Gomez CR, Luque AG, Ebner G. Microbiological study of external otitis in Rosario City, Argentina. Mycoses 2003; 46: 312–5.
7. Perfect JR. Fungal virulence genes as targets for antifungal chemotherapy. Antimicrob Agents Chemoter 1996; 40: 1577–83.
8. De Bernardis F, Mondello F, San Milan R, Ponton J, Cassone A. Biotyping and virulence properties of skin isolates of *Candida parapsilosis*. J Clin Microbiol 1999; 37: 3481–6.
9. Ghannum MA, Abu Elteen KH. Pathogenicity determinants of *Candida*. Mycoses 1990; 33: 265–82.
10. Cutler JE. Putative virulence factors of *Candida albicans*. Annu Rev Microbiol 1991; 45: 187–218.
11. Aoki S, Ito-Kuwa S, Nakamura Y, Masuhara T. Comparative pathogenicity of wild-type strains and respiratory mutants of *Candida albicans* in mice. Zol Bakt 1990; 273: 332–43.
12. Price MF, Wilkinson ID, Gentry LO. Plate method for detection of phospholipase activity in *Candida albicans*. Sabouraudia 1982; 20: 7–14.
13. Staib F. Proteolysis and pathogenicity of *Candida albicans* strains. Mycopathol Mycol Appl 1969; 37: 346–8.
14. Macdonald F, Odds FC. Purified *Candida albicans* proteinase in the serological diagnosis of systemic candidosis. JAMA 1980; 243: 2409–11.
15. Shaller M, Schackert C, Korting HC, Januschke E, Hube B. Invasion of *Candida albicans* correlates with expression of secreted aspartic proteinases during experimental infectio of human epidermis. J Invest Dermatol 2000; 114: 712–7.
16. Negi M, Tsubai R, Matsui T, Ogawa H. Isolation and characterization of proteinase from *Candida albicans*: Substrate specifity. J Invest Dermatol 1984; 83: 32–36
17. Sohnle PG, Frank MM, Kirkpatrick CH. Mechanisms involved in elimination of organisms from experimental cutaneous *Candida albicans* infections in guinea pigs. J Immunol 1976; 117: 523–30.
18. Hube B. *Candida albicans* secreted aspartyl proteinases. Curr Top Med Mycol 1996; 1: 55–69.
19. Yamamoto T, Nohara K, Uchida K, Yamaguchi K. Purification and characterization of secretory proteinase of *Candida albicans*. Microbiol Immunol 1992; 36: 637–41.
20. Serda Kanatrcioglu A, Yucel A. Phospholipase and protease activities in clinical *Candida* isolates with reference to the source of strains. Mycoses 2002; 45: 169–5.

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