Protease activities of Candida spp. isolated from otitis externa: preliminary result

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INTRODUCTION.

Otomycosis is a superficial, subacute or chronic infection of the outer ear canal characterized by inflammation, pruritus, pain and scaling. Otitis externa (OE) and otitis media (OM) in children are very common pediatric disorders. The bacteria, considered the most common cause of OM, are Streptococcus pneumoniae, Haemophilus influenzae and Moraxella catarrhalis¹⁻². Fungal infections of the ear (described as mycosis of the auditory canal in adults) are caused by Candida spp. and Aspergillus spp. have been studied³⁻⁸, but fungal ear infections in children have not been studied⁹. Candida spp. is an important pathogen in immunocompromised patients causing infections affecting skin, mucosa and deep organs. Due to the increasing incidence of Candida infections, there is great interest in Candida virulence factors, which are in turn important in the establishment of the strategies for control and prevention of candidiasis¹⁰. The majority of Candida spp. have the ability to produce a variety of enzymes, but extracellular protease production is considered to be important for skin colonization and host tissues penetration¹¹. Aspartyl acid protease is the most thoroughly studied proteinase enzyme in Candida spp.¹², and there are a number of publications investigating protease production in Candida spp. isolated from different sources³. We have identified research on protease activity in Candida spp. isolated from the external ear canal in adults with OE¹³, however, according to our knowledge, studies on protease activity of Candida spp. that cause ear infections in children are lacking.

Therefore, in the study presented below, we have investigated the role of Candida spp. in clinically suspected otomycosis in children and in vitro protease activity of Candida spp. isolated from the bony portion of the external ear.

METHODS

From April 2001 to March 2003, a total of 23 children suffering from otomycosis were examined at the Outpatient Otology Department, ranging in age from 13 to 18 years (mean 16.3 years).

The specimens were taken by cotton swab from the bony portion of the external ear. All clinical specimens were inoculated onto Sabouraud Dextrose Agar slants (Torlak, Belgrade, Serbia and Montenegro) and incubated for seven days at 26°C and 37°C, and examined macroscopically (i.e. visually) daily. Suspected cultures were examined microscopically in order to confirm Candida spp. All Candida isolates were identified by germ tube serum formation test, chlamydospore formation on cornmeal agar and by a battery of fermentation and assimilation tests (API 20°C 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 48 hours at 37°C.
The protease production was determined according to Aoki\(^{15}\) using a test medium consisting of agar plates containing bovine serum albumin (BSA). 60 ml of solution contained 0.04 g MgSO\(_4\) X 7H\(_2\)O, 0.5 g K\(_2\)HPO\(_4\), 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 N 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 a 10 l suspension of each strain was inoculated on the plates in triplicate. After incubation at 37\(^\circ\)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\(^{16}\) in terms of the ratio of 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 P\(_z\) value was obtained with three separate samples of each strain. The \textit{C. albicans ATCC 24433, C. parapsilosis DSMZ 5784} and \textit{C. kruzei DSMZ 6128} were used as positive and negative control strains (ATCC-American Type Culture Collection, DSMZ-Deutsche Sammlung von Mikroorganismen and Zellkulturen). The statistics could not be performed for tested strains due to the small number of isolates tested in each species.

**RESULTS**

In 23 children suffering from otomycosis, 7 \textit{Candida} strains were isolated from the bony portion of the external ear (34.78%). Four different species were determined: \textit{C. parapsilosis 3/8, C. guillermondii 2/8, C. albicans 2/8 and C. famata 1/8} (Table 1.). The protease activity of \textit{Candida} spp. strains was observed three days after inoculation on the BSA medium by area of brightness around the colony. Seven out of eight isolates (87.5%) had protease activity. All tested \textit{C. albicans, C. famata} and \textit{C.guillermondii} strains demonstrated \textit{in vitro} protease production, while only one out of two \textit{C. parapsilosis} isolates were protease positive (Table 1). The mean P\(_z\) value of triplicate of each tested strain of \textit{Candida spp.} had protease activity values ranging from 0.61 to 0.78 (P\(_z\) average 0.69). The most common symptoms were pain and swelling. The most frequently otomicroscopical and otoendoscopical findings were redness of the skin and white layers. Otoendoscopical findings of the ear hyperemia in a patient with otomycosis was caused by \textit{C. parapsilosis} (protease negative strain) (Figure 1A), and white debris in a patient with otomycosis was caused by \textit{C. guillermondii} (protease positive strain) (Figure 1B).

**DISCUSSION**

Fungal OE is sporadic and can be caused by a wide variety of fungi, the majority of which are saprobes such as \textit{Aspergillus} spp. or fungal normal flora of the skin. Since \textit{Candida} spp. is known to occur as a commensal on the skin of a healthy individual, the mere recovery of fungus from the clinical samples cannot conclusively establish the diagnosis of mycotic disease. Therefore, the emphasis is laid on possible virulence factors such as protease production, which is involved in the infections caused by \textit{Candida} spp. The constitutive hydrolytic enzymes of \textit{Candida} spp. avoid the invasion of host tissues, and, in this investigation, the majority of tested \textit{Candida} spp. had positive protease activity. These findings suggest that protease production may play an important role in the patho-
genesis of otomycosis caused by Candida spp. It is possible that protease enzymes enhance the ability of Candida spp. to colonize the skin and penetrate the host cells, which could be important in establishing the cause of the ear infection. It is known that the secreted proteases are an important virulence factor in Candida spp. cases of skin, mucosal and deep organ infections and may help the yeast invasion through the keratin protective layer and facilitate the initiation of the infection of the ear canal. Local lesions create conditions favoring fungal growth and development of mycosis. Most infections are present in patients who previously had medical treatment of the external canal and in those who have undergone surgical procedures, where local lesions, such as congestion, increased vascular permeability, higher temperature and acid pH, create favorable conditions for the growth of fungi. It is possible that the ability of yeast to adhere to the skin cells is proportional to acid protease production, which probably modifies the cell membranes of the host to accept the attachment of the fungus or modifies the surface of the yeast cell in a way that promotes attachment.

There has been published research investigating protease production in Candida spp. isolates from various sites on the body and this enzyme activity seems to be related to Candida virulence in the pathogenesis of the invasive candidiasis. The strains with higher proteolytic activity are considered more virulent. De Bernardis reported high in vitro protease activity in all C. parapsilosis strains isolated in patients with vaginitis. Yamamoto discovered that the majority of C. tropicalis and C. parapsilosis isolates had proteolytic activity while none of the tested C. glabrata strains secreted the enzyme. Kanatrcioglu and Yucel observed that 78.9% of Candida isolates from various body sites were protease positive.

In further research, it is necessary to clarify the contribution of protease activity to Candida virulence associated with otomycosis, and to use this information in the development of new therapeutic interventions, while protease and other enzymes activity in Candida species isolated from adults and children with ear infections should be additionally investigated.

**SUMMARY**

**PROTEAZNA AKTIVNOST IZOLOVANE CANDIDA SPP. KOD ZAPALJENJA SPOLJAŠNJEG SLUŠNOG HODNIKA: PRELIMINARNI REZULTATI**

Mikotičnu infekciju uva uglavnom uzrokuju Candida i Aspergillus. Mogući faktori virulencije kod Candida spp. jesu enzimi; proteaze, fosfolipaze, fosfotaze i esteraze. Po dosadašnjim saznanjima proteazna aktivnost u brisu Candida izolovanog kod pacijenata sa otomikozom nije istraživana. Cilj rada bio je određivanje proteazne in vitro aktivnosti kod 8 uzoraka brisa Candida spp. (C. parapsilosis, C. famata, C. guillermondii and C. albicans) izolovanih kod dece sa otomikozom. Najveći broj izolovanih uzoraka 7/8 (87.5%) bio je proteaza pozitivan. Proteazna aktivnost je varirala od Pz 0.61 dp 0.78. Neophodna su buduća istraživanja u definisanju doprinosa proteazne produkcije u virulenciji kandidom uzrokovane otomikoze.

**REFERENCES**


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**TABELA 1**

| PROTEASE ACTIVITY IN DIFFERENT CANDIDAE SPECIES ISOLATED IN CHILDREN WITH OTOMYCOsis |
|---|---|---|
| **Tested strains** | **Positive strains** | **Protease activity** |
| Candida spp | n | n | Pz+/+SD |
| C. Parapsilosis | 3 | 2 | 0.607+/-.180 |
| S. Guillermondii | 2 | 2 | 0.671+/-.112 |
| C. Albicans | 2 | 2 | 0.713+/-.132 |
| C. Fumata | 2 | 1 | 0.781+/-.213 |
| *C albicans ATCC24433 | 1 | 1 | 0.612+/-.098 |
| *C parapsilosis DSMZ5784 | 1 | 0 | 0.720+/-.121 |

*control strains*