SUSCEPTIBILITY PROFILE OF CANDIDA SPP. ISOLATED FROM HUMANS AND DOGS WITH STOMATITIS TO THE ESSENTIAL OIL OF THYMUS VULGARIS

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Candida spp. form a part of human and animal oral cavity flora. However Candida spp. is the main cause of dental related stomatitis in humans and stomatitis in dogs. Stomatitis treatment implies the use of azoles and polyenes to which yeasts build up resistance. The research is directed to the use of natural compounds such as essential oils.

The aim of this paper is to define the antifungal activity of thyme oil on 15 clinical strains of Candida spp., isolated from humans and dogs and to determine if there is a difference in susceptibility between human and dog isolates.

Sampling in patients with stomatitis was done by swabbing the denture or oral mucosa swab while sampling in dogs was done by swabbing the oral cavity mucosa after stomatitis has been diagnosed. In order to investigate the antifungal activity of thyme oil in vitro, microdilution method was used.

Thyme oil expressed antifungal effects on all investigated strains. Also, our data show that the values of minimum fungicide concentration (MFC) and minimum inhibitory concentration (MIC) are lower in human strains. Explanation is that in most cases, stomatitis in humans is asymptomatic and thus not treated, so Candida strains have not developed resistance. On the other hand, stomatitis in dogs is followed by a marked clinical picture and treated is by antimicotics (mostly by azoles), therefore resistant Candida strains are more likely to occur.

Key words: Candida, dogs, essential oil, stomatitis, Thymus vulgaris
INTRODUCTION

Yeasts of genus Candida are residential microorganisms which very often can be found in both human (Carvalhinho, 2012) and animal oral cavity. Apart from the oral cavity, Candida is present in some other human and animal moist areas such as: skin, inner ear, conjunctival canal, mouth, digestive system and perianal area. Candida yeasts along with other microorganisms are microbes of healthy living beings, but in specific conditions they can cause opportunistic infections (Cleff et al., 2005).

Candida albicans (C. albicans) is the main etiological factor of oral candidiasis in humans and other small animals. Apart from C. albicans, other kinds of oral yeasts may be included in various pathogenic processes. In the available literature, the following are found: C. glabrata, C. krusei, C. tropicalis, C. dubliniensis, C. parapsilosis in humans or C. parapsilosis, and C. guilliermondii and C. tropicalis in dogs (Moreti et al., 2006; Cleff et al., 2007; Pozzatti, 2007; Yurayart et al., 2011).

There are different clinical manifestations of oropharyngeal candidiasis in humans, including pseudomembranous candidiasis (thrush) and erythematous forms such as denture related stomatitis, which is often recurrent (Cannon et al., 1995). Wearing prosthetic appliances is commonly associated with denture stomatitis, which is characterized by inflamed mucosa covered by denture, more often under the upper denture (Wilson, 1998). Patients with denture related stomatitis may complain of burning sensation, discomfort, or bad taste. However patients very often do not have any symptoms. There are many pre-dilutional factors that influence denture stomatitis, including age of denture, denture cleanliness, continuous constant denture wearing, denture trauma which appears as a consequence of wearing dentures, smoking, dietary factors, pH of denture plaque (Soya et al., 2004, 2006). Denture stomatitis is a disease of fungal and bacterial origin, and C. albicans is most frequently isolated yeast from the patients' oral cavities (Baena-Monroy et al., 2005). Denture is more often a reservoir of microorganisms from which they can spread and cause diseases to other organs.

Stomatitis in dogs is characterized by erythematous oral mucous membrane which is ulcerous and covered with pseudo membranes, with halitosis and hypersalvation (McKcever and Klausener, 1986).

Opportunistic infections in dogs develop as a consequence of deteriorating defense mechanisms, immunosuppression and prolonged antibiotic treatment (Moretti et al., 2006; Cleff et al., 2007; Ong et al., 2010; Yurayart et al., 2011). Different Candida species are very important pathogenic factors in small animals and they are linked to urinary infections, (Ozawa et al., 2005) skin lesions (Moretti et al., 2004), otitis and systematic infections (Brown et al., 2005).

Today candidiosis is a problem of public health due to resistance to conventional antifungal medicines and there are some reports on relapsing disease especially in immunocompromised patients (Sanglard and Kvota, 2002; Kolombo and Guimaraes, 2003; Pozzatti et al., 2010).
In general, oral \textit{C. albicans} isolates have high levels of susceptibilities to a range of antifungal agents (Kuriyama \textit{et al.}, 2005), but some studies have shown high levels of azoles resistance in \textit{C. albicans} strains isolated from the throat and mouth (Paniaqua \textit{et al.}, 2002; Manifredi \textit{et al.}, 2006). Owing to their antibacterial effects, essential oils are the alternative for antibiotics in the pharmaceutical industry (Bakkali, 2008). The increase of \textit{C. albicans} resistance to antifungal medicines encourages the research of new therapeutic alternatives such as essential oils (Giordani \textit{et al.}, 2004; Cavaleiro \textit{et al.}, 2006).

Studies on essential oils have a goal to examine active compounds with a wide spectrum of effects, low toxicity and low prices (Rochette \textit{et al.}, 2003). Treatment of fungal infection in veterinary medicine is limited to conventional polyenes and triazoles. Additionally, these medicines have adverse effects and relatively high prices, especially when they are used for animals (Sanglard, 2002; Mondelo \textit{et al.}, 2003; Pressler \textit{et al.}, 2003; Rochette \textit{et al.}, 2003; Lukman \textit{et al.}, 2007).

The aim of this study is to:
1. determine antifungal activity (minimum inhibitory concentration – MIC and minimum fungicide concentration – MFC)
2. determine if there is a difference in susceptibility profile of \textit{Candida} spp. isolated in both humans and dogs

\textbf{MATERIAL AND METHODS}

\textit{Essential oils}

In the research, the essential thyme oil used (Citychem, Belgrade, Serbia), was obtained by water vapour distillation under of \textit{Thymus vulgaris} flowers. The percentage of particular components of thyme oil is given in Table 1.

\textbf{Table 1. Components of thyme oil}

<table>
<thead>
<tr>
<th>Substance</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymol</td>
<td>46.0</td>
</tr>
<tr>
<td>(\rho)-cymene</td>
<td>27.5</td>
</tr>
<tr>
<td>(\gamma)-terpinene</td>
<td>9.0</td>
</tr>
<tr>
<td>Linalool</td>
<td>6.5</td>
</tr>
<tr>
<td>Carvacrol</td>
<td>2.5</td>
</tr>
<tr>
<td>(\beta)-myrcene</td>
<td>2.4</td>
</tr>
<tr>
<td>Terpinen-4-ol</td>
<td>1.5</td>
</tr>
</tbody>
</table>

\textit{Fungal strains}

The study has included the research of 15 clinical isolates of \textit{Candida} spp. isolated in seven patients with diagnosed dental related stomatitis (5 \textit{C. albicans},
2 C. glabrata, 2 C. krusei and 1 C. tropicalis) and five dogs with stomatitis (3 C. albicans, 1 C. parapsilosis and 1 C. krusei).

The sampling in patients was done by swabs taken from their dentures (in patients with dental stomatitis) or by oral mucosa swabs. The sampling in dogs was done by mucosa swabs of the oral cavity when stomatitis has been diagnosed.

Swabs were transported to the microbiological laboratory in a period no longer than 30 minutes from the moment of sampling. Material was seeded on CHROMagar Candida plates (CHROMagar, Paris, France) and incubated at 37°C for 48 hours. The identification of Candida strains was done according to the specific appearance of the colonies as defined by the manufacturer.

**In vitro antifungal activities of thymes oil**

Susceptibility of strains to thyme oil has been investigated by the microdilution method according to the standardized protocol by The European Committee on Antimicrobial Susceptibility Testing – EUCAST (EUCAST). Serial oil dilutions were prepared in RPMI 1640 with 2% of glucose, in the concentration range 0.1 = 16 µL mL⁻¹.

Before testing, 15 Candida isolates, together with two reference strains (C. albicans ATCC 24433 and C. krusei ATCC 6258) were cultivated on Sabouraud dextrose agar plates (Oxoid, Basingstoke, United Kingdom) at 37°C during 24 hours. Yeast suspension has been prepared in sterile 0.9% NaCl and adjusted to 0.5 McFarland standard of turbidity (which fits the size of inoculums of 1-5×10⁶CFU/ml).

Each chamber of the microtitration plates was inoculated by adding 100 µL of yeast suspension and 100 µL of corresponding concentration of thyme oil. The test has also included three controls: positive control/growth control (fungi in PRMI 1640 without thyme oil), negative control (fungi in solution 2 µg/mL of amphotericine B) and sterility control (only RPMI 1640 without yeast suspensions).

Microtitration plates were incubated at 37°C. The results have been read visually after 24 and 48 hours on the basis of chamber’s content turbidity. MIC is defined as the lowest concentration of solution which causes a significant decrease in turbidity comparing to the positive control.

Determination of Minimum fungicide concentration (MFC) is done by seeding 20 µl from all clear wells on SDA incubated at 37°C for 24-48 hours.

**RESULTS**

Essential thyme oil has manifested antifungal effects to all tested strains. The susceptibility results presented as MIC and MFC values are shown in Table 2 for Candida strains isolated from humans and Table 3 for strains isolated from dogs.

Strains of the species C. albicans have shown the greatest susceptibility to thyme oil, while between other Candida species difference in MIC/MFC values has not been noticed.
Table 2. The results of Candida spp. susceptibility isolated from humans

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Species</th>
<th>MIC (µL mL⁻¹)</th>
<th>MFC (µL mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>36A</td>
<td>C.albicans</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>62A</td>
<td>C.albicans</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>80A</td>
<td>C.albicans</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>82A</td>
<td>C.albicans</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>87A</td>
<td>C.albicans</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>90T</td>
<td>C.tropicalis</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>49G</td>
<td>C.glabrata</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>60G</td>
<td>C.glabarata</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>33K</td>
<td>C.krusei</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>43K</td>
<td>C.krusei</td>
<td>0.5</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 3. Susceptibility results of Candida spp. isolated from dogs

<table>
<thead>
<tr>
<th>Strain number</th>
<th>Species</th>
<th>MIC (µL mL⁻¹)</th>
<th>MFC (µL mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A12</td>
<td>C.albicans</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>A2</td>
<td>C.albicans</td>
<td>2.5</td>
<td>5.0</td>
</tr>
<tr>
<td>A6</td>
<td>C.albicans</td>
<td>2.5</td>
<td>5.0</td>
</tr>
<tr>
<td>K4</td>
<td>C.krusei</td>
<td>5.0</td>
<td>10</td>
</tr>
<tr>
<td>P15</td>
<td>C.parapsilosis</td>
<td>1.25</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Figure 1. Comparison of Gm values for human and dog isolates
On the basis of our results it may be concluded that *Candida* spp. isolated from humans shows higher susceptibility to thyme oil compared to *Candida* spp. isolated from dogs. Comparison of geometric mean (GM) values for human and dog isolates is given in Figure 1.

**DISCUSSION**

The effect of essential oils and their main components on *Candida* spp. have been confirmed in *in vitro* and *in vivo* studies (Giordani *et al.*, 2004; Tamperi *et al.*, 2005; Duarte *et al.*, 2005; Pinto *et al.*, 2006; He *et al.*, 2007; Palmeira-de Oliveira *et al.*, 2009). Their activity can usually be presumed on the basis of their chemical content. Essential oils have antifungal activity since they contain high content of phenol. *Thymus* spp. is very rich in phenol (thymol). *Thymus zygis* is classified as a fungicide due to its powerful effect on *C. albicans* (Pina-Vaz, 2004). Various studies have shown that thyme oils manifest the highest antifungal activity compared to other essential oils (Omran, 2009).

Scanning by SEM, Braga and del Sasso (2008) have shown that the effect of thymol causes the change of susceptibility of the cell membrane, degradation of cellular wall, leaking of cell content, coagulation and cell lysis (Bakkali *et al.*, 2008; Palmeira de Olivera *et al.*, 2009). Concentrations below the MIC values significantly inhibited germ tube formation, an important virulence factor (Pina-Vaz, 2004).

Hammer (1999) has also defined MIC of thyme oil for *C. albicans* from 0.03% (0.3 µL mL⁻¹) by using the microdilution method. In this study the value was 0.02% (0.2 µL mL⁻¹) for human isolates of *C. albicans*.

In the study of Omran *et al.* (2010) where antifungal activity of thyme oil was investigated by disc diffusion method, MIC for *Candida* spp. was 2 mg/mL=0.02%. *C. albicans* showed the highest susceptibility, followed by *C. krusei* and *C. glabata* (Omran *et al.*, 2009). In our research, *C. albicans* strains also expressed the highest susceptibility. *C. krusei* is less susceptible to thyme oil and to other essential oils due to its cellular structure, as well as the intensified action of cell pumps which disturb the entrance of essential oils into the cell (Mondelo *et al.*, 2003; Pozzatti, 2007).

Our study also demonstrated higher susceptibility of human *Candida* spp. comparing to the strains *Candida* spp. isolated from dogs, which could be explained in a way that dental related stomatitis in patients very often is not treated. Therefore, human strains of *Candida* spp. have not developed resistance. On the other hand, in dogs, stomatitis has been accompanied by a stronger clinical picture, and is treated by antimicotics (mostly azoles) therefore resistant profiles of *Candida* spp. are more likely to occur.
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SADRŽAJ