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Candida Distribution in Onychomycosis and *in vitro* Susceptibility to Antifungal Agents

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ABSTRACT The aim of this study was to determine distribution of the *Candida (C.)* species in onychomycosis and analyses *in vitro* susceptibility to fluconazole and itraconazole. In recent years, cases of onychomycosis in Lithuania caused by *Candida* have increased significantly. In the period between 2009 and 2016, a total of 8149 clinical cases (outpatients and inpatients) were investigated at the Vilnius University Hospital Santaros Clinics (VUH SC). *Candida* yeasts were identified using VITEK 2 (BioMerieux, France) and IVD Maldi biotyper 2.3 (Bruker Daltonik GmbH, Germany), automated systems for identification of yeasts. The antifungal susceptibility to the *Candida* species were determined by disc diffusion. *Candida* spp. were the most frequently isolated pathogens in onychomycosis during the investigation period. The main species in onychomycosis were *C. albicans* (38.6%), followed by *C. krusei* (33.7%), *C. tropicalis* (11.1%), *C. parapsilosis* (7.9%), and other *Candida* (8.7%). The different antifungal susceptibility patterns among *Candida* species confirm the need to perform antifungal susceptibility *in vitro* testing of yeasts from patients with onychomycosis.

KEY WORDS: onychomycosis, prevalence, *Candida*, antifungal agents

INTRODUCTION

Derived from the Greek words "Onychos" (meaning nail) and "Mykēs" (fungus), onychomycosis is a term that describes fungal infections of the fingernails and/or toenails. It is caused by different types of fungi, and the affected nails become brittle, thin, disfigured, and dichromic (1-5). The literature available on the epidemiology of onychomycosis is varied. Onychomycosis accounts for approximately 50% of all nail fungal infections and affects 2.0-18.5% or more of the world's population (6-7). The reported prevalence of onychomycosis is up to 14% in North America, 20% in East Asia, and 26.8% in Europe (8). Epidemiology of infectious *Candida (C.)* species was not well documented in Lithuania. Several studies have reported the prevalence of *Candida* species in dermatomycosis and onychomycosis (9-11).

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The genus *Candida* is one of the most prominent causes of onychomycosis around the world and the *Candida* species are considered one of the most important causes of fingernail onychomycosis, especially in women. *Candida albicans* is the most prevalent species, followed by *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. lusitaniae*, and *C. krusei* (5,12).

Treatment of onychomycosis is necessary, often prolonged, and is associated with potential adverse drug reactions; this infection can also affect quality of life and can lead to a secondary infection if left untreated (4,8). The approved treatment strategies to deal with onychomycosis involve the use of topical, oral, and combination therapy (3,13). Itraconazole and fluconazole are the most widely available antifungal agents used for systemic treatment of onychomycosis (14).

The aim of this study was to determine *Candida* species distribution in onychomycosis and analyze *in vitro* susceptibility to fluconazole and itraconazole during the period from 2009 to 2016 in Lithuania.

PATIENTS AND METHODS

Between 2009 and 2016, a total of 8149 outpatients and inpatients with clinical suspicion of onychomycosis were investigated at the Vilnius University Hospital Santaros Clinics (VUH SC). The investigation was performed at the Laboratory of Microbiology of the Centre of Laboratory Medicine, VUH SC, and the Nature Research Centre. The specimens were obtained from clinically abnormal nails. The pathological material (nail fragments) was plated on Sabouraud Agar containing chloramphenicol (Oxoid, England), dermatophyte (DTM) agar (Liofilchem, Italy), and corn meal agar (Sifin diagnostics Gmbh, Germany) and cultivated in an incubator for 3-14 days at a temperature of 28±2 °C. *Candida* yeasts were identified using VITEK 2 (BioMerieux, France) and IVD Maldi biotyper 2.3 (Bruker Daltonik GmbH, Germany), automated systems for identification of yeasts.

Candida yeast morphology was examined from cultures grown on yeast morphology agar (Difco, USA) for two days at 25 °C. The cultures were examined microscopically using a Leica DM 5000B with differential interference contrast microscopy (×1000 magnification) and a digital camera, Leica DFC 450 (Leica Microsystems, Germany).

The Neo-Sensitabs (Rosco Diagnostica, Taastrup, Denmark) and Mast (MAST DIAGNOSTICA, Germany) discs were used in this study. To determine the antifungal susceptibility patterns of *Candida spp.*, a disk of each antifungal drug, including fluconazole FLU (25 µg/disk) and itraconazole ITR (10 µg/disk), were used. Disk diffusion assay and the interpretation of results were performed according to the manufacturer's instructions and M44-A2 guidelines for yeasts (15-17).

The data were presented as absolute numbers and percentages. The prevalence of *Candida* yeasts in onychomycosis, as well as changes in their susceptibility to fluconazole and itraconazole, were calculated using a linear model and determination coefficient, R^2. The minimum significance level was set at $P<0.05$.

All statistical analyses were performed with Statistica for Windows ver. 6.0 software 18 (18).

RESULTS

Among the 8149 clinically suspected cases of onychomycosis, 4229 (51.89%) patients were confirmed to be affected with onychomycosis. The positive rate percentage among all patients examined during the study period was in the range 43.32% to 66.05%. A total 4229 onychomycosis causative agents were isolated and identified during investigation period. The isolated causative agents were classified into three groups: yeasts, dermatophytes, and non-dermatophytes fungi. In our study, *Candida* yeasts were found to be the most frequent causative agents of onychomycosis (detected in 2044 patients, 48.33%), followed by dermatophytes (1461, 34.55%) and non-dermatophytes (724, 17.12%).

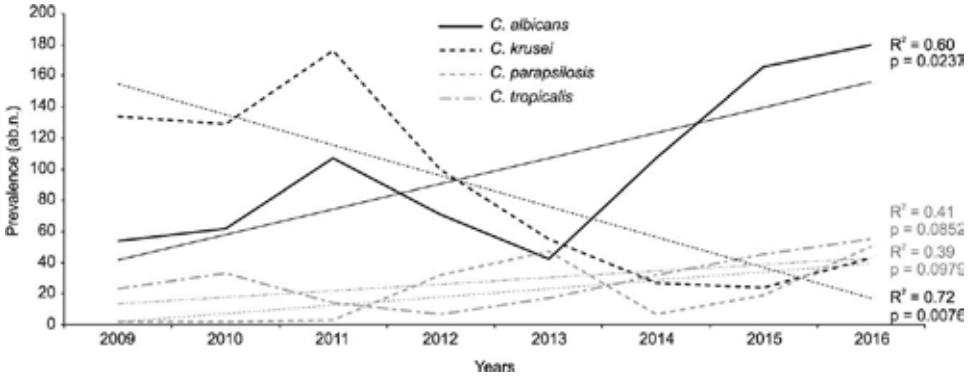

Figure 1. Distribution of main *Candida* species in onychomycosis.

A total of 16 *Candida* species were identified from the patients' material. Among *Candida* yeasts, the most commonly isolated organisms were *C. albicans* (n=789; 38.6%) followed by *C. krusei* (*Issatchenkia orientalis*) (n=688; 33.7%), *C. tropicalis* (n=226; 11.1%), and *C. parapsilosis* (n=162; 7.9%) (Figure 1). Accounting for 8.7%, other species from this genus were *C. glabrata*, *C. famata* (*Debaryomyces hansenii*), *C. lusitaniae* (*Clavispora lusitaniae*), *C. zeylanoides*, *C. guilliermondii* (*Meyerozyma guilliermondii*), *C. intermedia*, *C. apicola*, *C. pelliculosa* (*Wickerhamomyces anomalus*), *C. lambica*, *C. rugosa* (*Diutina rugosa*), *C. duobushaemulonis*, and *C. sphaerica* (*Kluyveromyces lactis*). The current valid taxonomic denominations for genera and species were listed in parallel to the reported species names (19-20).

Analyzing the results over time, we found a highly significant change in the pattern of the isolated *Candida* species, with *C. krusei* predominating in the first five study years (2009-2013) (R^2 = 0.72, *P*=0.0076) and *C. albicans* over the 2014-2016 period (R^2 = 0.60, *P*=0.0237). Over the eight-year period, *C. tropicalis* isolation rate increased (with the exception of the 2011-2013period), though no significant difference was observed (R^2 = 0.3915, *P*=0.0979). *C. parapsilosis* was detected only in two or three cases during the 2009-2011period. There was also an upward trend in the number of *C. parapsilosis* from 2012 to 2013, a decrease in 2014, and an increase over the 2015-2016 period (R^2 = 0.4149, *P*=0.0852). Other species from this genus (*C. intermedia*, *C. pelliculosa*, *C. rugosa*, *C. duobushaemulonis*, *C. sphaerica*, *C. apicola*, *C. famata*, *C. guilliermondii*, *C. lusitaniae*, *C. zeylanoides*, and *C. glabrata*) were isolated only in a small number of cases of onychomycosis, and no significant differences were observed in their prevalence (*P*>0.05).

Figure 2 and Figure 3 summarize the *in vitro* susceptibility of 1155 *Candida* isolates to fluconazole and 969 *Candida* isolates to itraconazole as determined by disk diffusion testing. Analyzing the results over time, we found a highly significant change in the pattern of *Candida* yeasts susceptible to fluconazole (R^2 = 0.5994, *P*=0.0244) and resistant to fluconazole (R^2 = 0.7904, *P*=0.0031). However, the differences among *Candida* yeasts with intermediate sensitivity to fluconazole were not statistically significant (R^2 = 0.41, *P*=0.0839). The number of *Candida* yeasts susceptible to fluconazole gradually increased – a significant difference was observed from 2009 to 2015 (R^2 = 0.71, *P*=0.0141). These results probably depend on the distribution of species. The rate of *C. albicans* yeasts susceptible to fluconazole isolation significantly increased over the eight-year period, while a significant decrease was observed in *C. krusei* yeasts resistant to fluconazole.

No significant difference was observed among *C. albicans* isolates (resistant – R^2 = 0.06, *P*=0.5714; intermediate – R^2 = 0.16, *P*=0.3199; susceptible – R^2 = 0.07, *P*=0.5358). The differences among *C. krusei* yeasts susceptible to fluconazole and those resistant to fluconazole were not statistically significant (resistant – R^2 = 0.17, *P*=0.3002; susceptible – R^2 = 0.29, *P*=0.1713), but a significant difference was observed (R^2 = 0.61, *P*=0.0456) in the proportion of *C. krusei* isolates intermediately sensitive to fluconazole. During the eight-year period, the isolation rate of *C. parapsilosis* yeasts was irregular, and therefore no significant difference was observed (*P*>0.05). A statistically significant difference was established in *C. tropicalis* yeasts resistant to fluconazole and intermediately sensitive to fluconazole: resistant – R^2 = 0.62, *P*=0.0208, intermediate – R^2 = 0.50, *P*=0.0490. However, no significant difference was observed in the isolation rate increase of *C. tropicalis* yeasts susceptible to fluconazole (R^2 = 0.20, *P*=0.2682).

The susceptibility of *Candida* yeasts to itraconazole is presented in Figure 3. Analyzing the susceptibility to itraconazole results over time, a significant

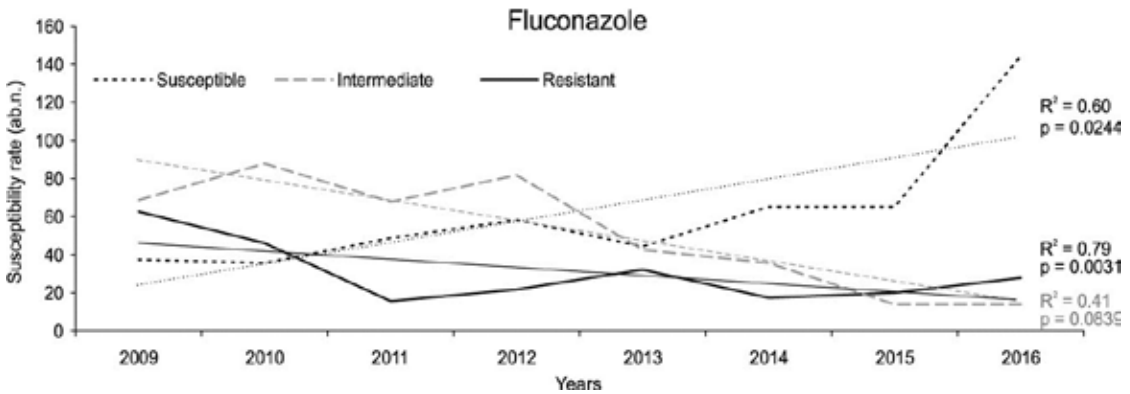

Figure 2. The susceptibility of *Candida* yeasts to fluconazole.

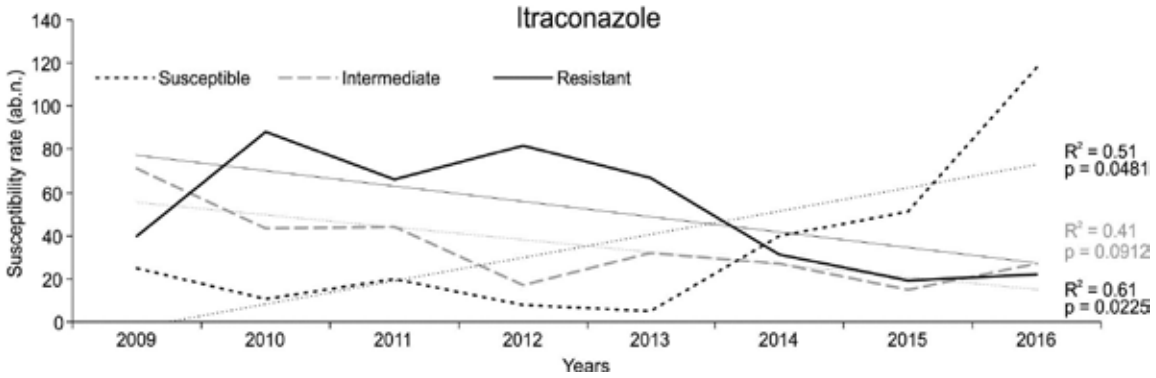

Figure 3. The susceptibility of *Candida* yeasts to itraconazole.

increase in *Candida* yeasts susceptible to itraconazole (R^2 = 0.51, *P*=0.0481) was observed, along with a significant decrease in *Candida* yeasts intermediately sensitive to itraconazole (R^2 = 0.61, *P*=0.0225). However, no significant difference was observed in the decrease of *Candida* yeasts resistant to itraconazole (R^2 = 0.41, *P*=0.0912). There was an increase in the number of *Candida* yeasts susceptible to itraconazole in 2014, coinciding with the period that *C. albicans* were the most frequent species in onychomycosis. During the eight-year period, no significant differences were observed in the decrease in the number of *C. albicans* yeasts with intermediate itraconazole sensitivity (R^2 = 0.85, *P*=0.0011), or those that were resistant (R^2 = 0.07, *P*=0.5249), and susceptible (R^2 = 0.17, *P*=0.3199) to itraconazole. The *in vitro* susceptibility results of *C. krusei* yeasts were not statistically significant (resistant – R^2 = 0.27, *P*=0.1833; intermediate – R^2 = 0.02, *P*=0.7439; susceptible – R^2 = 0.39, *P*=0.0930). There were no significant differences in the *in vitro* susceptibility results of *C. parapsilosis* yeasts (*P*>0.05), which were probably related to the variability of the isolation rate of *C. parapsilosis* yeasts over the eight-year period. In addition, statistically significant differences were established in the decrease of *C. tropicalis* yeasts intermediately sensitive (R^2 = 0.62, *P*=0.0208) and resistant (R^2 = 0.50, *P*=0.0490) to itraconazole. However, no significant difference was observed in the increase of *C. tropicalis* yeasts susceptible to itraconazole (R^2 = 0.20, *P*=0.2682).

DISCUSSION

Onychomycosis is a major problem in dermatology due to its widespread occurrence. Its prevalence has increased since World War I, and today onychomycosis is considered a "disease of civilization", characterized by extreme chronicity and resistance to therapy (21). In this study, we performed the first large-scale surveillance study of the distribution of *Candida* species in Lithuania. We examined 8149 outpatients and inpatients that were suspected of having onychomycosis between 2009 and 2016. Onychomycosis caused by *Candida* yeasts was confirmed in 48.33% of patients. Similar results were obtained in Poland (22,23), Brazil (24), Iran (25,26), and Croatia (27). Literature data indicates that yeasts could be responsible for an increase in mycosis incidents in recent years (23). The yeast species most frequently isolated from patients with onychomycosis were *C. albicans* (38.6%), followed by *C. krusei* (33.7%), *C. tropicalis* (11.1%), and *C. parapsilosis* (7.9%). Studies have reported that the most frequent *Candida* species, especially *C. albicans*, *C. krusei*, *C. tropicalis*, and *C. parapsilosis*, account for most of the cases of onychomycosis worldwide (6,22-28). The epidemiology and etiological agents of onychomycosis vary in different geographic areas (4,8).

Onychomycosis often has a significant impact on the quality of life in those patients who seek treatment. Apart from cosmetic problems, the disorder may result in pain while walking as well as difficulties in nail cutting and finding suitable footwear (29,30).

Not many studies in Lithuania have focused on the susceptibility of *Candida spp.*, which are responsible for onychomycosis (31). *Candida* yeasts are morphologically, physiologically, and genetically specialized. There is a possibility that the causative agents of onychomycosis could soon become resistant to one or more antifungal agents. Itraconazole and fluconazole are the most widely available antifungal agents used for the systemic treatment of onychomycosis (13).

In our study, the pattern of *C. albicans* susceptibility to fluconazole was as follows: the majority of isolates (68.3%) were susceptible, while 18.8% of isolates were intermediately sensitive, and 12.9% were resistant. *In vitro* susceptibility testing of fluconazole demonstrated that resistance to fluconazole in *C. parapsilosis* and *C. tropicalis* was 31.3% and 25.9%, respectively.

During the eight-year period, resistance to itraconazole in *C. albicans* isolates was 28.3%. In the present study, a high number of *C. krusei* isolates (59.0%) resistant to itraconazole was found. *In vitro* susceptibility testing of itraconazole demonstrated that the resistance to fluconazole among *C. parapsilosis* and *C. tropicalis* was 38.3% and 22.4%, respectively.

This tendency towards resistance has also been noticed by other researchers. Some have reported that *C. krusei* were genetically resistant to a certain antifungal drug and it was therefore recommended for use in treatment (32-34). Other studies have also reported an increasing risk of fluconazole-resistant *Candida* yeasts (35,36).

Today, the increase in tourism and immigration has influenced the distribution of some species and can quickly change the epidemiological profile in a given geographical area. We think that culture testing and the identification of pathogens with susceptibility testing are important steps in helping clinicians choose the correct therapy to treat onychomycosis.

CONCLUSION

In the period between 2009 and 2016, *Candida* yeasts were found to be the most frequent causative agents of onychomycosis in Lithuania. *C. albicans* was the most frequently isolated species from patients with onychomycosis. *Candida* species distribution showed a highly significant change in the pattern of causative agents in onychomycosis: the isolation rate of *C. albicans* increased, while *C. krusei* decreased. The different antifungal susceptibility patterns among *Candida* species confirm the need to perform antifungal susceptibility *in vitro* testing of yeasts from patients with onychomycosis.

Ethical approval

Ethical approval was not required as all procedures were part of our routine care. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional committee and with the 1964 Helsinki Declaration and its later amendments.

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