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Onychomycosis - Sampling, Diagnosing as Efficiant Part of Hospital Pharmacology

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SUMMARY

Introduction Onychomycosis is a fungal infection of one or more nails. Causes of onychomycosis are dermatophytes, yeasts and non-dermatophyte molds, but the most common cause is *Trichophytonrubrum* (*T. rubrum*) from the group of dermatophyte fungi..

The aims Using sampling determination of the most common clinical type of onychomycosis, lokalization and involvement of the nail plate, and monitoring the efficacy of methods/tests in the diagnosis of nail onychomycosis.

Material and methods This paper is a part of academic IV phase study. The study included 30 patients with onychomycosis. Each sample was seeded on Sabouraud Dextrose Agar (SDA) and Diluted SDA (D-SDA) at 28°C and 37°C, as well as the Dermatophyte Test Medium (DTM) at 28°C. Identification of isolated fungi to the level of genus/species has been based on macroscopic and microscopic characteristics by KOH and Blancophor fluorescent dye. PCR were performed to detect *T. rubrum*-specific and pan-dermatophyte multiplex PCR product. Informed consent was obtained from all patients.

Results The most common clinical form was subungual lateral distal onychomycosis (DLSO)of the hands and feet pollex fingernails, while the size of the involvement of the nail plate was 1/2 - 1/3 in the majority of patients. Cultivation gave a positive result in 50% of cases and the most commonly isolated microorganism was the *T. rubrum*.For negative cultures (50%) the PCR was carried out which demonstratedhigh sensitivity and *T. rubrum* remained the most frequently detected.

Conclusions Using the methods of cultivation and PCR, onychomycosis was confirmed in 28 (93.3%) patients. Cultivation gave a negative result in 50% of cases, while the PCR was positive in 86.6%. Our research shows the highest incidence of *T. rubrum* (60%). In continuation of this study will be analyzed the choice and effectiveness of therapy.

Keywords: dermatophytes, nail, cultivation, PCR , *T. Rubrum*

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INTRODUCTION

The term onychomycosis comes from a Greek word "νύχι" (nail), "μύκης" (mushroom) and it represents a fungal infection of one or more nails. Based on various studies prevalence of onychomycosis is aproximately 2% to 13% of general population [1,2]. Predisposing factors of onychomycosis are: age, sex, occupation, climate, previous onychomycosis, inadequate hygiene of legs, sweating, inadequate footwear, sports and trauma, contact with soil and animals. The prevalence was significantly higher in patients with immunodeficiency, compromised peripheral circulation, diabetes, psoriasis as well as the patients on long-term antibiotic therapy [3-7].

Causes of onychomycosis

Onychomycosis is caused by dermatophytes, yeasts and non-dermatophyte molds, but certainly the most common cause are the fungi of the dermatophyte group. Dermatophytes are strictly pathogenic fungi which have keratinophilic and keratinolytic features and affect the skin, hair and nails. Dermatophytes include three genera: *Trichophyton*, *Microsporum* and *Epidermophyton*, which are divided by ecology into anthropophilic, geophilic and zoophilic types [8]. These diseases are highly contagious and can be transmitted by direct and indirect contact [8].

Onychomycosis is most frequently caused by *T. rubrum*, *T. interdigitale* and *E. floccosum* from the group of dermatophytes causing about 90% of onychomycosis on the feet and 50% of onychomycosis on the hands [9]. The most common cause is the anthropophilic species *T. rubrum*. Various causes can often give an identical clinical presentation, especially in advanced cases of onychomycosis.

Virulence factors and immune response

The main effect of dermatophytes is through their endoproteases which can be classified into two large protein families: serin proteases and metalo-proteases [10]. A humoral and cell-mediated immunity occurs as a defensive response of the host. The immune response to *Trichophyton* species is particularly unusual because it can cause immediate hypersensitivity and delayed hypersensitivity reaction in different individuals [11].

Clinical forms

Depending on the location of the primary infection and its spread, *Tinea unguium* (onychomycosis) can be divided in four subtypes:

- Distal and lateral subungual onychomycosis (DLSO)
- Proximal subungual onychomycosis (PSO)
- White superficial onychomycosis (WSO)
- Total dystrophic onychomycosis (TDO)

Diagnostic methods

The first important step in diagnosing onychomycosisis is sampling. Many studies have shown that proper sampling of the nail is very important for the correct diagnosis. Methods for collecting the infected nails are: cutting the free edge of the nail, scratching hyperkeratotic subungual material and scraping the surface of the nail with a scalpel [16].

After proper sampling the diagnosis of onychomysosis usually involves making a direct microscopic preparations with 10-30% potassiumhydroxide (KOH) in dimethylsulfoxide (DMP) which allows identification of fungal elements but cannot make the identification to the level of species. Cultivation remains the gold standard in the diagnosis of onychomycosis based up on which it is possible to identify the level of genus or species in most routine laboratories. It is performed by seeding the material on Sabouraud Dextrose Agar (SDA) and Dermatophyte Test Medium (DTM). Further more, the metod of molecular diagnostics can also be used, most frequently the Polymerase Chain Reaction (PCR) [14, 15].

European Mycologycal Association suggested in the year of 2013 the methodological laboratory procedures for the diagnosis of onychomycosis which will be included in the European standard protocols. The protocol recommends PCR as a first method of choice for onychomycosis of the feet, and PCR and conventional mycologycal methods for onychomycosis of the hands.

Onychomycosis therapy

Proper diagnosis and laboratory confirmation of onychomycosis are needed for the use of antimycotic therapy and the most effective hospital pharmacology results.

Therapy can be local/topycal, systemic/oral or combined. Topycal treatment consists of removing of the infected nail material and persistent application of topycal anti-

fungal drugs over several weeks/months [16]. Systemic antimycotic therapy is required if more than 50% of the nail is affected, but it is expensive, has a variety of adverse effects such as: disorders of various liver enzymes, interactions with other drugs and many others [17].

THE AIM

By sampling determination the most common clinical type of onychomycosis, localization and involvement of the nail plate, and monitoring the efficacy of methods/tests in the diagnosis of nail onychomycosis.

MATERIAL AND METHODS

This paper is a part of the prospective academic (non-commercial) phase IV study, carried out in the Institute for microbiology and immunology, Medical Faculty, University of Belgrade, and Esthetic center ″Dr VAIS″. Study has been approved by the Esthetic center ″Dr VAIS″ Ethics Board and conducted in compliance with the EU clinical trials directives [18]. Informed consent was obtained from all patients.

Patients with clinical symptoms suspicious of the onychomycosis, confirmed by a dermatology specialist, were tested on onychomycosis till collecting 30 patients with nails onychomycosis confirmed. Samples were collected from October 1st 2013 to December 31st 2013, from two medical centers: Esthetic center ″Dr VAIS″ in Belgrade and Niš.

Diagnosing procedure included:

Sampling the nail - Depending on the type of onychomycosis part of the nail plate was sampled in several ways: by cutting the distal free edge of the nail, scratching the and scraping the surface of the nail with a scalpel (by which we received the hyperkeratotic material from deeper layers) [16]. All samples were collected in sterile containers and labeled properly.

Laboratory methods - Each sample of the nail was cultivated on five mediumes:

- SDA at 28°C and 37°C
- D-SDA at 28°C and 37°C
- DTM at 28°C

Period of incubation was completed in three weeks at the indicated temperatures and samples were controlled once a week to detect fungal growth. The samples with no fungal growth after four weeks were considered negative.

Identification of isolated fungi to the level of genus/species has been based on macroscopic and microscopic characteristics. We used KOH and Blancophor fluorescent dye for making preparations for microscopy. Preparations were observed by light microscope and fluorescent microscope at magnification 10x and 40x for detecting and identifying fungal elements.

For the identification of dermatophytes we used Dermatophytes Multiplex PCR (Statens Serum Institute, Denmark) that detects specific *T. rubrum* and pan-dermatophytic multiplex PCR product.

PCR method involved the following steps as instructed by the manufacturer:

- Preparing the nail samples for each patient
- Preparing Master Mix mixture
- PCR amplification in the Thermal Power Device Termojet (Eurogentec, Searing Belgium) The mixtures were exposed to a temperature of 94°C for five minutes, for denaturation of DNA; followed by a 45 temperature cycles consisted of: 1. Denaturation of DNA at the temperature of 94°C lasting for 30 seconds; 2. Reproduction at thetemperature of 60°C lasting for 30 seconds; 3. Binding of the primers to the matrix (Ta annealing) at the temperature of 72°C lasting for 30 seconds and 4. Elongationat the temperature of 72°C lasting for 3 minutes.
- Electrophoresis and visualization ofthe polymerse chain reaction products

For visualization of the products of PCR we performed electrophoresis in 1.5% agarose gel and brightening of the gel with ultraviolet light. The size of the resulting DNA products was compared to the size of the DNA control fragment from the PCR kit for *T. rubrumand* pan-dermatophytic marker.

RESULTS

Thirty patients with positive results were included in the study [Table 1], 21 (70%) female average age 54 years and 9 (30%) male average age 38 years. In clinical findings DLSO prevailed (86.6%), followed by PSO (10%) and WSO (3.4%). In 83% of patients onychomycosis was found on the feet, while 17% of patients had changes on their hands. In most cases, thumbs were affected both on the hands (57%) and the feet (49.3%), including percentage involvement of each finger on the right and

Number	Gender	Types of onychomycosis	Hand right/left 1,2,3,4,5	Foot right/left 1,2,3,4,5	Involvement of the nail
1.	F	DLSO	/	R 1-5	>2/3
2.	F	DLSO	/	R 1; L 1	1/3 - 2/3
3.	F	DLSO	/	R 1	>2/3
4.	F	DLSO	/	R 1	1/3 - 2/3
5.	F	DLSO	L 1	/	1/3 - 2/3
6.	F	DLSO	/	R 1	1/3 - 2/3
7.	M	WSO	/	R 1-5; L 1-5	1/3 - 2/3
8.	M	DLSO	/	R 1; L 1	1/3 - 2/3
9.	M	DLSO	/	R 2-5	1/3 - 2/3
10.	F	DLSO	/	R 1	1/3 - 2/3
11.	M	DLSO	/	R 1; L 1	1/3 - 2/3
12.	F	DLSO	/	L 1	<1/3
13.	M	DLSO	/	R 1; L 1	>2/3
14.	F	DLSO	/	R 1; L 1	1/3 - 2/3
15.	F	DLSO	/	R 1,2,5; L 1,2,5	1/3 - 2/3
16.	F	DLSO	/	R 1-5; L 1-5	1/3 - 2/3
17.	M	DLSO	/	R 1	>2/3
18.	F	DLSO	/	L 1	1/3 - 2/3
19.	M	DLSO	/	R 1; L 1-2	1/3 - 2/3
20.	F	DLSO	/	R 1-5; L 1-5	1/3 - 2/3
21.	F	PSO	L 1	/	<1/3
22.	F	DLSO	/	R 1	1/3 - 2/3
23.	M	DLSO	L 1,3	/	<1/3
24.	F	DLSO	/	R 1; L 1	>2/3
25.	M	PSO	R 1	/	<1/3
26.	F	DLSO	/	R 1,3,4	1/3 - 2/3
27.	F	DLSO	/	R 1; L 1	1/3 - 2/3
28.	F	PSO	L 3,4	/	1/3 - 2/3
29.	F	DLSO	/	R 1; L 1	1/3 - 2/3
30.	F	DLSO	/	R 1; L 1	<1/3

Table1. Types of onychomycosis, localization and involvement of the nail plate

F - female;
M - male;
DLSO - distal lateral subungual onychomycosis;
PSO - proximal subungual onychomycosis;
WSO - white superfitial onychomycosis

the left foot [Table 1, Figure 1].

The size of involvement of the nail plates was different in different patients [Table 1]. In 66.6% of patients the nail plate involvement was 1/3- 2/3, while the percentage of nail involvement of <1/3 and >2/3 was the same (16.7%) [Picture 1].

After cultivating on three different cultures (SDA, D-SDA and DTM) at different temperatures [Picture 1], we obtained 15 positive results. As the most common isolate in 60% of cases *T. rubrum* was isolated [Figure 2]. For 15 samples (50%) where there was no growth found, we performed PCR. *T. rubrum* was detected in 11 patients (73.3%), pandermatophyte marker (PD) was found in 2 samples (13.3%), which shows that the cause was other than *T. rubrum* and two samples were negative (13.3%). The results are shown in Figure 1.

DISCUSSION

Onychomycosisis widespread in general population. Most often itoccurs in adults and rarely in children [19]. In this study, the average age of patients was 46 years, which matches the results of a large number of studies that show the average age of patients 40-50 years [20,21]. The large number of patients included in our study were female (70%), matching the results of various studies where the uncomfortable shoes and household tasks were the main reasons for female morbidity [20,22,23]. However, our re-

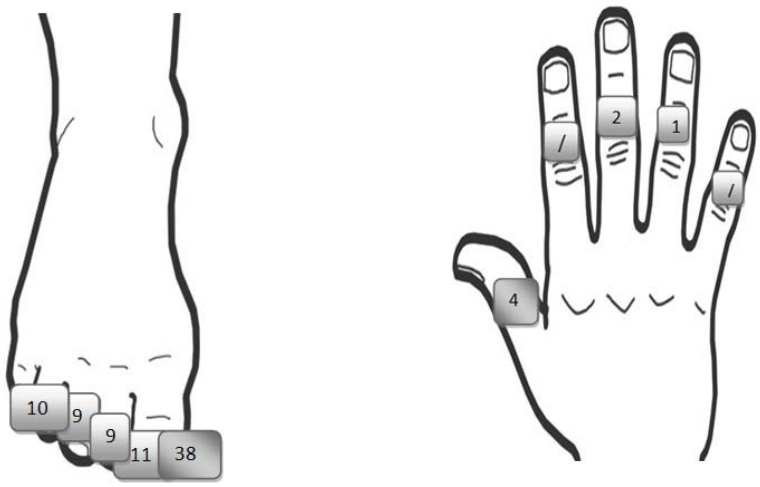
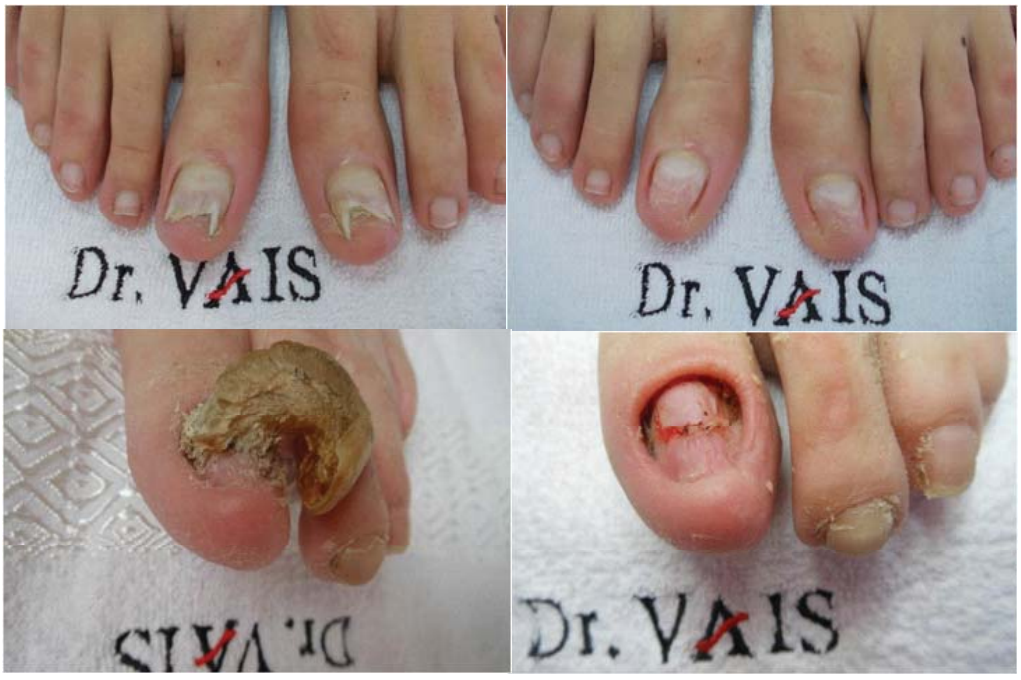


Figure 1. The incidence of onychomycosis on certain nails of feet and hands



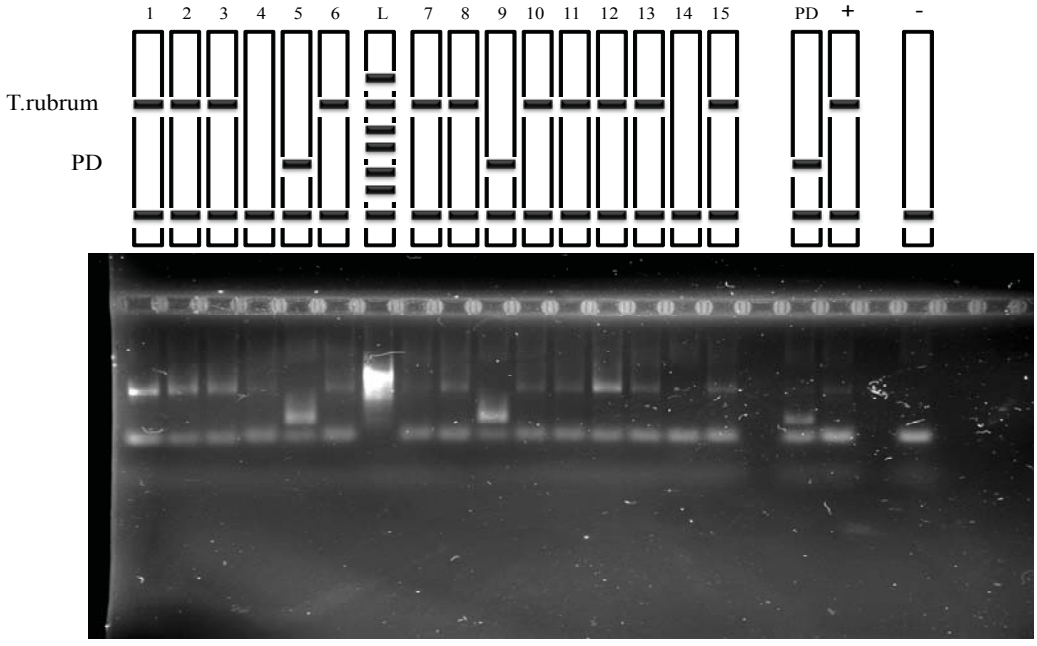
Picture 1. Some of the nails cultured in this study

sults do not match the studies where the main reasons for male morbidity are trauma and active sports [4].

Numerous studies have shown that the most common clinical typeis DLSO, which matches the result of our study, where DLSO was diagnosed in 86.6% [8].Other studies have shown that DLSO and TDO are representing-more than 90% cases of onychomycosis, which is also inaccordance with our results [24]. Toenails are 25 times more infected than fingernails. The most frequently affected nails are the first and second toenails because they suffer the mosttrauma and preasure from the shoes [9]. The results of our study match earlier results where the feet thumb nails were affected in even 56.7%.

Cultivation is still considered to be the gold standard, although numerous studies have shown the sensitivity that ranges from 25-75% [5,7]. According to the recommendations, the sample should be seeded on SDA and at least one more medium. In thisstudy we cultivated the samples on the SDA, D-SDA and DTM. SDA is a standard mycological medium which is used for isolation of the causes of onychomycosis and which sensitivity ranges from 50-70% [5]. D-SDA has less glucose compared to standard SDA, which allows better sporulation of the fungi making the identification much easier. DTM is a medium that is used for isolation of dermatophytes. Their growth becomes visible when the yellow color of the medium changes to red, due to the presence

Figure 2. PCR results



of alkaline metabolites as a result of dermatophyte growth [25].This study showed that dermatophyte isolates have the biggest sensitivity to the DTM (33.4%). In most studies the common cause of onychomycosis is *T. rubrum*. It is stated that *T. rubrum* and *T. mentagrophytes* causes nearly 90% ofonychomycosis followed by *E. floccosum* [9,26]. Our research shows the highest incidence of *T. rubrum*(60%).

PCR is a method that is increasingly used in the diagnosis of the onychomycosis. Recommendations are for PCR to be the metod of choice particulary for diagnosing feet onychomycosis. For onychomycosis ofhands the conventional methods are recommened also. The sensitivity of PCR varies in different studies, ranging from 40-95% [21,27, 28]. In this study, sensitivity was very high 86.7%, inwhich 73.3% identified *T. rubrum*. Out of 30 patients included in this study with clinically suspected onychomycosis, using the methods of cultivation and PCR, onychomycosis was confirmed in 28 patients (93.3%). Cultivatinggave negative results in 50% of cases, while the PCR was positive in 86.6%. In addition to the PCR results being significantly faster than cultivation (expected in 2-3 days), where the results are issued after 3 weeks and longer, the main advantage of PCR methodis higher sensitivity compared to the other methods used in diagnosing onychomycosis.

CONCLUSIONS

The most common clinical type is DLSO. It was diagnosed in 86.6%. This study have shown that DLSO and TDO are representing more than 90% cases of onychomycosis. Our research shows the highest incidence of *T. rubrum* (60%). This study showed that dermatophyte isolates have the biggest sensitivity to the DTM (33.4%). PCR is a method that is increasingly used in the diagnosis of the onychomycosis. Its sensitivity was very high 86.7%, in which 73.3% identified *T. rubrum*. In continuation of this study will be analyzed the choice and effectiveness of therapy.

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Onihomikoze – uzorkovanje i dijagnostika su efikasni deo bolničke farmakologije

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KRATAK SADRŽAJ

Uvod Onihomikoza predstavlja gljivičnu infekciju jednog ili više noktiju. Izazivači onihomikoza su dermatofiti, kvasnice i nedermatofitne plesni. Najčešći izazivači su gljive iz grupe dermatofita, a među njima je najčešći izazivač *Trichophyton rubrum* (*T. rubrum*).
Cilj Uzorkovanjem identifikacija klinički najčesceg tipa onihomikoze, lokalizacija i zahvaćenost onihomikozom na izmenjenim nokatnim pločama, i monitoring efikasnosti metoda/testova u dijagnostici nokatne onihomikoze.
Materijal i metode Ovaj rad je deo IV faze akademske studije. U studiju je uključeno 30 pacijenata sa onihomikozom. Svaki uzorak je zasejavan na *Sabouraud Dextrose Agar* (SDA) i *Diluted SDA* (D-SDA) na 28°C i 37°C, kao i *Dermatophyte Test Medium* (DTM) na 28°C. Inkubacija je trajala tri nedelje nakon čega su pravljeni nativni preparati sa kulture i posmatrani svetlosnim mikroskopom u cilju uočavanja i identifikacije gljivičnih elemenata. Za identifikaciju dermatofita korišten je PCR koji detektuje specifični *T. rubrum* i pan-dermatofitni mulipleks PCR produkt. Svi pacijenti potpisali su Informativnu saglasnost.
Rezultati Najčešći klinički oblik je bila distalna lateralna subungvalna onihomikoza sa najvećom zahvaćenošću prvih noktiju šake i stopala, dok je veličina zahvaćenosti nokatne ploče iznosila 1/3 - 2/3 kod većine pacijenata. Kultivisanje je dalo pozitivan rezultat u 50% slučajeva, a najčešći izolat je bio *T. rubrum*. Za negativne kulture (50%) rađen je PCR koji je pokazao visoku osetljivost, a *T. rubrum* je takođe najčešće detektovan.
Zaključak Primenom metoda kultivisanja i PCR, onihomikoza je potvrđena kod 28 (93,3%) ispitanika. Kultivisanje je dalo negativan rezultat u 50% slučajeva, dok je PCR bio pozitivan u 86,6%. Naše istraživanje pokazalo je najveću učestalost *T. rubrum* (60%). U nastavku ove studije analiziraćemo izbor i efikasnost terapije.

Ključne reči: onihomikoza, dermatofiti, kultivisanje, PCR, *T. rubrum*

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