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Application of PCR methodology in modern diagnostics of fungal infections caused by dermatophytes

Dissertation summary

for the degree of Doctor of Education and Science

Doctoral Program:
"Dermatology and Venereology"
Professional field 7.1. Medicine
Area of higher education 7. Health and Sport

Scientific supervisor: Assoc. Prof. Liliya Zisova, PhD

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Content:

Abbreviations

Latin abbreviations:

PCR - Polymerase chain reaction

Real-time PCR - Real-time polymerase chain reaction

T. - Trichophyton

M. - Microsporum

E. - Epidermophyton

C. - Candida

SDA - Sabouraud Dextrose Agar

PAS - Periodic Acid-Schiff

Sol. A - Solution A

Sol. B - Solution B

DB - Dilution buffer

IC - Internal control

PC1 - Positive control 1

PC2 - Positive control 2

NC - Negative control

MCA - Melting curve analysis

ITS - Internal transcribed spacer

HI - Hyphal Index

Spp - Species

DG - DermaGenius

Cyrillic abbreviations:

DNA - deoxyribonucleic acid

KOH - potassium hydroxide

OM - onychomycosis

DLOM - distal-lateral onychomycosis

PCOM - superficial white onychomycosis

POM - proximal onychomycosis

TDOM - total dystrophic onychomycosis

NPD - non-dermatophytic moulds

DM - diabetes mellitus

I. Introduction

Dermatophytes are a group of keratinophilic fungi with the ability to infect human skin, nails and hair. Three anamorphic genera are known - *Trichophyton*, *Microsporum* and *Epidermophyton*. The infections they cause are mainly in the stratum corneum of the epidermis and skin appendages, but their occurrence and development are far from being only a cosmetic problem. Their treatment is tied to prolonged use of topical and/or systemic antifungals, which is costly and has side effects. In some at-risk groups, fungal infections can be the start of serious complications if therapy is not started in time. Such are, for example, immunocompromised patients, patients with diabetes mellitus, patients with malignancies.

Therefore, the diagnosis of dermatophytosis is of utmost importance, both to initiate appropriate therapy as quickly as possible and to prevent future complications.

In the present scientific work the conventional methods for the diagnosis of dermatomycoses are compared with the innovative for mycology in Bulgaria technique of real-time PCR. Conventional methods include direct microscopic examination with potassium hydroxide (KOH) followed by culture examination on selective medium. Although they have been well established in clinical practice for decades, they still have disadvantages - lack of specificity of the direct examination, long growth time (3-4 weeks) in culture examination, great experience is needed to identify fungal morphology, etc. These disadvantages can be compensated by the use of newer tests with greater sensitivity, specificity and taking much less time to obtain a result - molecular biological techniques. Such a method in our study is real-time multiplex PCR.

In this thesis, the methods listed are used to examine and compare material from patients with suspected mycosis of the scalp (tinea capitis), mycosis of the beard (tinea barbae), mycosis of the skin (tinea corporis, tinea pedis, tinea manuum, tinea cruris) and onychomycosis (tinea unguium or onychomycosis).

We also compared the etiologic agents and incidence of onychomycosis in patients with and without diabetes mellitus using direct microscopic examination, culture, histology, and real-time PCR. Thus, the advantages of using real-time PCR in clinical practice are demonstrated.

The progress of mycological research and statistical data obtained from molecular biological techniques encourage the development of new algorithms in the diagnosis of fungal infections.

Evaluation of real-time PCR assays for the detection of dermatophytes is an important component of the ultimate goal of greater use of this method for the diagnosis of fungal infections. Promising studies in this area have been published in recent years.

In 2017, Sharma et al (2017), published a pilot study comparing the use of direct microscopy and culture with the PCR technique. Their study involved 40 patients (25 with suspected fungal skin and nail infection and 15 controls). Their reported results for culture and PCR were as follows: sensitivity 24% and 48%, specificity 100% and 93.3%, respectively.

Marie-Pierre Hayette et al (2019) published in the journal Medical Mycology their work comparing real-time PCR methodology (DermaGenius® Nail multiplex real-time PCR) with convectional methods (culture and histological examination) for the investigation of fungal nail infections. Tests were performed on 138 nail materials using the DermaGenius® Nail real-time PCR kit, which has the ability to detect *T. rubrum*, *T. interdigitale* and *C. albicans* directly from nail materials. Analysis of their results showed the following sensitivity and specificity values - histology 81.1% and 100%, culture 71.6% and 100%, real-time PCR 80% and 74.4%. It should be emphasized that only positive histology and/or positive culture were used as the standard for positive testing in this study, as the aim was to evaluate the real-time PCR method. The best results were reported from a combination of real-time PCR and histology with 94.7% sensitivity.

Sherin M. Emam et al (2016) published a paper in the Alexandria Journal of Medicine where they did a study on the sensitivity and specificity of direct microscopy, convectional PCR, and real-time PCR compared against culture, again on nails. They conducted the studies on 80 patients between the ages of 22 and 77. Culture testing was positive for isolated dermatophytes in 30 (37.5%) patients - *T. mentagrophytes* (14 cases), *T. rubrum* (8 cases), *T. verrucosum (*4 cases), *T. schoenleinii* (4 cases), and moulds were isolated in 8 cases (10%) - *Aspergillus niger*. The results obtained against culture were as follows - for direct microscopy - 80% sensitivity, 16% specificity, conventional PCR methodology - 73.3% and 40%, and real-time PCR - 93.3% and 40%, respectively.

In their study, Wisselink et al (2011) in the Journal of Microbiological Methods compared the results of culture and real-time PCR in nail, hair and skin materials. The studies were conducted on 1437 patients. The results of this voluminous study showed a significantly increased detection rate of dermatophytes by real-time PCR (48.5%) compared to culture (26.9%). The calculated sensitivity of real-time PCR was 97%.

The Bulgarian experience with PCR methodology is the study of V. Stavrakieva (2005), which compares direct microscopic and culture studies with several different conventional PCR techniques (including ready-made kits for performing the methodology). The study was performed on 191 patients with suspected onychomycosis and 25 healthy individuals. The values of direct examination, culture and PCR for sensitivity, specificity were as follows: 96.8% and 73.3%, 27% and 100%, 95.6% and 100%, respectively.

Gong et al (2016) published in the journal Mycopathologia their results comparing 165 nail materials by direct microscopy, culture, conventional PCR and real-time PCR. The results obtained for sensitivity and specificity of each test were: direct microscopy 85% and 99.7%, culture 57.1% and 99.8%, conventional PCR 83.7% and 99.6%, and real-time PCR 92.4% and 97.3%, respectively. Of these 165 patients, 70 had suspected mycosis. In these patients, the positive results for the four tests were 68 (97.1%) for direct testing, 49 (67.1%) for plating, 54 (77.1%) for conventional PCR, and 61 (87.1%) for real-time PCR.

Bergman et al (2013) published their study in the journal Mycology, in which they developed and validated a real-time PCR method for the detection of all dermatophyte species (pan-dermatophyte PCR), including identification of the two most common causative agents of dermatophyte infections, *T. rubrum* and *T. interdigitale* by duplex PCR and compared it with the results of a conventional culture of 202 nail, skin and hair materials. The results were as follows: 103 (51%) of the samples were positive for fungal DNA as reported by PCR, while only 79 (39%) of the samples were positive on culture. The sensitivity and specificity of the culture were 77% and 97%, and of the PCR were 92% and 99%, respectively.

In their 2021 study, Cuchí-Burgos et al. report their results comparing 152 materials with direct microscopic examination, culture examination, and real-time PCR performed with the same kit we are using in the current study, the DermaGenius® 2.0 Complete kit. Their results reported a sensitivity of 93.3% for real-time PCR, while only 64.4% for culture.

In another study, again conducted with the DermaGenius® 2.0 Complete kit, Ndiaye et al. (2021) compared the results between real-time PCR and conventional methods (direct and culture) in the examination of 129 hair materials from patients with suspected tinea capitis. The sensitivity and specificity of real-time PCR were 89.3% and 75.3%, making them significantly more sensitive than culture studies ($p<0.001$).

The diagnosis of superficial fungal infections is the subject of intensive research in many countries around the world. The diagnostic methods used are varied, including both conventional and more innovative techniques, but the common goal is to find the best approach to diagnose mycoses. This mainly involves accurate identification of the causative agents as well as obtaining results as quickly as possible. In some patient groups, such as immunosuppressed or diabetic patients, initiation of timely therapy is of great importance to avoid possible complications associated with mycotic infections.

The use of real-time PCR in the diagnosis of dermatophyte infections is a relatively new approach in mycology and as such has been tested by time and experience of its use. A growing number of studies have presented results comparing conventional methods with real-time PCR or conventional PCR with real-time PCR, respectively. The results are promising, but the method has not yet established itself as the new gold standard in the diagnosis of superficial fungal infections caused by dermatophytes. To date, no in-depth study has been conducted in Bulgaria to evaluate real-time PCR methodology against conventional diagnostic methods - direct microscopic, culture and histological examinations in dermatophyte infections of hair, skin and nails.

Also, at present, data from studies on the incidence and etiologic agents in patients with onychomycosis with diabetes mellitus (DM) versus patients with onychomycosis without DM are limited. And those that evaluate these indicators using simultaneous real-time PCR, histology, culture and direct microscopy and comparing them are lacking.

All this motivated us to carry out these studies in the present research work. To this end, we used one of the few recognized and already widely used real-time PCR assays, the DermaGenius® Complete real-time PCR kit, with the ability to detect 11 dermatophytes and *Candida albicans*.

II. Aim and objectives

1. Aim

The aim of this dissertation is to demonstrate the utility and importance of real-time PCR for the diagnosis of dermatophyte infections.

2. Objectives

In order to achieve the set objective, the following tasks were formulated:

2.1 To compare real-time PCR results with direct KOH examination, culture and histology in all patients participating in the study.

2.2 To compare the results of real-time PCR with direct and culture examinations in patients with suspected mycotic infections of the scalp and beard.

2.3 To compare the results of real-time PCR with direct and culture studies in patients with suspected mycotic skin infections.

2.4 To compare the results of real-time PCR with direct KOH, culture and histological examinations in patients with suspected onychomycosis.

2.5 To compare the etiological agents and incidence of onychomycosis in patients with and without diabetes mellitus by direct microscopic examination, culture, histology and real-time PCR.

The null hypothesis (H0) states that there is no difference in sensitivity, specificity and efficacy of conventional methods compared to real-time PCR.

III. Materials and Methods

1. Clinical materials

The current prospective and comparative study was conducted at the Department of Dermatology and Venereology, Medical University - Plovdiv, Bulgaria for the period from March 2019 to January 2022. A total of 196 patients (103 females, 93 males) aged 2 to 90 years with clinical symptoms suggestive of fungal infection affecting the scalp, beard, skin or nails were included.

Unit of observation: Patients with suspected onychomycosis, skin mycosis, scalp mycosis or beard mycosis.

Of the 196 patients, 144 had suspected onychomycosis (tinea unguium), 29 had clinical symptoms suggestive of fungal infection affecting the skin (tinea corporis, tinea cruris, tinea pedis, tinea manuum), 21 had suspected scalp mycosis (tinea capitis), and 2 had suspected beard mycosis (tinea barbae) (Figure 1).

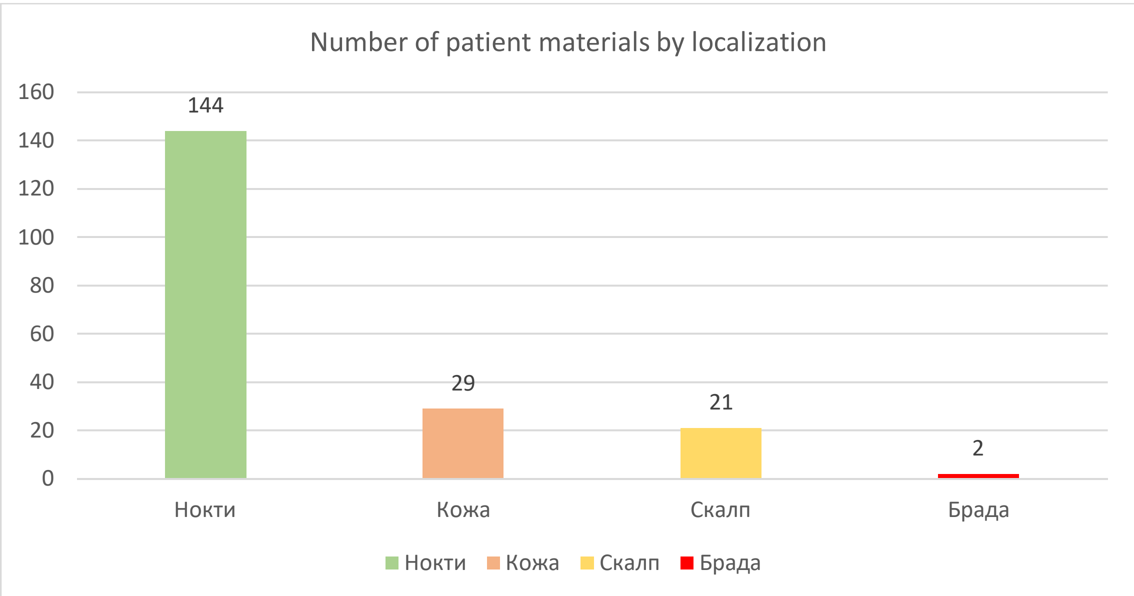

Figure 1 - Distribution of materials from patients according to their localization.

For **tasks 1, 2, 3 and 4**, materials were collected from patients' hair, skin and nails, respectively. Patients had clinical signs suggestive of mycosis as follows:

1.1 For scalp and beard mycosis (n=23) - itchy patches on scalp or beard with hair loss, with or without the presence of erythema and desquamation. In tinea barbae possible presence of papules and pustules (Figure 2, 3 and 4).

Figure 2 - Tinea capitis. *Figure 3 - Tinea capitis.* *Figure 4 - Tinea barbae.*

1.2 For mycosis fungoides (n=29) - for tinea corporis and tinea cruris - erythematous rash with an annular or round shape, with a periphery that is slightly raised, with the presence of an active edge, paler centre, with the presence or absence of itching, small vesicles or pustules; for tinea pedis - small, dense vesicles filled with clear fluid; reddened, scaly patches, most commonly on the arch of the foot or laterally; presence of erythema, macerations and/or erosions in the interdigital area and skin under the toes; for tinea manuum, presence of desquamation, hyperkeratosis and sometimes presence of vesicles, in older forms, the hands may appear as 'callused' (Figure 5, 6, 7 and 8).

Figure 5 - Tinea corporis

Figure 6 - Tinea corporis

Figure 7 - Tinea pedis

Figure 8 - Tinea pedis

1.3 For onychomycosis (n=144) - nail plate thickening, discoloration, subungual hyperkeratosis, onycholysis, dystrophic nails, longitudinal and transverse lines (Figure 9, 10, 11, 12, 13 and 14).

Figure 9 - DLOM

Figure 10 - DLOM

Figure 11 - DLOM

Figure 12 - TDOM

Figure 13 - TLOM

Figure 14 - TDOM

Patients with suspected onychomycosis were classified according to the following most common clinical presentations of OM: distal-lateral subungual onychomycosis (DLOM), superficial white onychomycosis (SWOM), proximal onychomycosis (POM), and total dystrophic onychomycosis (TDOM). The number of nails that were affected was designated as follows: mild grade (≤4 nails affected), moderate grade (5-8 nails affected), and severe grade (≥9 nails affected).

Patients with suspected onychomycosis had no clinically or histologically diagnosed diseases known to damage the nail plate (e.g., psoriasis, lichen planus, etc.).

For **task 5,** patients with suspected onychomycosis with and without diabetes were studied.

1.4 For onychomycosis with and without diabetes - divided into 2 groups as follows:

1.4.1 Group I - diabetics with suspected onychomycosis (n=51). Group I included patients with type 1 and type 2 diabetes, over 18 years of age. The diabetic patients were referred from the Clinic of Endocrinology, University Hospital "Sts. M. Orbettsova and dr. Becheva within the intra-university interdepartmental research project № DPDP.07/20 on "Application of classical and real-time PCR methods in the study of onychomycosis in patients with diabetes".

1.4.2 Group II - patients without diabetes and suspected onychomycosis (n=93). Group II included patients from the Clinic of Skin and Venereal Diseases, University Hospital "St. The group consisted of inpatients and outpatients.

1.2 Collection of clinical material and preparation for investigations

Before taking material from the affected areas, they were cleaned with 70% ethyl alcohol to remove possible contaminants from the surface.

The maximum amount of material was taken as it was divided and used to perform 3 or 4 studies, respectively. All samples collected from patients (n=196) were divided into 3 parts to perform direct microscopic examination, culture examination, and real-time PCR. For patients with suspected onychomycosis, where sufficient and appropriate material could be collected, it was divided into 4 parts (n=86). Histological examination with PAS staining was also performed on the fourth portion of the sample.

The collection of materials was carried out in the mycological laboratory of the Clinic of Dermatology and Venereology, University Hospital "St. George" - Plovdiv with the assistance of Sr. lab. Antonina Ivanova.

2. Methods

2.2 Clinical methods

2.2.1 History and clinical examination

A history was taken from each patient regarding current complaints, onset, development and duration of changes, past history of fungal infection, trauma, contact with domestic and/or street animals, family history of fungal infection and previous treatment. For patients with suspected onychomycosis and the presence of diabetes mellitus - possible complications (such as peripheral neuropathy, retinopathy, nephropathy, etc.).

2.3 Laboratory methods

2.3.1 Direct microscopic examination

The assay was performed on all samples (n=196) by placing the materials on a chemically clean slide, then dropping 1-2 drops of 10% potassium hydroxide (KOH), mixing it with the sample, and incubating the as-prepared preparations for 30 min-1 h to lyse the cellular elements from the stratum corneum of the epidermis. The sample was then examined under microscope at lower (x10) and higher magnification (x40) for the presence of fungal elements - hyphae, spores, pseudo hyphae.

2.3.2 Cultural study

Cultures were reseeded in all patients (n=196), regardless of the results obtained by direct microscopic examination. Samples were placed on 2 culture media, Sabouraud Dextrose Agar (SDA) with Ceftriaxone and Sabouraud Dextrose Agar (SDA) with Chloramphenicol and Cycloheximide. They were then incubated at a temperature between 28ºC - 36ºC and checked regularly for 4 weeks for the presence of growth. If no growth was reported after 4 weeks, the study was assumed negative. Identification of the growth obtained was done macroscopically and microscopically.

2.3.3 Macroscopic identification of the culture was made by observing the morphological characteristics of the colony - size, shape, consistency, edges, colour of the culture (surface and bottom), growth time, type of growth - whether mossy, cottony, smooth etc., and the presence or absence of pigments (Figure 15 and 16).

Fig. 15 - Growth of T. interdigitale.

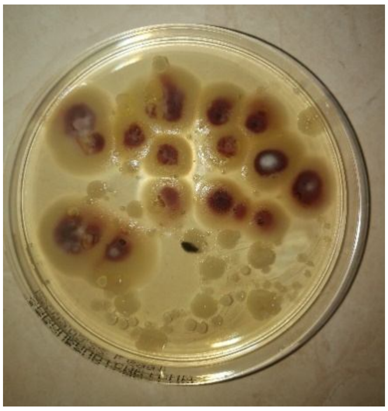

Fig. 16 - Growth of T. rubrum.

2.3.4 Microscopic identification of the cultures was performed by examining the microscopic characteristics of dermatophytes, yeasts and moulds on the native preparation and determining species affiliation based on the morphological structure of spores, macro- and microconidia.

2.3.5 Species identification of *Candida* yeasts

In cases where the culture result was *Candida spp*, an additional Chromium agar test (HiCrome™ Candida Differential Agar, Ridacom) was performed to differentiate the most common *Candida* species, *C. albicans, C. glabrata, C. krusei* and *C. tropicalis*. The remaining *Candida* species were indicated as *C. non-albicans*.

The identification of the direct microscopic and culture examinations were performed in the mycological laboratory of the Clinic of Dermatology and Venereology, University Hospital "St. Prof. Dr. Lilia Zisova, PhD and Sr. Lab. Antonina Ivanova.

2.3.6 Histological examination

Histology was only performed on a proportion of the nail material where possible (n=86). Nail clippings were sent for histopathological examination in containers of 10% buffered formalin. Periodic Acid-Schiff (PAS) staining was used. Each material was then observed under a microscope at lower (x10) and higher (x40) magnification for the presence of fungal elements in purplish red color - hyphae, spores, pseudo hyphae (Figure 17 and 18).

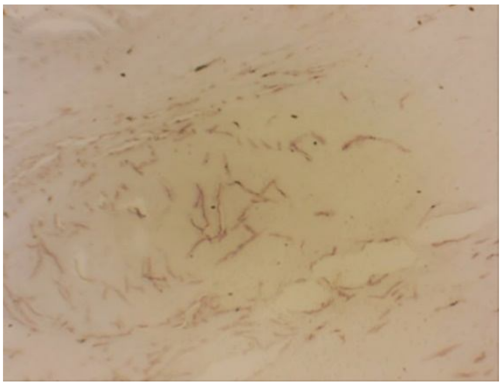

Fig. 17 - Hyphae of hist. preparation (x10).

Fig. 18 - Hyphae of hist. preparation (x40).

The histology examinations were performed in the histological laboratory of the Department of General and Clinical Pathology, Medical University of Plovdiv with the assistance of Prof. Dr. Veselin Belovezhdov, MD and Dr. Veselin Chonov within the inter-university interdepartmental research project № DPDP.07/20 on "Application of classical and real-time PCR methods in the study of onychomycoses in patients with diabetes".

2.3.7 Real-time PCR

Real-time polymerase chain reaction was performed on all samples (n=196) included in the study. It was performed with the DermaGenius® 2.0 Complete multiplex real-time PCR kit (PN-401, PathoNostics, The Netherlands), which has the ability to detect 12 pathogens - *Trichophyton rubrum, (T. rubrum), Trichophyton interdigitale (T. interdigitale), Trichophyton tonsurans (T. tonsurans), Trichophyton mentagrophytes (T. mentagrophytes), Trichophyton soudanense (T. soudanense), Trichophyton violaceum (T. violaceum), Trichophyton benhamiae (T. benhamiae), Trichophyton verrucosum (T. verrucosum), Microsporum canis (M. canis), Microsporum audouinii (M. audouinii), Epidermophyton floccosum (E. floccosum)* and *Candida albicans (C. albicans)* in nail, skin and hair materials. The kit contains the following reagents - PCR mix 1, PCR mix 2, Taq polymerase, dilution buffer (DB), internal control (IC), PCR positive control 1 (PC1), PCR positive control 2 (PC2). The assay was performed according to the manufacturer's instructions.

2.3.7.1 Preparation

Materials taken from infected parts of nails, skin or hairs were placed in sterile 1.5 ml tubes (Eppendorf tubes) with a lid in an amount covering only the bottom of the tube.

2.3.7.2 DNA extraction

The PathoNostics extraction kit (PN-502) containing solution A (solution A) and solution B (solution B) was used for DNA extraction.

The resulting supernatant is transferred to a new sterile 1.5 ml tube, avoiding the transfer of solid particles left by nails, hairs or squams. The temperature at which the resulting sample is kept is 4ºC when the following steps are performed on the same day, or at -20ºC for longer storage.

2.3.7.3 Preparation of real-time PCR mix

PCR mix 1 and 2 are prepared in a volume of 20 µl each containing for 1 sample - 10 µl PCR mix 1 or PCR mix 2 respectively, 1,5 µl Taq polymerase and 8,5 µl dilution buffer, then mixed gently. Run in 0.2 ml PCR tubes and completely on ice or cooling blocks.

2.3.7.4 Addition of isolated DNA to PCR mixes

Add 5 µl of DNA from each sample to PCR mix 1 and PCR mix 2, making a total of 25 µl for each sample. For the negative control (NC) add 5 µl of dilution buffer and for the positive control (PC) add 5 µl of PC1 and PC2 to PCR mix 1 and PCR mix 2, respectively.

2.3.7.5 Starting the real-time PCR program

Once each PCR tube is placed on the plate of the real-time PCR instrument, the PCR cycling program assigned to run with that kit is selected and the process is started.

The total running time from the start of the process to the analysis of the results is 3 h and 50 minutes.

DTprime Real-time Detection Thermal Cycler was used for the amplification and analysis of the melting curve (Figure 19 and 20).

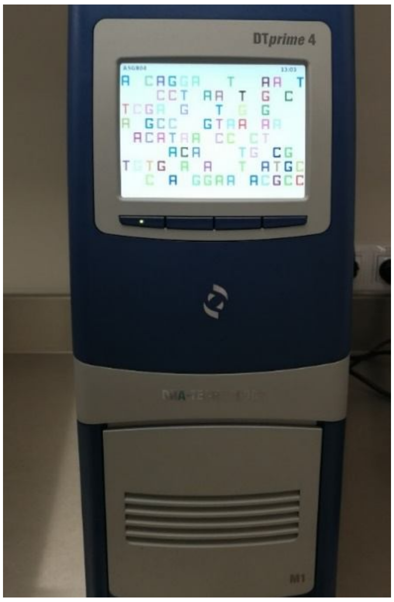

Fig. 19 - DTprime 4.

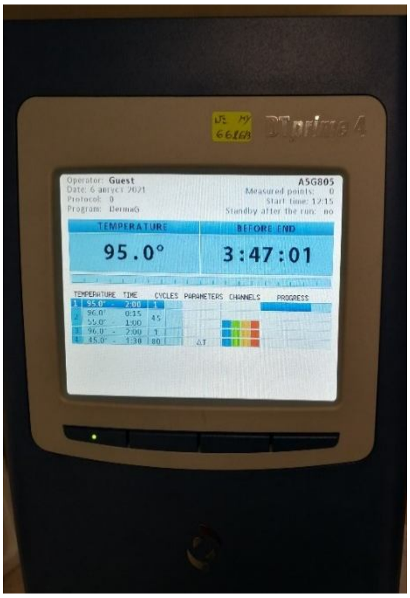

Fig. 20 - DTprime 4 (just launched).

2.3.7.6 Data analysis and interpretation of results

Data analysis is started by checking the Ct values determined using the threshold function and checking the amplification curves (Figure 21). Interpretation of the results was performed using the tables provided by the manufacturer, which contained information on which pathogens each channel corresponded to, for PCR mix 1 and PCR mix 2, respectively.

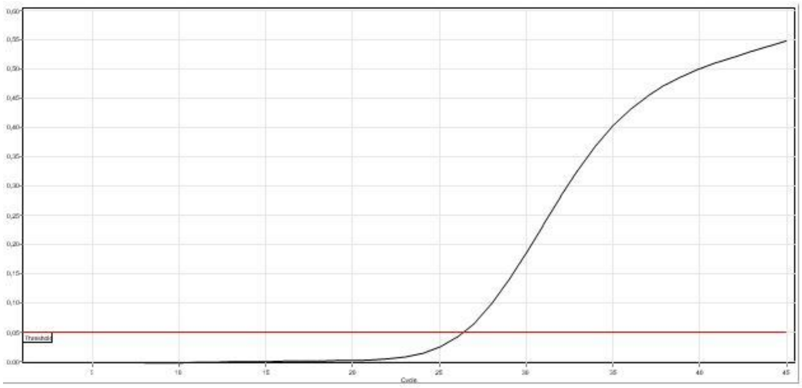

Figure 21 - Example of an amplification curve.

For the melting curve analysis, tables were also provided to accurately differentiate the eleven dermatophytes and *Candida albicans* according to their melting temperature (Figs. 22, 23 and 24).

DermaGenius® 2.0 Complete T_m-values (°C): PCR mix 1					
Channel	**Target**	**RGQ**	**LC480**	**CFX**	**MIC**
Green 465-510 FAM	*C.albicans*	63.0-66.0	62.5-65.5	61.5-64.5	63.0-66.0
Yellow 533-580 HEX	*T. interdigitale*	69.5-72.5	70.0-73.0	68.0-71.0	70.5-73.5
	T. tonsurans	74.5-77.5	75.0-78.0	73.0-76.0	75.5-78.5
	T. mentagrophytes	77.0-79.5	77.5-80.5	75.5-78.0	77.5-80.0
Orange 533-610 ROX	*T. rubrum / T. soudanense*	56.5-59.5	57.5-60.5	55.0-58.0	57.5-60.5
	T. violaceum	66.5-69.5	68.0-71.0	65.5-68.5	67.5-70.5
Red 618-660 Cy5	Internal Control	No melting peak	No melting peak	No melting peak	No melting peak

Figure 22 - Melting temperature values: PCR mix 1 (the column below the CFX channel is relevant for reporting the exact causative agent).

DermaGenius® 2.0 Complete T_m-values (°C): PCR mix 2					
Channel	**Target**	**RGQ**	**LC480**	**CFX**	**MIC**
Green 465-510 FAM	*T. benhamiae*	69.0-72.0	69.0-72.0	66.0-69.0	69.0-72.0
	T. verrucosum	74.5-77.5	74.0-77.0	71.0-74.0	74.0-77.0
Yellow 533-580 HEX	*M. canis*	67.0-70.0	69.0-72.0	65.5-68.5	69.0-72.0
	M. audouinii	58.5-61.5	59.0-62.0	56.5-59.5	60.5-63.5
Orange 533-610 ROX	*E. floccosum*	68.5-71.5	68.5-71.5	67.0-70.0	69.5-72.5
Red 618-660 Cy5	Internal Control	No melting peak	No melting peak	No melting peak	No melting peak

Figure 23 - Melting temperature values: PCR mix 2 (the column below the CFX channel is relevant for reporting the exact causative agent).

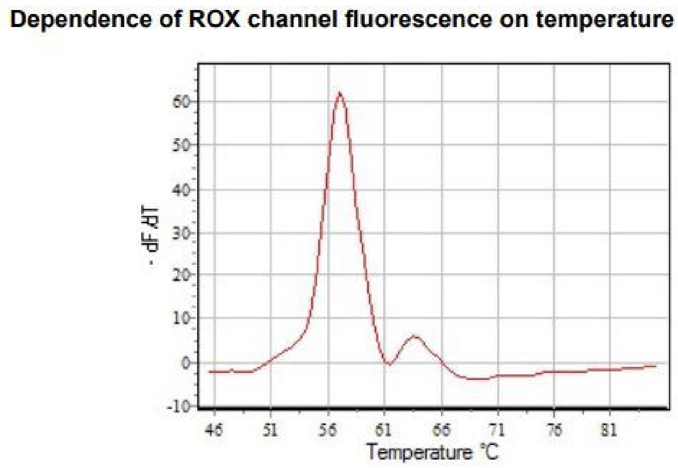

Figure 24 - Melting curve for the detection of T. rubrum in the ROX (orange) channel.

Negative and positive controls must be accounted for in advance to ensure the reliability of the results obtained.

The development of the real-time PCR samples was carried out at the Centre for Applied Science and Innovation (CASI), Sofia with the assistance of Ridacom Ltd., Department of Microbiology - PCR Laboratory, Medical University of Plovdiv and Department of Medical Biology - Technology Center, Medical University of Plovdiv with the assistance of Valentin Dichev, PhD.

2.4 Statistical methods

Statistical data processing was performed by:

2.4.1 Analysis of frequency distributions

This method was used to describe quantitative variables with normal and near-normal distributions. The Kolmogorov-Smirnov test was used to test the variation series for normality of distribution.

2.4.2 Alternative analysis

The method was used to estimate frequency distributions in qualitative and grouped data.

2.4.3 Non-parametric analysis

The $\chi 2$ method (Pearson's criterion) was used to analyze the presence of association, relationships and dependencies between the studied traits. The $\chi 2$ criterion was used to test hypotheses of no significant effect of a factor in multiple tables, and Fisher's exact test was used in quadruple tables. The McNemar test was used to calculate and compare the sensitivity, specificity, and efficacy of each diagnostic test.

2.4.4 Graphical analysis

Proprietary tables, charts and graphs, Microsoft Excel and Microsoft Word were used for the graphical presentation of the data.

Statistical software SPSS version 19 for Windows was used. Values at the significance level of $p<0.05$, with a probability of significance greater than 0.95 (95% confidence interval), were considered statistically significant.

Statistical processing was performed by Assoc. Prof. Dr. Tsonka Miteva-Katrandjieva, PhD, Department of Social Medicine and Public Health, Medical University Plovdiv.

3. Determination of a standard for a positive sample

In this thesis, true fungal infection was defined as: clinical signs of mycosis, combined with at least one of the following tests: positive culture (for dermatophyte or *C. albicans*), positive histology, or positive real-time PCR.

4. Ethical aspects

The study was approved by the Ethics Committee of the Medical University - Plovdiv at the meeting № 7/01.10.2020. Participants signed an informed consent to provide the necessary clinical samples and to perform the relevant investigations before inclusion in the study. The requirements of the Declaration of Helsinki on Ethics in Science were met. The studies were performed in accordance with the requirements of good clinical practice and Bulgarian laws and regulations for clinical and scientific research involving human subjects.

5. Monitoring bodies

The research was carried out by the principal investigator (the PhD student) - Dr. Gergana Vazheva, under the direct supervision of the PhD student's supervisor - Assoc. Prof. Dr. Lilia Zisova, MD, with the approval of the Head of the Department of Dermatology and Venereology, Medical University of Plovdiv - Prof. Dr. Hristo Dobrev, MD and the cooperation of:

-senior laboratory technician at the Mycological Laboratory of the Clinic of Skin and Venereal Diseases, University Hospital "St. George"- Plovdiv;

- Head of Clinic and medical staff of the Clinic of Endocrinology and Metabolic Diseases, University Hospital "St. George" - Plovdiv;

- Heads of Department and medical staff of the Department of General and Clinical Pathology, Department of Microbiology and Department of Medical Biology, Medical University of Plovdiv;

- Staff of the Centre for Applied Science and Innovation (CASI), Sofia Sofia.

Part of the research and the results obtained were carried out within the inter-university interdepartmental research project № DPDP.07/20 on "Application of classical and real-time PCR methods in the study of onychomycosis in patients with diabetes", with project leader Assoc. Prof. Dr. Lilia Zisova, PhD and main investigator Dr. Gergana Vazheva, funded by the Medical University of Plovdiv.

IV. Results and Discussion

1. Results of a prospective comparative study evaluating the results of direct microscopic, culture, histological and real-time PCR examinations in patients with suspected dermatophyte infection of the scalp, beard, skin and nails (n=196)

1.1 General characteristics of patients

The study included 196 patients aged 2 to 90 years, mean age 49.13 ± 1.56, standard deviation (SD) 21.85. The median age was 54.50 and the mode age was 55 years. Of all 196 patients, 93 (47.4%) were male and 103 (52.6%) were female (Figure 25).

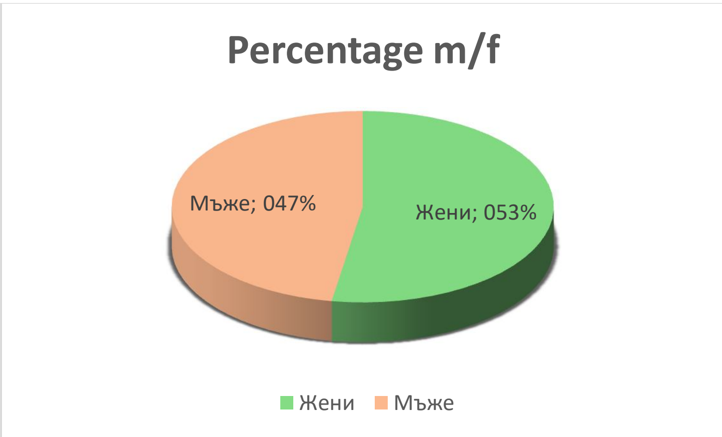

Figure 25 - Percentage of males and females of all patients.

1.2 Distribution of samples according to the materials collected from patients and their localization on the patients' body

The patient materials used for the study consisted of squamous and capilicium hairs (n=21), squamous and beard hairs (n=2), squamous skin (n=29), and nail clippings (n=144), distributed in percentages of 10.7%, 1.0%, 14.8%, and 73.5%, respectively (Table 1).

Table 1 - Distribution of materials.

Material	Number (n)	Percentage %
Squams and hairs of capilicium	21	10,7
Scabs and beard hairs	2	1,0
Leather scrubs	29	14,8
Nail clippings	144	73,5
Total	196	100,0%

According to the body localization from which the materials were collected, the largest number of materials were from lower limbs (including toenails and skin from lower limbs), 147 (75%), followed by materials from capitulum, 21 (10.7%), hands (including nails and skin), 12 (6.1%), truncus materials, 11 (5.6%), inguinal folds, 3 (1.5%), and beard, 2 (1.0%).

1.3 Distribution of patients according to the age of pathological changes (skin, nails, scalp, beard)

The largest number of patients had their complaints 1 to 5 years before the examination - 61 (31.1%). Patients with complaints up to 3 months before the examination were 44 (22.4%), those with symptoms up to 6 months were 11 (5.6%), up to 1 year were 24 (12.2%), between 6 and 10 years were 34 (17.3%), and those over 10 years, respectively, were 22 in number (11.2%) of all cases (Table 2).

Table 2 - Duration of pathological changes on scalp, skin and nails.

Duration of pathological changes	Number of patients	Percentage (%)
Up to 3 months	44	22,4
Up to 6 months	11	5,6
Up to 1 year	24	12,2
1 - 5 years	61	31,1
6 - 10 years	34	17,3
Over 10 years	22	11,2
Total	196	100,0

1.4 Presence of diabetes

Patients with diabetes mellitus were 51 (26.0%) and all had suspected onychomycosis. They were included in the ongoing DPD07/20 project study, comparing the incidence and etiological causes of patients with suspected onychomycosis with and without diabetes mellitus. The remaining 145 patients (74.0%) did not have diabetic disease.

1.5 Standard for a positive sample

Clinical signs of mycosis combined with at least one of the following tests were considered for the deficiency of a positive sample for true fungal infection: positive culture (for dermatophyte or C. albicans), positive histology, or positive real-time PCR. Against the standard thus set, the total number of positive samples in the present study was 124 (63.27%). The number of negative tests was 72 (36.73%).

A total of 6 (3.06%) samples were invalid on real-time PCR, 2 of which were positive on culture or histology and were included with the true positive samples, and 4 (2.04%) of the samples were negative on culture and histology and were therefore included with the negative samples.

To accurately calculate the sensitivity, specificity and efficacy of the method, these 6 invalid samples were not included in the calculation of these parameters for the real-time PCR method.

1.6 Results of the conducted studies

In order to compare the results of real-time PCR with direct, culture and histological examinations in mycotic infections of all patients in the study, the sensitivity, specificity and efficacy of each of the tests performed were investigated.

1.6.1 Direct microscopic examination results for all patients (n=196)

Positive results were 87 in number (44.39%) of all samples and negative results were 109 (55.61%) respectively (Table 3).

Table 3 - Results for all patients from direct microscopic examination.

Result of direct examination	**Number (n)**	**Percentage (%)**
Positive	87	44,39
Negative	109	55,61
Total	196	100,0

The sensitivity for direct microscopy was 63.71%, specificity 88.89%, and efficacy 72.96%, according to the established standard ($p<0.001$) (Table 4).

Table 4 - Sensitivity, specificity and efficacy of direct microscopy for all patients.

Type of study	Sensitivity (%) Standard (+) n=124	Specificity (%) Standard (-) n=72	Efficacy (%) Standard n=196	McNemar test
Direct research	n=79 (**63,71%**)	n=64 (**88,89%**)	n=143 (**72,**96%)	**<0,001**

1.6.2 Culture results for all patients (n=196)

The positive results obtained for dermatophytes and *C. albicans* from the culture study were 72 (36.73%), while the negative results were 124 (63.27%). Completely sterile inoculations remained 52 samples (26.53%) and the negative samples against the set standard were the remaining 72 samples (36.73%) where the results were yeast *C. non-albicans* and/or non-dermatophytic moulds (NDM) (Table 5).

Table 5 - Culture result for all patients (n=196).

Result of culture	**Number (n)**	**Percentage (%)**
Positive (incl. dermatophytes and *C. albicans*)	72	36,73
Negative (sterile petri)	52	26,54
Negative (non-dermatophytic moulds and *C. non-albicans)*	72	36,73
Total	196	100,0

Of the positive seedling samples, 52 (72.22%) were pure cultures of dermatophytes. Of these, *T. rubrum* in 20 samples (38.46%), followed by *M. canis* - 13 (25%), *T. mentagrophytes* - 9 (17.30%), *M. ferrugineum* - 3 (5.76%), *M. audouinii* and *T. soudanense* 2 samples each (3.84%) and *T. tonsurans* and *T. violaceum* 1 sample each (1.92%) (Figure 26).

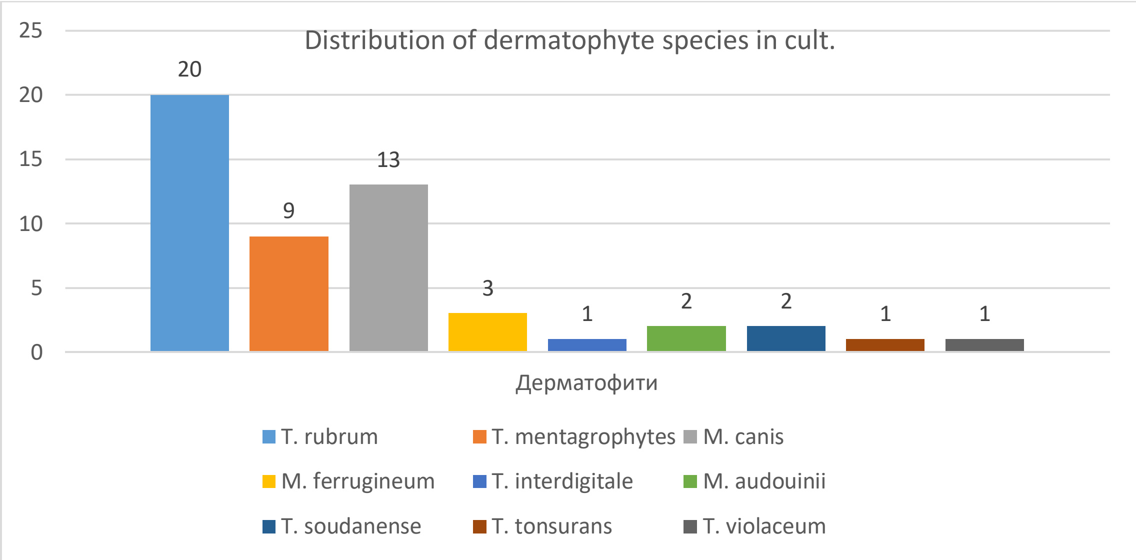

Figure 26 - Distribution of dermatophyte species in culture.

The remaining positive results were on pure *C* cultures. *albicans* - 3 (4.16%) and mixed infections - 17 (23.61%), distributed as follows. *C. rubrum* and *C. non-albicans* - 3 samples, *T. rubrum* and *C. albicans (C. rubrum and C. albicans* - 1, *T. rubrum, C. albicans* and mould - 1, *T. rubrum* and mould - 3, *T. rubrum, C. non-albicans* and mould - 1, *T. mentagrophytes* and *C. albicans* - 1, *T. mentagrophytes* and *C. non-albicans* - 1, *T. mentagrophytes* and mould - 2, *T. interdigitale* + mould - 1, *M. ferrugineum* and mould - 1 and *C. albicans* with mould - 2 samples (Figure 27).

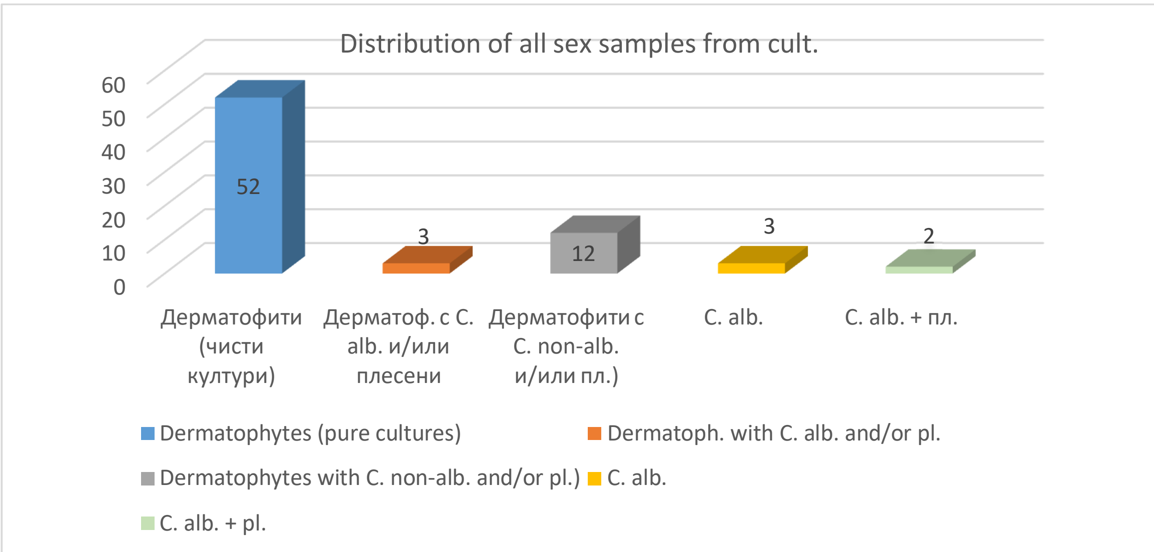

Figure 27 - Distribution of all positive cult samples (n=72).

The following *Candida* species were identified on Chromium agar. *albicans* (8 samples), *C. krusei* (7 samples*), C. glabrata (*6), *C. tropicalis (*1) in pure and mixed cultures. Of these, only *C. albicans* entered the positive sample standard, the remaining species were designated as *C. non-albicans*.

Complete growth of non-dermatophytic moulds (NDM) was observed in 45 sowings. Of these, *Rhodotorula spp, Aspergillus spp, Scopolariopsis spp, Alternaria spp, Penicillium spp* were most commonly identified. The remaining negative samples (27) had yeast growth *C. non-albicans* or mixed growth of *C. non-albicans* with NPD.

The sensitivity, specificity and efficacy of the culture assay for all samples tested are shown in Table 9. Their values were 58.06% sensitivity, 100% specificity and 73.47% efficacy (p<0.001) (Table 6).

Table 6 - Sensitivity, specificity and efficacy of the culture test for all samples.

Type of study	Sensitivity (%) Standard (+) n=124	Specificity (%) Standard (-) n=72	Efficacy (%) Standard n=196	McNemar test
Cultural study	n=72 (**58,06%**)	n=72 (**100**,0%)	n=144 (**73,47%**)	**<0,001**

1.6.3 Results of histological examination

Histological examination was performed on nail material only (n=86) and are detailed in subsection 4.6.4 Results of histological examination of patients with suspected onychomycosis.

1.6.4 Real-time PCR results for all samples (n=196)

The reported positive results of real-time PCR were 108 (55.10%) and negative results were 82 (41.84%). There were 6 invalid samples for the study (3.06%), which was due to sample inhibition during the PCR process due to larger amount of material or material with high human DNA content (nails) (Table 7).

Table 7 - Results for all real-time PCR samples.

Real-time PCR result	Number (n)	Percentage (%)
Positive	108	55,10
Negative	82	41,84
Invalid	6	3,06
Total	196	100,0

Among the positive results, DermaGenius (DG) detected mostly dermatophytes - 100 (92.59%), but also *C. albicans* - 3 (1.38%). Mixed infections of two dermatophytes or dermatophyte and *C. albicans* were 5 in number (4.63%) (Figure 28).

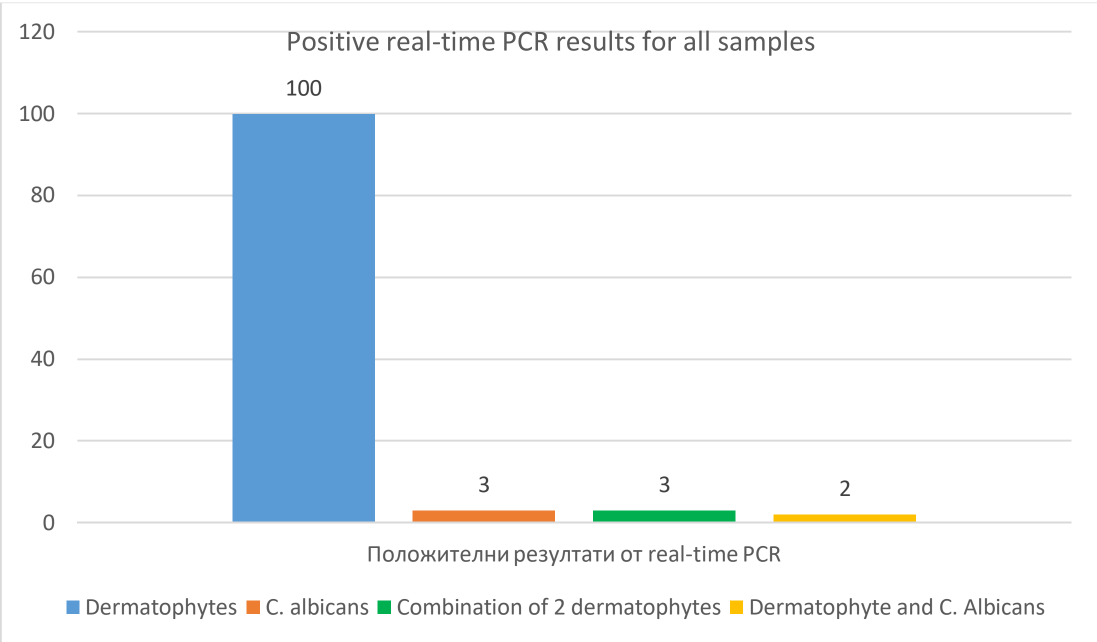

Figure 28 - Positive real-time PCR results for all samples.

The most common representative of dermatophytes was *T. rubrum* with 55 samples (50.93%), followed by *T. interdigitale* - 23 (21.30%), *M. canis* - 17 (15.74%), *T. mentagrophytes* - 3 (3.78%), *C. albicans* - 3 (3.78%), *E. floccosum* - 1 (0.93%), *T. tonsurans* - 1 (0.93%). Mixed signals for *T. rubrum* and *T. interdigitale* in 3 samples (3.78%), and *T. rubrum* and *C. albicans* in 2 (1.85%) (Figure 29).

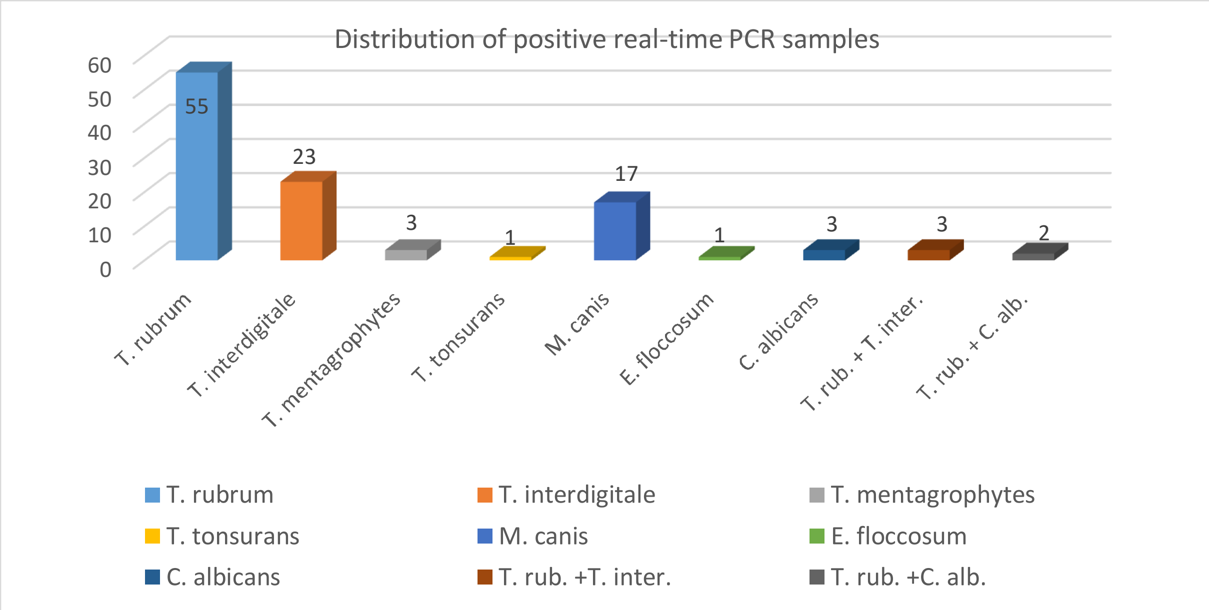

Figure 29 - Distribution of positive real-time PCR samples for all samples.

The reported sensitivity for the real-time PCR was 88.52%, and the specificity and efficacy were 100% and 92.63%, respectively (Table 8).

Table 8 - Sensitivity, specificity and efficacy of real-time PCR for all samples.

Type of study	Sensitivity (%) Standard (+) n=122	Specificity (%) Standard (-) n=68	Efficacy (%) Standard n=190	McNemar test
Real-time PCR	n=108 (**88,52%**)	n=68 (**100,0%**)	n=176 (**92,63%**)	**<0,001**

With the results obtained, the null hypothesis (H_0), which states that there is no difference in sensitivity, specificity and efficacy of conventional methods compared with real-time PCR, was rejected.

1.7 Discrepancies in results

1.7.1 Inhibition of real-time PCR samples

Real-time PCR was inhibited in 6 cases out of 196 (3.06%). In these cases, the Ct values of the internal control (IC) were too high compared to those of the negative control (NC), in which no causative agent was detected. This suggests the presence of a PCR inhibitor, which most commonly occurs with a large amount of material, solid material (most commonly nails) or a large amount of human DNA in the sample. Among these 6 samples, 1 was positive on histological examination and 1 was positive for *C. albicans* on culture examination. The remaining 4 were negative.

1.7.2 Inconsistencies of real-time PCR with culture testing

Due to inhibition (1 case) or negative result (10 cases) - (5.61%), DermaGenius (DG) PCR missed the detection of 1 *T. rubrum*, 2 *T. mentagrophytes*, 2 T. *soudanense*, 1 *T. tonsurans*, 1 *T. violaceum*, 1 *M. audouinii*, 2 *C. albicans* present in the detection kit, and 1 M. ferrugineum, which is not included in the kit.

On the other hand, the culture test missed the diagnosis of 24 cases (12.24%) due to a negative result, and 23 cases (11.73%) where there was yeast growth *C. non-albicans* and/or non-dermatophytic moulds where real-time PCR had positive results. A total of 30 *T. rubrum,* 10 *T. interdigitale*, 2 *T mentagrophytes*, 3 *M. canis*, 1 *E. floccosum* and 1 mixed infection *of T. rubrum* in combination with *C. albicans*. Figure 33 shows the results of the positive real-time PCR samples and the culture results for dermatophytes, *C. albicans, C. non-albicans* and non-dermatophyte moulds (NDM), illustrating the differences in the results obtained between the two studies (Figure 30).

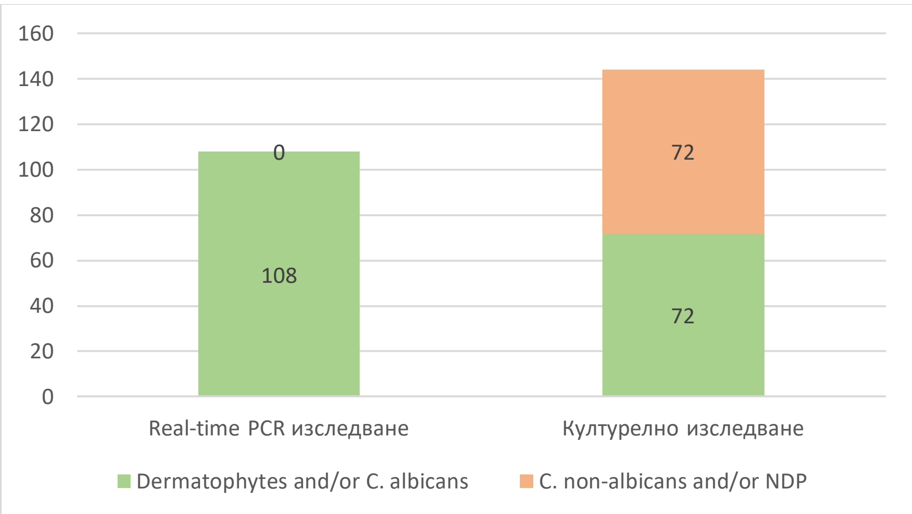

Figure 30 - Comparison of positive real-time PCR results versus positive culture results (by standard) and growth results of C. non-albicans and/or NDP.

1.7.3 Species identification discrepancies

Species identification discrepancies based on the culture and real-time PCR results were observed in 17 cases, shown clearly in Table 9.

Table 9 - Discrepancies in species identification between real-time PCR and culture.

Result of real-time PCR study	Result of cultural research
8 samples T. interdigitale	**7** samples T. mentagrophytes, **1** sample T. mentagrophytes + C. albicans
2 - T. interdigitale + T. rubrum	**1** - T. mentagrophytes, **1** - T. rubrum
1 - T. interdigitale	**1** - T. rubrum + C. albicans
1 - C. albicans	**1** - T. rubrum
1 - T. rubrum + C. albicans	**1** - C. albicans
1 - T. interdigitale	**1** - M. canis
1 - T. interdigitale	**1** - M. ferrugineum
1 - T. rubrum	**1** - M. ferrugineum
1 - M. canis	**1** - T. mentagrophytes

A total of 8 cases detected by real-time PCR as *T. interdigitale*, were diagnosed by culture as *T. mentagrophytes* (1 case of *T. mentagrophytes* + *C. albicans*); 2 cases of mixed infection of *T. interdigitale* in combination with *T. rubrum* by PCR, were identified by culture as 1 *T. mentagrophytes and* 1 *T. rubrum*; 1 case of *T. interdigitale* by PCR was diagnosed as a mixed infection of *T. rubrum* and *C. albicans* in culture; 1 case of *C. albicans* by PCR was diagnosed in culture as *T. rubrum*; 1 case of mixed infection of *T. rubrum* and *C. albicans* with DG resulted in only *C. albicans* on culture; 1 case of *T. interdigitale* by PCR was diagnosed as *M. canis* by culture; 1 case of *T. interdigitale* from PCR was diagnosed as *M. ferrugineum* from the culture study; 1 case of *T. rubrum* from real-time PCR was diagnosed as *M. ferrugineum* by inoculation and 1 case of *M. canis* from real-time PCR was diagnosed as *T. mentagrophytes* by culture. These results could be positively confirmed in favor of DG PCR by ITS (internal transcribed spacer) sequencing.

1.8 Established dependencies

Compared to the established standard for a true positive sample, two clear correlations related to gender and age of the patients stood out in the results obtained.

Of all the patients participating in the study, males were 93 (47.45%) in number and females were 103 (52.55%). Out of males, 71 (76.34%) were positive for fungal infection as per the established standard and 22 (23.66%) were negative. Out of females, 53 (51.46%) were positive and 50 (48.54%) were negative (Table 10).

Table 10 - Distribution of positive and negative samples by sex.

	All patients		
	Positive	Negative	Total
Men - number/(%)	71/(**76,34%)**	22/(**23,66%)**	93/(100%)
Women - number/(%)	53/(**51,46%)**	50/(**48,54%)**	103/(100%)
Total - number/(%)	124/(63,27%)	72/(36,73%)	196/(100%)

A statistically significant difference was found in positive and negative results with respect to gender ($\chi^2 = 13.025$, $p<0.001$).

All patients in the study were also divided into age groups over 20 years to determine whether there was an age dependence of positive and negative samples. The positive patients in the group of up to 20 years were 19 (15.32%), 20-39 years were 10 (8.07%), 40-59 years were 48 (38.71%), 60-79 years were 39 (31.45%) and above 80 years were 8 (6.45%) (Table 11).

Table 11 - Distribution of positive and negative samples according to age.

	All patients		
	Positive	Negative	Total
Up to 20 years (number) % of total positive samples	19 15,32%	7 9,72%	26 13,27%
20-39 years (number) % of total positive samples	10 8,07%	21 29,17%	31 15,82%
40-59 years (number) % of total positive samples	48 **38,71%**	18 25,0%	66 33,67%
60-79 years (number) % of total positive samples	39 **31,45**	23 31,94%	62 31,63%
Over 80 years (number) % of total positive samples	8 6,45%	3 4,17%	11 5,61%
Total % of total positive samples	124 100,0%	72 100,0%	196 100,0%

There was a statistically significant higher percentage of positive samples for mycotic infection in patients between 40-59 years and 60-79 years (χ^2 = 16.871, $p<0.002$).

2. Results of a prospective comparative study evaluating the results of direct microscopic, culture and real-time PCR examinations in patients with suspected dermatophyte infection localized to the scalp and beard (n=23)

2.1 General characteristics of the patients and the collected materials

Patients with suspected fungal infection caused by dermatophytes with localization on scalp and beard were 23 in number, aged from 2 to 56 years, mean age 13.57±3.03 years, standard deviation 14.52. The median was 8.00 and the mode of age was 8. Of these, 18 (78.3%) were male and 5 (21.7%) were female (Figure 31).

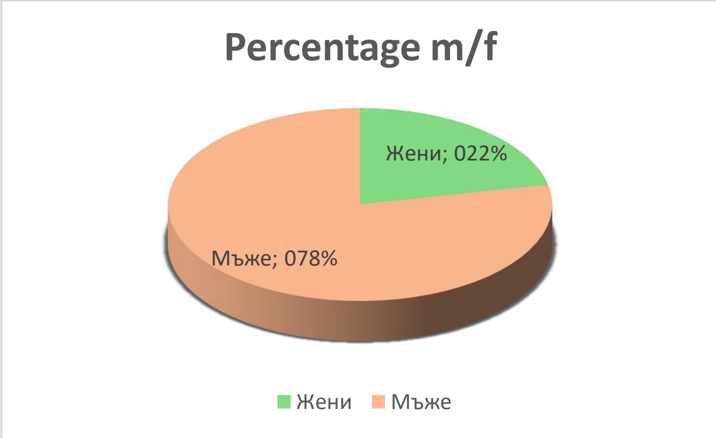

Figure 31 - Percentage of males and females among patients with suspected scalp and beard mycosis.

The materials collected from scalp, i.e., hairs and squams from capilicium, were 21 (93.30%) in patients between 2 and 27 years of age, and those from beard (hairs from beard and squams from skin in this area) were 2 (8.70%) in patients 55 and 56 years of age, respectively.

2.2 Distribution of patients according to the age of pathological changes (scalp and beard)

Most patients had complaints less than 3 months before the examination - 21 (91.4%), 1 patient had complaints for 6 months (4.3%) and 1 for 1 year (4.3%).

2.3 Standard for a positive sample

Compared to the set standard for a true positive result for mycosis, the number of positive samples of patients with suspected scalp and beard mycosis was 20 (86.94%). The number of negative tests was 3 (13.04%).

2.4 Results of studies conducted

2.4.1 Results of direct microscopic examination of patients with suspected scalp and beard mycosis (n=23)

Positive results were 17 in number (73.91%) of all samples and negative results were 6 (26.09%) respectively.

The sensitivity for direct microscopy was 85%, specificity 100%, and efficacy 86.96%, according to the established standard ($p>0.05$) (Table 12).

2.4.2 Culture results of patients with suspected scalp and beard mycosis (n=23)

The number of positive and negative culture results were 17 (73.91%) and 6 (26.09%), respectively, of which 5 had no growth and 1 sample had growth of NDP (*Rhodotorula spp*).

Out of the positive culture samples, all 17 (73.91%) were pure cultures of dermatophytes. Of these, *M. canis* in 12 samples (70.59%), followed by *T. rubrum* - 3 (17,65%), *T. mentagrophytes* - 1 (5,88%), *T. soudanense* - 1 (5.88%) (Figure 32).

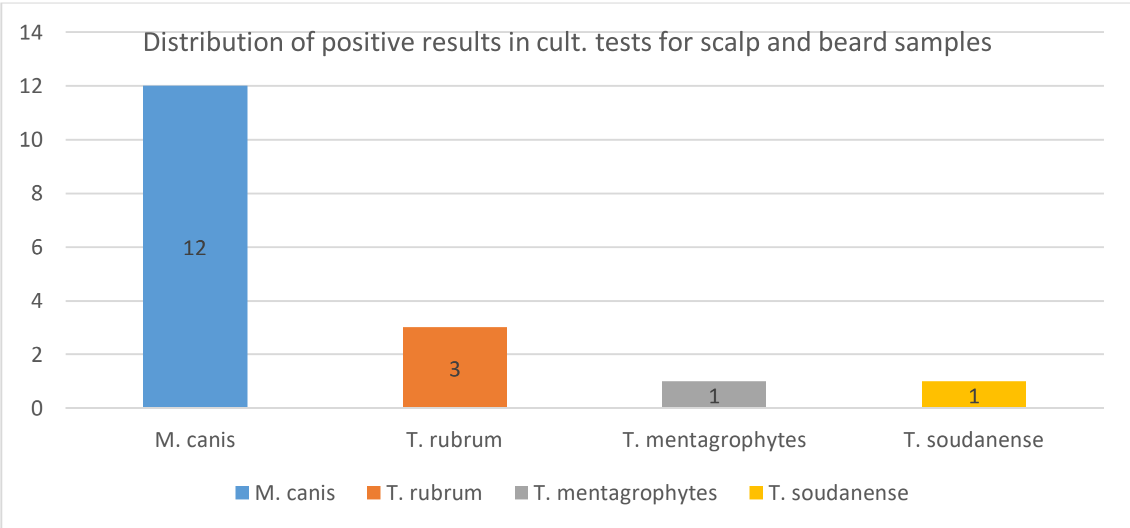

Figure 32 - Distribution of positive culture results in patients with suspected scalp and beard mycosis.

The sensitivity, specificity and efficacy of the culture test for scalp and beard samples were 85% sensitivity, 100% specificity and 86.96% efficacy ($p>0.05$) (Table 12).

2.4.3 Real-time PCR results for scalp and beard samples

The reported positive results of real-time PCR were 19 (82.61%) and negative results were 4 (17.39%).

Among the positive results, DermaGenius (DG) detects entirely dermatophytes - *M. canis* - 14 (73,68%), *T. rubrum* - 2 (10,53%), *T. mentagrophytes* - 2 (10,53%) and *T. tonsurans* - 1 (5.26%) (Figure 33).

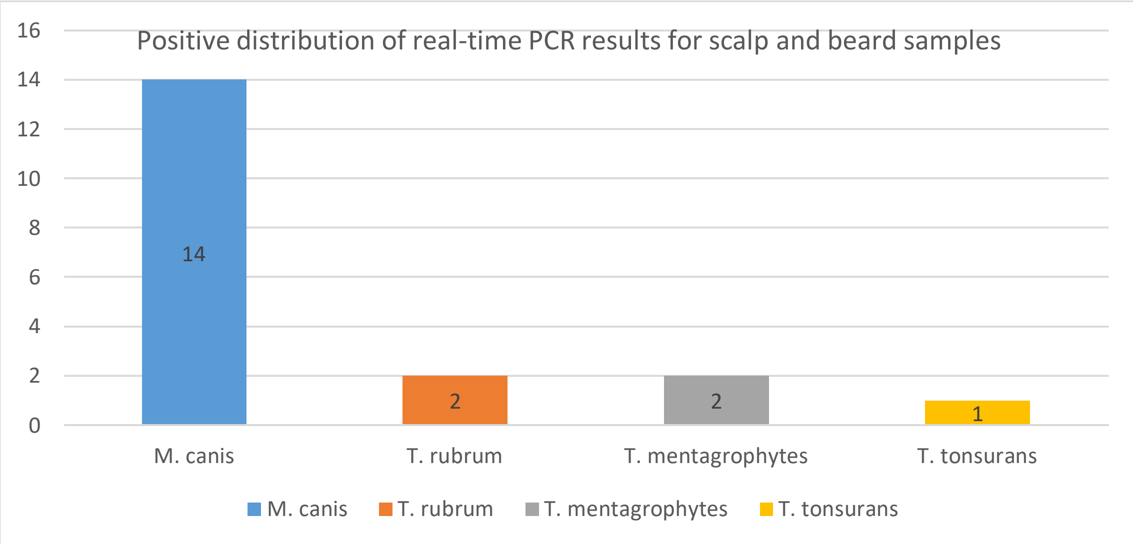

Figure 33 - Distribution of positive real-time PCR results for scalp and beard samples.

The reported sensitivity for the real-time PCR was 95%, and the specificity and efficacy were 100% and 95.65%, respectively (p>0.05) (Table 12).

Table 12 - Sensitivity, specificity and efficacy of direct, culture and real-time PCR results for scalp and beard samples.

	Direct examination	Culture	Real-time PCR
Sensitivity (%) Standard (+) n=20	n=17 (**85,**0%)	n=17 (**85,**0%)	n=19 (**95,**0%)
Specificity (%) Standard (-) n=3	n=3 (**100,**0%)	n=3 (**100,**0%)	n=3 (**100,**0%)
Efficacy (%) Standard n=23	n=23 (**86,**96%)	n=23 (**86,**96%)	n=22 (**95,65%**)
McNemar test	>0,05	>0,05	>0,05

3. Results of a prospective comparative study evaluating the results of direct microscopic examination, culture and real-time PCR in patients with suspected dermatophyte infection with skin localization (n=29)

3.1 General characteristics of the patients and collected materials for research

Patients with suspected fungal infection caused by dermatophytes with skin localization were 29 in number, aged from 5 to 84 years, mean age was 48.10±3.51, with standard deviation 18.89. The median was 53.00 and the mode of age was 43. Of these, 17 (58.62%) were male and 12 (41.38%) were female (Figure 34).

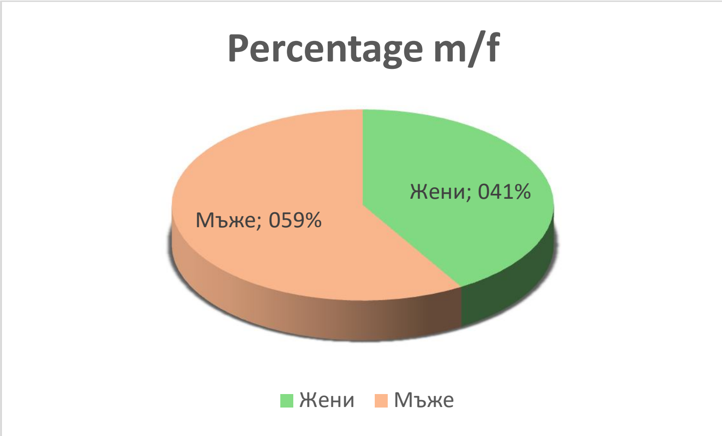

Figure 34 - Percentage of males and females among patients with suspected skin mycosis.

The materials were squams of skin distributed as follows: 4 (13.80%) from hands, 11 (37.93%) from legs, 11 (37.93%) from truncus, 3 (10.34%) from inguinal folds.

3.2 Distribution of patients according to the age of pathological skin changes

Most of the patients had complaints less than 3 months prior to the examination - 18 (62.07%), 2 patients had complaints for 6 months (6.90%) and 1 for one year (3.45%), 3 had complaints between 1 and 5 years (10.34%), 3 between 6 and 10 years (10.34%) and 2 patients had complaints for more than 10 years (6.90%).

3.3 Standard for a positive sample

Compared to the set standard for a true positive result for mycosis, the number of positive samples of patients with suspected skin mycosis was 21 (72.41%). The number of negative tests was 8 (27.59%).

Of the negative samples, 1 sample (3.45%) was invalid, which was also negative on culture, and therefore was not included in the calculation of sensitivity, specificity and efficacy of real-time PCR.

3.4 Results of the conducted studies

In order to compare the results of real-time PCR with direct and culture studies in mycotic skin infections, the sensitivity, specificity and efficacy of each assay were investigated.

3.4.1 Results of direct microscopic examination of patients with suspected skin mycosis (n=29)

The positive results of direct microscopic examination were 22 in number (75.86%) and the negative results were 7 (24.14%), respectively.

The sensitivity for direct microscopy was 85.71%, the specificity was 50%, and the efficacy was 75.86%, according to the established standard (p>0.05) (Table 13).

3.4.2 Culture results of patients with suspected skin mycosis (n=29)

The number of positive and negative culture results obtained were 10 (34.48%) and 19 (65.52%), respectively, of which 13 had no growth and 6 samples had yeast *C* growth. *non-albicans* and/or NDP.

Of the positive seedling samples, 8 (80%) were pure cultures of dermatophytes. Of these, 2 samples were *T. rubrum* (20%), 2 *T. mentagrophytes* (20%), 1 - *T. soudanense* (10%), 1 *T. interdigitale (*10%), 1 *M. canis* (10%), 1 *M. ferrugineum (*10%). The remaining 2 positive samples were of mixed infections - 1 *T. rubrum* + *C. non-albicans* and 1 *T. rubrum* + *C. non-albicans* + NDP (Figure 35).

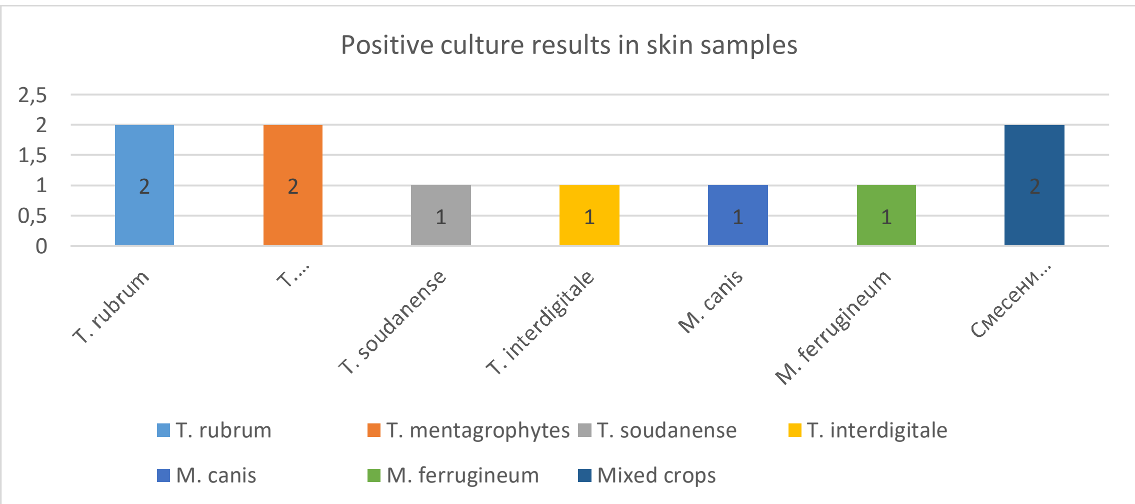

Figure 35 - Distribution of positive culture results in skin samples.

The sensitivity, specificity and efficacy of the culture test for skin samples were as follows: 47.62% sensitivity, 100% specificity and 62.07% efficacy ($p<0.05$) (Table 13).

Table 13 - Sensitivity, specificity and efficacy of direct and culture results for skin samples.

Type of study	Sensitivity (%) Standard (+) n=21	Specificity (%) Standard (-) n=8	Efficacy (%) Standard n=29	McNemar test
Direct research	n=18 **(85,71%)**	n=4 **(50,0%)**	n= 22 **(75,86%)**	>0,05
Cultural study	n=10 **(47,62%)**	n=8 **(100,0%)**	n=18 **(62,07%)**	<0,05

3.4.3 Real-time PCR results for skin samples

There were 20 positive real-time PCR results (68.97%), 8 negative (27.59%) and 1 invalid sample (3.45%).

Among the positive results, DermaGenius (DG) detects dermatophytes completely. The most common was *T. rubrum* - 12 (41.38%), followed by *M. canis* - 3 (10.34%), *T. mentagrophytes* - 1 (3.45%), *T. interdigitale* - 2 (6.90%), *E. floccosum* - 1 (3.45%) and 1 mixed signal from *T. rubrum* + *C. albicans* (3.45%) (Figure 36).

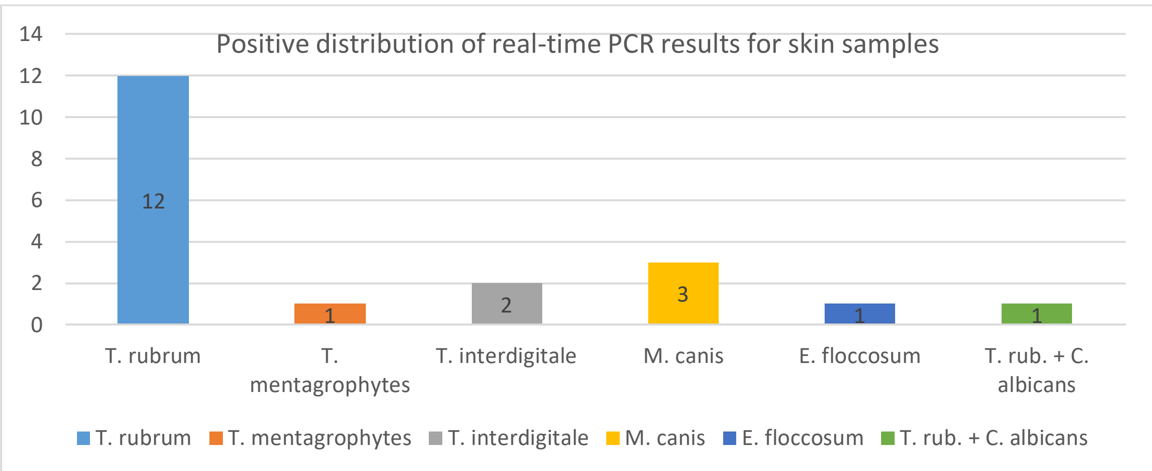

Figure 36 - Distribution of positive real-time PCR results from skin samples.

The reported sensitivity for the real-time PCR was 95.24%, and the specificity and efficacy were 100% and 85.71%, respectively (p>0.05) (Table 14).

Table 14 - Sensitivity, specificity and efficacy of real-time PCR results for skin samples.

Type of study	Sensitivity (%) Standard (+) n=21	Specificity (%) Standard (-) n=7	Efficacy (%) Standard n=28	McNemar test
Real-time PCR	n=20 (**95,42%**)	n=7 (**100**,0%)	n=24 (**85,71%**)	>0,05

4. Results of a prospective comparative study evaluating the results of direct microscopic, culture, histological and real-time PCR examinations in patients with suspected dermatophyte infection with nail localization (n=144)

4.1 General characteristics of the patients and materials collected for research

Patients with suspected fungal infection caused by dermatophytes with nail localization were 144 in number, aged from 3 to 90 years, mean age 55.01±1.47 years, standard deviation 17.62. The median age was 57.00 and the mode age was 60. Of these, 58 (40.28%) were male and 86 (59.72%) were female (Figure 37).

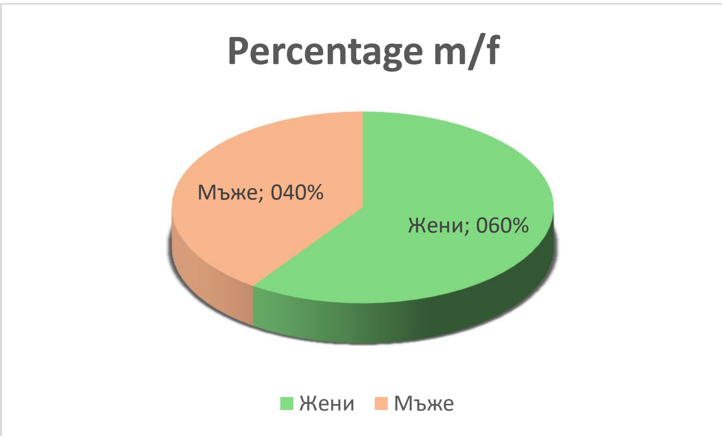

Figure 37 - Percentage of males and females among patients with suspected onychomycosis.

The materials collected were clippings and scrapings of fingernails and subungual material distributed as follows: 8 (5.56%) from fingernails, 136 (94.44%) from toenails.

4.2 Extent of involvement and localisation of nail plate changes

According to the number of nails that were affected, nail changes were divided into three grades - mild (when <4 nails were affected), moderate (between 5 and 8 nails) and severe (>9 nails). There were 105 (72.92%) patients with mild, 3 (2.08%) with moderate and 36 (25%) with severe degree of involvement, respectively (Table 15).

Table 15 - Degree of nail involvement.

Degree of involvement	Number (n)	Percentage (%)
Mild	105	72,92
Average	3	2,08
Severe	36	25
Total	144	100,0

According to the type and location of nail plate involvement, there were 135 patients with distal-lateral involvement (DLOM) (93.75%), 1 patient with superficial white OM (0.69%), and 8 patients in total dystrophic OM (5.56%) (Table 16).

Table 16 - Type of nail plate involvement.

Type of nail plate involvement	Number (n)	Percentage (%)
Distolateral	135	93,75
White superficial OM	1	0,69
Total dystrophic OM	8	5,56
Total	144	100,0

4.3 Distribution of patients according to the age of pathological changes (by nail)

Most of the patients had complaints between 1 and 5 years prior to the examination - 58 (40.28%), followed by patients with complaints between 6 and 10 years - 31 (21.53%), 22 with complaints up to 1 year prior to the examination (15.28%), 20 with complaints more than 10 years (13.89), 8 up to 6 months (5.56%) and 5 less than 3 months prior to the examination (3.47%).

4.4 Presence of diabetes

Of all 144 patients with suspected onychomycosis, 93 (64.58%) had no diabetes mellitus and the remaining 51 had diabetes mellitus (35.42%). The patients with diabetes mellitus were included in the ongoing study under PhD project No. DPDP07/20. All of them were controlled diabetic patients with the disease duration described in Table 17.

Table 17 - Disease duration in patients with DM.

Duration of DM	Number (n)	Percentage (%)
1 to 5 years	16	31,37
6 to 10 years	20	39,22
Over 10 years	5	9,80
Over 15 years	10	19,61
Total	51	100,0

4.5 Standard for a positive sample

Compared to the set standard for a true positive result for mycosis, the number of positive samples of patients with suspected onychomycosis was 83 (57.64%). The number of negative tests was 61 (42.36%).

5 samples (3.47%) were invalid for real-time PCR, 2 of them were positive on culture or histology, but 3 samples were negative on the other tests. These 5 samples were not included in the calculation of the sensitivity, specificity and efficacy of real-time PCR.

4.6 Results of the conducted studies

In order to compare the results of real-time PCR with direct, culture and histological tests in mycotic nail infections, the sensitivity, specificity and efficacy of each of the tests were investigated.

4.6.1 Results of direct microscopic examination of patients with suspected onychomycosis (n=144)

Positive results of direct microscopy were 48 in number (33.33%) of all samples, and negative results were 96 (66.67%), respectively.

The sensitivity for direct microscopy was 53.01%, specificity 93.44%, and efficacy 70.14%, according to the established standard ($p<0.001$) (Table 18).

4.6.2 Culture results of patients with suspected onychomycosis (n=144)

The number of positive and negative culture results obtained were 45 (31.25%) and 99 (68.75%), respectively, of which 34 (23.61%) had no growth and 65 (45.14%) samples had yeast *C* growth. *non-albicans* and/or NDP.

Of the positive seedling samples, 27 (60%) were pure cultures of dermatophytes. Of these, 15 samples were *T. rubrum* (33.33%), 6 *T. mentagrophytes* (13.33%), 1 - *T. tonsurans* (2.22%), 1 *T. violaceum* (2.22%), 2 *M. audouinii* (4.44%) and 2 *M. ferrugineum* (4.44%). Positive for *C. albicans* were 3 samples (6.67%). The remaining 15 positive samples were mixed growth - 1 *T. rubrum* + *C. albicans*, 1 *T. mentagrophytes* + *C. albicans*, 2 *C. albicans* + NDP, 2 *T. rubrum* + *C. non-albicans,* 3 *T. rubrum* + NDP*,* 1 *T. rubrum* + *C. non-albicans* + NDP*,* 2 *T. mentagrophytes* + NDP, 1 *T. mentagrophytes* + *C. non-albicans,* 1 *T. interdigitale* + NDP and 1 *M. ferrugineum* + NDP (Figure 38).

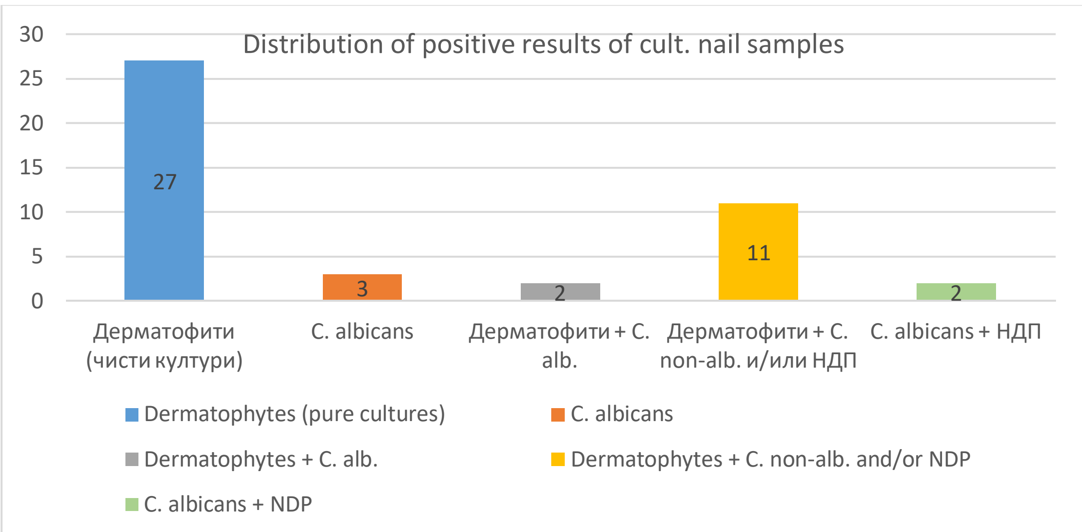

Figure 38 - Distribution of positive culture tests of nail samples.

The following *Candida* species were identified on Chromium agar: 8 *C. albicans*, 7 *C. krusei*, 6 *C. glabrata* and 1 *C. tropicalis*. Only *C. albicans* enters the positive sample standard of the current study. All other species are designated as *C. non-albicans*.

Complete growth of non-dermatophytic moulds (NDM) was observed in 39 plots. Of these, *Rhodotorula spp, Aspergillus spp, Scopolariopsis spp, Alternaria spp, Penicillium spp* were most commonly identified. The remaining negative samples (26 samples) had growth of *C. non-albicans* or mixed yeast growth of *C. non-albicans* with NPD.

The sensitivity, specificity and efficacy of the culture test for nail samples were 54.22% for sensitivity, 100% for specificity and 73.61% for efficacy (p<0.001) (Table 18).

Table 18 - Sensitivity, specificity and efficacy of direct microscopic and culture examinations for nail samples.

Type of study	Sensitivity (%) Standard (+) n=83	Specificity (%) Standard (-) n=61	Efficacy (%) Standard n=144	McNemar test
Direct research	n=44 (**53,01%**)	n=57 (**93,44%**)	n=101 (**70,14%**)	<0.001
Cultural study	n=45 (**54,22%**)	n=61 (**100,0%**)	n=106 (**73,61%**)	<0.001

4.6.3 Real-time PCR results of patients with suspected onychomycosis (n=144)

The reported positive real-time PCR results were 69 (47.92%), negative 70 (48.61%) and there were 5 invalid samples (3.47%).

Among the positive results, DermaGenius (DG) detected 62 dermatophytes (89.85%), 3 samples with *C. albicans* (4.35%) and 4 mixed signals (5.80%) containing *T. rubrum* + *T. interdigitale* (3 samples) and *T. rubrum* + *C. albicans* (1 sample).

The most common dermatophyte was *T. rubrum* - 41 (59.42%), followed by *T. interdigitale* - 21 (30.43%) (Figure 39).

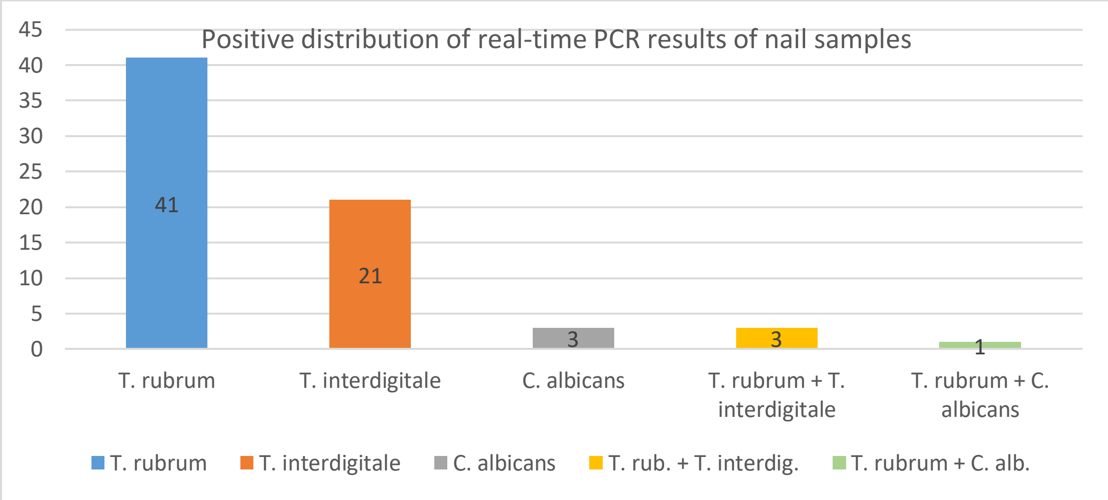

Figure 39 - Distribution of positive real-time PCR results of nail samples.

The values obtained for sensitivity, specificity and efficacy were as follows: 85.19% for sensitivity, 100% for specificity and 91.37% for specificity ($p<0.001$) (Table 19).

Table 19 - Sensitivity, specificity and efficacy of real-time PCR for nail samples.

Type of study	Sensitivity (%) Standard (+) n=81	Specificity (%) Standard (-) n=58	Efficacy (%) Standard n=139	McNemar test
Real-time PCR	n=69 (**85,19%**)	n=58 (**100,0%**)	n=127 (**91,37%**)	<0,001

With the results obtained, the null hypothesis (H_0), which states that there is no difference in sensitivity, specificity and efficacy

of conventional methods compared with real-time PCR, was rejected.

4.6.4 Results of histological examination of patients with suspected onychomycosis (n=86)

Material for histological examination was collected in 86 patients. Of these, 49 were positive (56.98%) and 37 (43.02%) were negative.

In 6 cases of positive histology, 5 were negative and 1 was invalid on real-time PCR. On the other hand, in 3 cases where real-time PCR was positive, histology was negative.

The sensitivity, specificity and efficacy of histology for the nail samples were 85.96%, 100% and 90.70%, respectively ($p<0.001$) (Table 20).

Table 20 - Sensitivity, specificity and efficacy of histological examination for nail samples.

Type of study	Sensitivity (%) Standard (+) n=57	Specificity (%) Standard (-) n=29	Efficacy (%) Standard n=86	McNemar test
Histological examination	n=49 (**85,96%)**	n=29 (**100,0%)**	n=78 (**90,70%)**	<0,001

4.6.5 Combination of real-time PCR and histology

Combining the histological with real-time PCR assays yielded the highest sensitivity, specificity and efficacy results of 96.26%, 100% and 97.62%, respectively (p>0.05) (Table 21).

Table 21 - Sensitivity, specificity and efficacy of combining histological and real-time PCR assays.

Type of study	Sensitivity (%) Standard (+) n=55	Specificity(%) Standard (-) n=29	Efficacy(%) Standard n= 84	McNemar test
Histology + real-time PCR	n=53 (**96,36%)**	n=29 (**100,0%)**	n=82 (**97,62%)**	>0,05

5. Results of studies on the incidence and etiological causes in patients with onychomycosis with and without diabetes (n=144)

5.1 General characteristics of patients

Patients with onychomycosis with and without diabetes mellitus were divided into 2 groups:

- **Group I** (n=51) of patients with diabetes mellitus and suspected onychomycosis;

- **Group II** (n=93) patients without DM but with suspected OM.

In the group of diabetic patients with suspected onychomycosis, 51 patients aged 44 to 86 years, mean age 63.88 years, standard deviation 10.788 were included. With a median of 62.00 and a mode of age of 55. Of these, 27 (52.94%) were male and 24 (47.06%) were female.

The group of patients without diabetes but with suspected onychomycosis included 93 patients aged 3 to 90 years, mean age 50.15 years, standard deviation

18.752. With a median of 51.00 and a mode of age of 68. Of these, 31 (33.33%) were male and 62 (66.67%) were female.

5.2 Incidence and etiological causes in the two groups of patients

Statistical analyses based on the results of real-time PCR and culture against the true-positive standard set in the present study were performed to estimate the frequency and etiologic agents in the two groups of patients and whether there was a difference between them.

Table 23 shows the results for the etiologic agents from the real-time PCR study of group I and group II patients (Table 22).

Table 22 - Real-time PCR results for etiological agents for group I and group II.

Etiological agent real-time PCR	Group I Number (%)	Group II Number(%)	Total number (%)
T. rubrum	19 (37,25%)	22 (23,66%)	41 (28,47%)
T. interdigitale	9 (17,65%)	12 (12,90%)	21 (14,58%)
T. rubrum + T. interdigitale	1 (1,96%)	2 (2,15%)	3 (2,08%)
T. rubrum + C. albicans	0 (0,0%	1 (1,08%)	1 (0,69%)
C. albicans	1 (1,96%)	2 (2,15%)	3 (2,08%)
Invalid result	0 (0,0%)	5 (5,38%)	5 (3,47%)
Negative result	21 (41,18%)	49 (52,69%)	70 (48,61%)
Total	51 (100,0%)	93 (100,0%)	144 (100,0%)

Patients with the etiological agent *T. rubrum* in group I were 19 (37.25%) and those in group II were 22 (23.66) of all positive samples in the respective group. The presence of *T. interdigitale* from group I had 9 (17,65%), from group II - 12 (12,90%), mixed signals for *T. rubrum* + *T. interdigitale* for group I - 1 (1,96%), for group II - 2 (2,15%), mixed signal for *T. rubrum* + *C. albicans* only 1 (1.08%) in group II, *C. albicans* in group I - 1 sample (1,96%), and in group II - 2 (2,15%). Invalid samples were 5 (5.38%) in number only in group II, and negative results were 21 (41.18%) in group I and 49 (52.69%) in group II.

According to these data, there was no significant statistical difference between the etiological agents in the two groups of patients ($\chi^2 = 6.847$, $p>0.335$).

The culture results showed small differences, but again no significant statistical difference was found between the two groups of patients ($\chi^2 = 1.996$, $p>0.736$) (Table 23).

Table 23 - Culture results for the etiological agents for Group I and Group II.

Etiological causative agent culturally studied.	Group I Number (%)	Group II Number(%)	Total number (%)
Dermatophyte	12 (23,5%)	24 (25,8%)	36 (25%)
Dermatophyte + C. albicans	2 (3,9%)	1 (1,1%)	3 (2,1%)
C. albicans	1 (2,0%)	4 (4,3%)	5 (3,5%)
NDP and/or C. albicans	23 (45,1%)	43 (46,2%)	66 (45,8%)
Negative result	13 (25,5%)	21 (22,6%)	34 (23,6%)
Total	51 (100,0%)	93 (100,0%)	144 (100,0%)

5.3 Established dependencies

Regarding the presence or absence of diabetes mellitus and the positive result for fungal causative agent, the following correlations emerged for the group of diabetic patients with OM and the group with OM only:

It was found that 72.55% of the diabetic patients had positive result for fungal nail infection, whereas in the non-diabetic group, only 49.46% had positive result for mycosis fungoides (Table 24). The results were statistically significant ($\chi^2 = 7.190$, $p<0.007$).

Table 24 - Results for positive and negative tests for OM versus presence or absence of DM.

Presence of diabetes mellitus - yes/no	Score by set standard		Total
	Positive	Negative	
Yes - number (%)	37 (**72,55%)**	14 (27,45%)	51 (100,0%)
No - number (%)	46 (**49,46%)**	47 (50,54%)	93 (100,0%)
Total	83 (57,64%)	61 (42,36%)	144 (100,0%)

The age of diabetes mellitus has also been found to be important in the development of onychomycosis. Patients with DM were divided into the following groups differentiating the age of DM - between 1 and 5 years, 6-10 years, 11-15 years and more than 15 years. DM patients positive for OM with a diabetes duration between 1 and 5 years were 62.5%, between 6 and 10 years were 85%, between 11 and 15 years were 60%, and over 15 years were 70% ($\chi^2 = 9.471$, $p<0.05$) (Table 25).

Table 25 - Results for positive and negative tests for OM versus age of diabetes.

Statute of limitations	Score by set standard		Total
	Positive	Negative	
1-5 years	10 (62,5%)	6 (37,5%)	16 (100,0%)
6-10 years	17 (85%)	3 (15%)	20 (100,0%)
11-15 years	3 (60%)	2 (40%)	5 (100,0%)
Over 15 years	7 (70%)	3 (30%)	10 (100,0%)
No diabetes	46 (49,5%)	47 (50,5%)	93 (100,0%)
Total	83 (57,6%)	61 (42,4%)	144 (100,0%)

V. Conclusion

In this dissertation, a study comparing real-time PCR methodology with conventional methods - direct microscopic, culture and histological examination of hair, skin and nail materials was performed to demonstrate the utility and value of real-time PCR for the diagnosis of dermatophyte infections.

The sensitivity, specificity and efficacy values obtained for direct microscopic, culture, histological and real-time PCR were as follows: 63.71%, 88.89% and 72.96% ($p<0.001$); 58.06%, 100% and 73.47% ($p<0.001$); 85.96%, 100% and 90.70% ($p<0.001$); 88.52%, 100% and 92.63% ($p<0.001$). With this, H_0 is rejected, thus the main objective of the dissertation is achieved.

Statistically significant correlations were also found, confirming the predominance of mycotic involvement in men and in patients aged between 40 and 79 years. Onychomycosis was also found to be more common in patients with diabetes mellitus.

In conclusion, real-time PCR is a technique with exceptional potential in the diagnosis of many infections and conditions. Its widespread use in a variety of medical fields is undeniable, the closest example being the Covid-19 pandemic. However, for many other fields, such as dermatology and mycology in particular, the technique has not yet established itself as the gold standard, although it has demonstrated its advantages many times. By conducting studies such as the present one, we have the opportunity to change and update the "face" of dermatology by introducing new algorithms in the diagnosis of superficial fungal infections.

Reasoning

1. Proper collection of material from the affected area and whether it is distributed over more than one study are critical to the results.

2. *Trichophyton rubrum* and *Trichophyton interdigitale* are the most common dermatophytes in onychomycoses.

3. Men are affected more often by fungal infections caused by dermatophytes (on nails, skin and hair) than women.

4. The sensitivity, specificity and efficacy of real-time PCR is higher than that of direct microscopic, culture and histological examinations.

5. Real-time PCR saves the 3-4 week wait that is required for culture testing and results are available within one business day.

6. With real-time PCR, it is possible to diagnose dermatophytes even when there is growth of non-dermatophyte moulds on the culture, which inhibit the growth of dermatophytes and prevent the culture from being read correctly.

7. The combination of real-time PCR and histological examination have the highest sensitivity, specificity and efficacy for the detection of onychomycoses.

8. Patients with diabetes mellitus are more likely to develop onychomycosis than patients without diabetes mellitus.

9. There is no statistically significant difference in the etiological agents of onychomycosis in patients with and without diabetes mellitus.

Contributions of the author

Original contributions

1. For the first time in the scientific literature, real-time PCR with the DermaGenius Complete multiplex real-time PCR kit is performed with hair, skin and nail materials and compared with direct microscopic, culture and histological examinations for the diagnosis of fungal infections caused by dermatophytes.

2. For the first time in the scientific literature, the incidence and etiologic causative agents of onychomycosis patients with and without diabetes mellitus are compared by real-time PCR, direct microscopic, culture, and histologic studies, simultaneously.

Contributions original for Bulgaria

1. DermaGenius® Complete real-time PCR kit is used for the first time in a scientific study in our country.

2. For the first time in our country, a comparative study of the sensitivity, specificity and efficacy of real-time PCR compared with direct microscopic and culture examinations in patients with suspected scalp and beard mycoses, as well as in patients with suspected skin mycoses.

3. For the first time in our country, a comparative study of the sensitivity, specificity and efficacy of real-time PCR compared with direct microscopic, culture and histological examinations in patients with suspected onychomycosis is performed.

4. For the first time, the frequency and etiological agents of onychomycosis in patients with and without diabetes mellitus were investigated by real-time PCR in our country.

5. For the first time in our country, a histological examination of nails for onychomycosis is included in a volumetric study.

Scientifically applied contributions

1. DermaGenius® Complete multiplex real-time PCR is used for the first time as the main method for the diagnosis of dermatophyte infections of hair, skin and nails in our country.

2. For the first time, an algorithm for the diagnosis of superficial fungal infections including real-time PCR is proposed to be introduced into clinical practice in our country.

Publications related to the dissertation

List of publications:

1. Classical and more modern methods versus PCR methodology in the diagnosis of dermatophyte infections, Lilia G. Zisova, **Gergana Z. Vazheva**, J. Dermatology and Venereology, no. 3, 2019; 8-14;

2. **Vazheva G**, Zisova L (2021) Tinea barbae profunda caused by *Trichophyton rubrum* - an autoinoculation from a primary Tinea pedis. Folia Medica 63(2): 292-296.

3. **Vazheva GZ**, Zisova LG, Becheva EA, Chonov VR, Dichev VD, Miteva-Katrandzhieva TM, Rachkovska ZV, Orbetzova MM, Belovezhdov VT (2022) In search of dermatophytes - frequency and etiology of fungal infections in patients with and without diabetes mellitus. Folia Medica 64(6): 922-931.

Congress participation:

1. Real-time PCR methodology in the present-day diagnostics of dermatophyte infections, Zisova L., **Vazheva G**. - E-poster - 29th EADV Virtual Congress, 29 -31^{thst} October, 2020;

2. Tinea barbae, caused by *Trichophyton rubrum* - autounoculation from a primary tinea pedis, **Vazheva G**., Zisova L. - E-poster - 29th EADV Virtual Congress, 29 -31^{thst} October, 2020;

3. Frequency and etiology of fungal infections in patients with and without diabetes mellitus - a modern view of mycological investigations, **Vazheva G**., Zisova L. - E-poster - 17th EADV Symposium, 12-14 May, 2022.

4. Application of real-time PCR in the diagnosis of dermatophyte infections of the hair, skin, and nails, **Vazheva, G.**, Zisova, L. – E-poster – EADV Symposium Seville, 18-20 May, 2023.

Summary

The diagnosis of superficial fungal infections is the subject of intensive research in many countries around the world. The diagnostic methods used are diverse, including both conventional and innovative techniques.

This dissertation presents a study comparing real-time PCR methodology with conventional methods - direct microscopic, cultural, and histological examinations of materials from hair, skin, and nails - in order to demonstrate the benefits and significance of real-time polymerase chain reaction (PCR) for the diagnosis of dermatophytic infections.

The values obtained for the sensitivity, specificity, and efficacy of direct microscopic, cultural, histological, and real-time PCR studies are as follows: 63.71%, 88.89%, and 72.96% ($p < 0.001$); 58.06%, 100%, and 73.47% ($p < 0.001$); 85.96%, 100%, and 90.70% ($p < 0.001$); 88.52%, 100%, and 92.63% ($p < 0.001$). Therefore, H0 is rejected, and the main objective of the dissertation is achieved.

Significant statistical dependencies were also established, confirming the predominant fungal involvement in men and in patients between 40 and 79 years of age. A more frequent occurrence of onychomycosis was also observed in patients with diabetes.

The use of real-time PCR in the diagnosis of dermatophytic infections is a relatively new approach in mycology and is subject to testing and experience from its use. In more and more studies, results are presented comparing conventional methods with real-time PCR or, respectively, conventional PCR with real-time PCR. The results are promising, but the method has not yet established itself as a new gold standard in the diagnosis of superficial fungal infections caused by dermatophytes.

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