Evaluation of a multiplex-PCR-based method for the rapid identification of dermatophytes in nail specimens from patients with suspected onychomycosis

Georgia Vrioni[1], Maria Vassiliki Kazani[2], Constantinos Tsiamis[1], Helen Papadogeorgakis[2], Athanassios Tsakris[1]

[1]Department of Microbiology, Medical School, National and Kapodistrian University of Athens, Greece;
[2]Department of Microbiology, Andreas Sygros Hospital of Cutaneous and Venereal Diseases, Athens, Greece

Summary

Onychomycosis is a common nail disorder that can mimic other nail conditions. Fungal infection of the nail requires a long term systemic antifungal treatment, which means that accurate diagnosis is mandatory. The standard procedure for fungal detection in nails requires direct microscopy and culture, but is still time consuming with high rates of false-negative results. Molecular assays offer an alternative diagnostic process that might resolve problems of the standard methodology. The aim of the current study was to evaluate a direct multiplex-PCR method in rapidly identifying dermatophytes in nail specimens. Two-hundred fifty-two nail specimens of clinically suspected onychomycosis cases were prospectively collected and equally divided for direct microscopy, culture and PCR analysis. PCR was performed using the Dermatophyte PCR kit (Statens Serum Institut Diagnostica, Denmark). The whole procedure was completed within 6 hours, including the extrac-

tion stage. As many as 86 (34.1%) specimens were PCR-positive for dermatophytes, while 79 (31.3%) were positive by any of the conventional methods (55 by both microscopy and culture, 23 only by microscopic examination, and one only by culture). Thus, by using the PCR assay, the number of positive specimens was increased by 7.1%. Furthermore, the percentage of those with a species-specific identification (*T. rubrum* or dermatophytes) was increased by 11.9% Interestingly, previous treatment uptake was not affecting PCR results since 12 specimens (4 negative with both conventional methods and 8 positive only by microscopy) from patients previously treated with antifungal regimens, were PCR-positive. The findings of this prospective study suggest that this rapid and convenient multiplex-PCR method is a promising complementary diagnostic tool for the management of patients with suspected onychomycosis.

Corresponding author
G. Vrioni,
Associate Professor,
Department of Microbiology,
Medical School,
National & Kapodistrian University of Athens
75 Mikras Asias, 115 27 Athens
Tel: +30 210 746 2011
Fax +30 210 746 2210
email address: gvrioni@med.uoa.gr

Key words
Onychomycosis, multiplex PCR, molecular diagnosis

Introduction

Onychomycosis, an infection of the nail apparatus on the fingers and toes, is mainly caused by dermatophytes but also by yeasts and non-dermatophyte molds (NDM) and is responsible for up to 50% of nail diseases.[1-2] Especially, the onychomycosis due to a dermatophyte is referred as "tinea unguium".[3] It concerns mainly the adult population, affecting roughly 2–3 % of the total population.[3-7] The Achilles Project, the largest survey conducted in 16 European countries on patients with foot disease, has estimated the prevalence of onychomycosis in 29.6 %.[4] Particularly, in Greece, according to studies carried out in different geographic regions, fungal infections of the nail are responsible for 15.7% to 24.3% of all nail diseases.[8-11]

The most common cause of onychomycosis is the cosmopolitan anthropophilic dermatophyte *Trichophyton rubrum* (prevalence 60-90% in toenail and 50% in fingernail infections), followed by the also anthropophilic *T. interdigitale* (formerly *T. mentagrophytes* var *interdigitale*).[1, 12-13] In Greece, the dermatophytes causing tinea unguium are *T. rubrum* (89-92%), and *T. interdigitale* (7-8%). Other species like *T. violaceum, T. tonsurans, Epidermophyton floccosum* and *Microsporum canis* are uncommon (1-2%).[8, 10-11]

The tendency of the global incidence of onychomycosis is going to rise, mainly due to the increasing number of high-risk persons with immunodeficiency.[1,14] Moreover, the current medical costs for treating onychomycosis are quite high, especially for the toenail infection or in the context of immunosuppression and are likely to increase in the future.[15-16] The fungal nail infections, even not life-threatening disease, can stigmatize the quality of life of the affected persons.[13, 17-19] Obviously, the early diagnosis of the onychomycosis is beneficial both in economic and emotional level.[13]

Unfortunately, clinical symptoms are not pathognomonic and onychomycosis can resemble other nail disorders. The microscopic examination of nail material (use of 10-30% potassium hydroxide-KOH or calcofluor mounts), the culture of the nails and the morphological identification of the fungus are the conventional procedure. According to the findings, the clinician prescribes the appropriate treatment for dermatophyte, yeast, NDM, or mixed infections.[14,16] The direct microscopy is a rapid and inexpensive method. But, in some cases it seems that is not useful for the determination for the viability or the identity of the fungal species. Also, the direct microscopy presents a percentage of 5-15% false-negative results.

The disadvantages of the culture are the time-consuming (up to 2–6 weeks) and, in the cases of previous unsuccessful or inappropriate treatment, the high percentage of false-negative results (30-50%).[3,12,20] Also, the species identification based on colony characteristics and the microscopic morphology may not allow the final identification of the fungus.[12,20] Thus, for evidence-based treatment of onychomycosis considerable improvement is needed.

The last decade molecular biological methods – such as PCR and matrix-assisted laser desorption ionization (MALDI–TOF MS-time of flight mass spectrometry) have been introduced to overcome the drawbacks of conventional methods for detecting fungi in nail specimens.[3,14,20] Different types of PCR have been performed: multiplexed, nested, real-time PCR, or combined with other techniques such as restriction fragment length polymorphism analysis to produce distinct banding patterns for dermatophyte and NDM species.[3,12,14,20] Recently, Serum Statens Institut (Copenhagen, Denmark) has launched a commercial PCR kit for the detection of dermatophytes in nail scrapings. The Dermatophyte PCR Kit promises to increase the sensitivity, the specificity and the speed of the diagnostic detection of dermatophytes in general and specifically *T. rubrum*.[21-26] The present study was undertaken to evaluate prospectively the performance of this commercial PCR assay in the routine clinical laboratory practice.

Methods

Routine examination

A total of 252 nail specimens from separate patients with suspected onychomycosis were prospectively received and examined between November 2013-November 2016 at two clinical laboratories; one at Andreas Sygros Hospital, Athens and the other at Medical School, University of Athens. Specimens were equally divided into three parts upon arrival for direct microscopic examination, culture and PCR analysis (PCR methods were performed in Microbiology Department of Medical School).

Direct microscopy of the sample for fungal elements was performed by placing the collected nail scrapings onto a clean glass slide with a drop of 20% KOH. In parallel, the second portion of the nail samples were cultured on Sabouraud Dextrose Agar 2% plates (containing cycloheximide 0.4g/L to inhibit growth of molds contaminants) to facilitate growth of dermatophytes. Sabouraud agar plate was also employed for culture of non-dermatophyte fungal species, e.g., yeasts and other molds. The plates were incubated at 30ºC for 3 weeks under controlled humidity. For the identification of the fungal isolates were used macro- and micromorphologic criteria.

PCR assay

The Dermatophyte PCR assay was run according to the manufacturer's recommendations for the dual detection of dermatophytes and *T. rubrum* in nail specimens. Briefly, the extraction of DNA was an easy two-step 15 minutes procedure directly from the nail scrapings. After DNA extraction a multiplex PCR was performed following the manufacturer's instructions. The primer mix contains two pairs of primers directed toward genes encoding chitin synthase 1-*chs1* for detecting dermatophytes generally (panDerm1 5′-GAAGAAGATTGTCGTTTGCATCGTCTC-3′ & panDerm2 5′-CTCGAGGTCAAAAGCACGCCAGAG -3′) and a second (*T. rubrum*-forward 5′-TCTTTGAACGCACATTGCGCC-3′ and *T. rubrum*-reverse 5′-CGGTCCTGAGGGCGCTGAA-3′) targeting internal transcribed spacer gene *its2* for the specific detection of *T. rubrum*. Dermatophyte and *T. rubrum* genomic DNA provided by the manufacturer served as positive controls (control 1 and control 2, respectively). Buffer mix was used as negative control. A plasmid originating from a microorganism other than *T. rubrum* was provided by the manufacturer as an internal control. It is required that the internal control be positive in all PCR-negative nail specimens, which ascertains the absence of inhibitory substances. DNA amplification was performed using a DNA Thermal Cycler (Perkin Elmer Cetus, Waltham, Massachusetts, USA). The presence of specific PCR products was examined by staining with ethidium bromide on 2% agarose gel electrophoresis using DNA molecular weight marker (Roche, Mannheim, Germany) (Fig1). The test was completed within 5 to 6 hours, including the extraction stage and agarose gel electrophoresis.

The diagnostic "gold" standard of the onychomycosis is the direct microscopy and/or culture of the specimens. PCR results were compared with those of conventional methods. In this study, the positive specimens identified by microscopy (hyphae seen) but negative by culture (no fungal grown) were considered as dermatophyte positive. Respectively, in the cases of positive cultures for NDM's they were considered as dermatophyte negative, regardless the result of microscopy.

Results

Examination of the 252 nail specimens revealed that 79 (31.3%) were positive by any of the conventional methods (55 by microscopy and culture, 23 only by

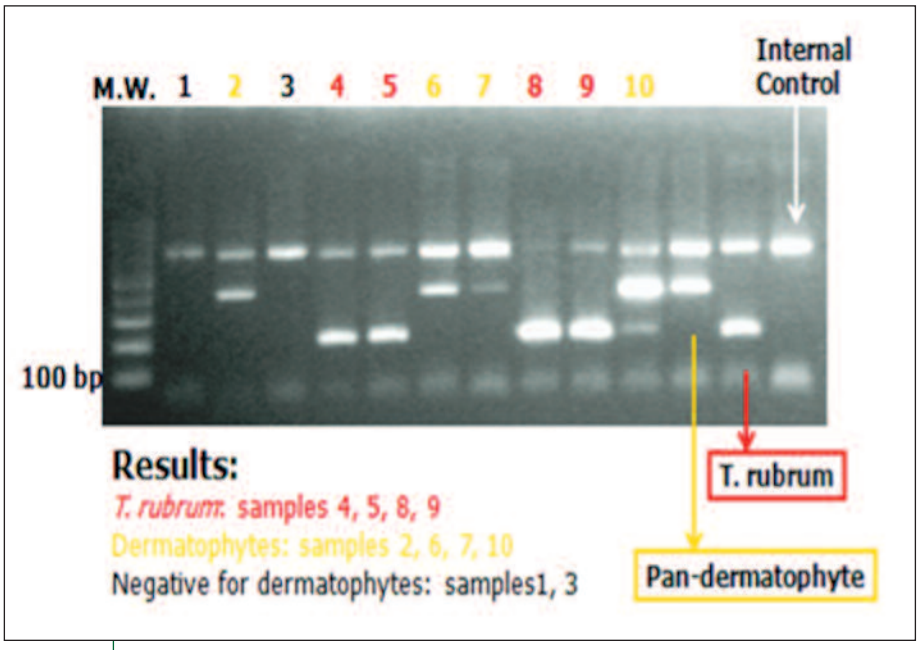


Figure 1 Agarose gel electrophoresis of the amplified multiplex PCR products of clinical specimens from patients with suspected onychomycosis (lanes 2-10). Positive controls for Pan-dermatophyte DNA (366 bp), *T. rubrum* DNA (203 bp), internal control (660 bp) and negative control (lane 1) are included.

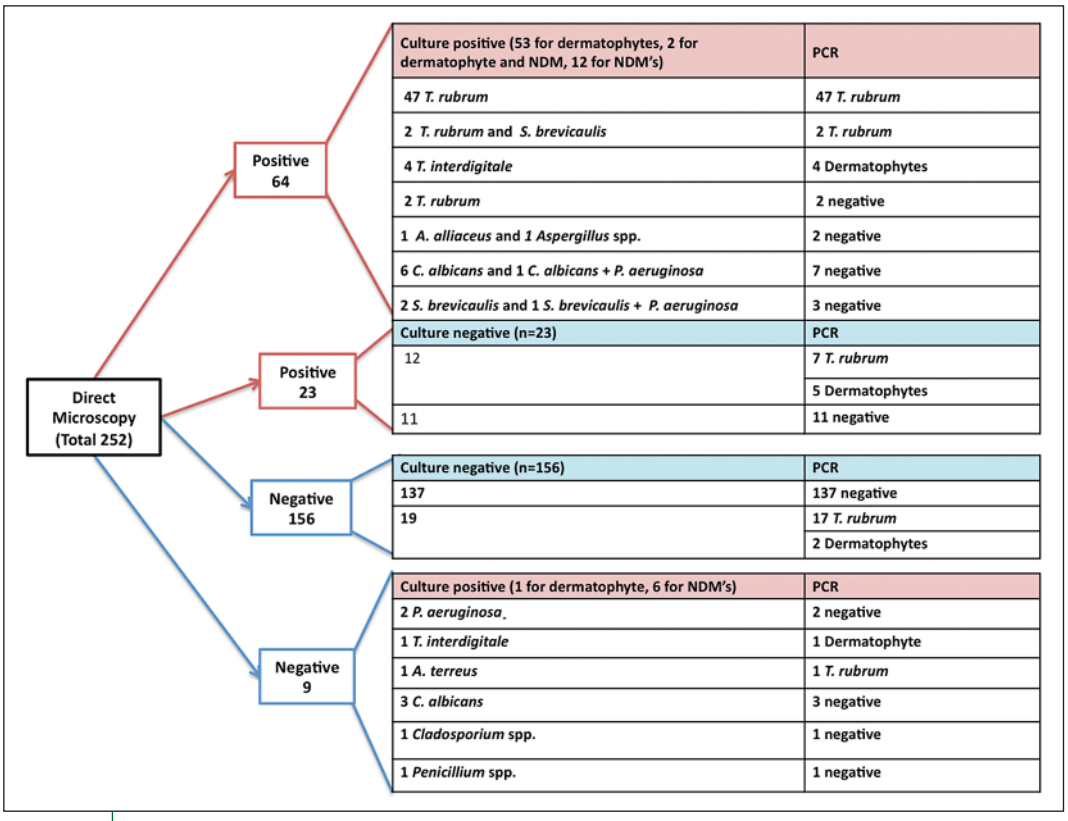
microscopy, and one only by culture), while 86 (34.1%) were PCR-positive for dermatophytes. Among 55 microscopy and culture positive specimens (for *Trichophyton* spp.), only 2 were not detected by PCR. Among 156 specimens negative by both conventional methods, 19 were PCR-positive (17 for *T. rubrum* and 2 for dermatophyte), while the rest of them were confirmed as PCR-negative. Of the 23 microscopy-positive but culture-negative specimens, 12 were PCR-positive (7 for *T. rubrum* and 5 for dermatophytes). From 19 specimens diagnosed by culture as NDM or *Candida* species, one yielded *T. rubrum* by PCR assay. Details regarding the performance of direct microscopy, culture and multiplex PCR are shown in Fig. 2.

The sensitivity, specificity, PPV and NPV of the multiplex-PCR was 83.5%, 88.4%, 76.7% and 92.1%, respectively. The number of positive specimens was increased by 7.1% with the use of the PCR assay (PCR performance gained 20 specimens negative by conventional methods, and it gave 2 false negative results in specimens positive by both microscopic examination and culture) (Fig2). Furthermore, the percentage of those with a species-specific identification (*T. rubrum* or dermatophytes) was increased by 11.9% [PCR performance detected and identified 25 *T. rubrum* and 7 dermatophytes in specimens positive only by microscopic examination (12) or negative by both conventional methods (20), while it missed only 2 specimens positive by both conventional methods]. The combination of PCR with the conventional methods yielded more positive results than the combination of conventional methods alone [99/252 (39.3%) vs 79/252 (31.3%), respectively). This positivity rate vary significantly from that of combined microscopy and culture ($p<0.01$).

Previous treatment uptake was not found to affect PCR results. In more detail, 12 specimens (4 negative with conventional methods and 8 positive only in microscopic examination) from patients previous treated with systemic or topical antifungal regimens were PCR-positive. Finally, no cross reactions were detected with NDM or *Candida* species identified in 17 nail specimens. One culture-positive specimen yielded *A. terreus* in culture, but in PCR it gave *T. rubrum*-positive result (Fig. 2).

Discussion

Onychomycosis is a common nail infection difficult to treat. Correct and specific diagnosis is of a major importance as it allows appropriate antifungal treatment to be promptly initiated. In addition to the classic methods (direct microscopy and fungal cultures) the mo-



Culture positive (53 for dermatophytes, 2 for dermatophyte and NDM, 12 for NDM's)	PCR
47 *T. rubrum*	47 *T. rubrum*
2 *T. rubrum* and *S. brevicaulis*	2 *T. rubrum*
4 *T. interdigitale*	4 Dermatophytes
2 *T. rubrum*	2 negative
1 *A. alliaceus* and *1 Aspergillus* spp.	2 negative
6 *C. albicans* and 1 *C. albicans* + *P. aeruginosa*	7 negative
2 *S. brevicaulis* and 1 *S. brevicaulis* + *P. aeruginosa*	3 negative

Culture negative (n=23)	PCR
12	7 *T. rubrum*
	5 Dermatophytes
11	11 negative

Culture negative (n=156)	PCR
137	137 negative
19	17 *T. rubrum*
	2 Dermatophytes

Culture positive (1 for dermatophyte, 6 for NDM's)	PCR
2 *P. aeruginosa*	2 negative
1 *T. interdigitale*	1 Dermatophyte
1 *A. terreus*	1 *T. rubrum*
3 *C. albicans*	3 negative
1 *Cladosporium* spp.	1 negative
1 *Penicillium* spp.	1 negative

Figure 1 Performance of direct microscopy, culture and multiplex PCR for the 252 nail specimens from patients with suspected onychomycosis

lecular methods have been added to the diagnostic procedures.[3] The commercial kit applied in this study (Dermatophytes Multiplex PCR), in contrast to PCR-based assays which can detect and identify only *T. rubrum*,[27] enables the diagnosis of infection caused by different dermatophytes. Additionally, the kit enables the identification of genus and species in *T. rubrum* infections. Also, in comparison to the classic methods, it increases the detection rates by 4.3 to 21.1%. [20-26] Similarly, the present data showed that implementation of PCR increased detection of dermatophytes by 7.1%. Moreover, Dermatophyte PCR assay enabled the specific identification of dermatophytes in nails specimens positive only by microscopy (12 specimens) or negative by all conventional methods (20 specimens), which it has been also observed in previous studies.[21,23,25-26] These findings were useful for the initiation of the appropriate treatment.[16,28] The investigated nail specimens in this study had been taken under routine conditions and the reason of their collection had been not only to prove a fungal infection, but also to exclude onychomycosis. Therefore, the high percentage of negative results with conventional methods and PCR is not surprising (144/252 specimens, 57.1%).

Discrepant results observed between conventional and PCR methods may be due to various reasons, such as insufficient material, sample quality variation, entrapment of fungus in the keratin, or previous treatment.[21,26] Twelve PCR-positive but culture-negative specimens were microscopy-positive, indicating that this difference was not due to contamination, which represents a potential risk for conventional PCRs. As for the 11 microscopic positive results of cultures and PCR-negatives specimens, a possible explanation is the inhomogeneous distribution of the fungal elements in the sample.[21] Furthermore, the small quantity of the examined material should be the reason of the two PCR-negative, but microscopic- and culture-positive specimens. The finding that one specimen, microscopic-negative, yielded *A. terreus* by culture and *T. rubrum* by PCR may be due to the rapidly overgrowth of contaminating or colonizing mold.[29] In our study, use of antifungal treatment was not an exclusion criterion, like in a previous study.[26] This did not affect PCR results and 12 specimens, although negative by conventional methods, yielded positive by PCR (4 from patients previous treated with systemic and 8 with topical antifungal regimens). Fortunately, the

presence of PCR-inhibitory substances was not a problem in our study, since internal control was positive in all cases.[27,30]

The interpretation of the NDM isolated from nail cultures is controversial. In our study, 18 specimens retrieved various NDM in culture and 12 of them were also positives in microscopic examination. So culture is necessary for identification of NDM that may be involved in nail infections.[3] According to re-defined six main criteria by Gupta and colleagues for diagnosis of onychomycosis due to NDM,[31] microscopic detection of fungi in the KOH preparation and isolation of the pathogen from culture, as well as repeated isolation, are essential for ruling out mere contamination. Moreover, the analysis of our results showed that the specificity of the molecular assay was excellent as none of the NDM positive specimens yielded positive results in Dermatophyte Multiplex PCR.

In our setting, specific detection of *T. rubrum* in parallel with pan-dermatophyte primers is sufficient as the vast majority of positive samples were identified as *T. rubrum* [51 out of 56 (91%) by culture, and 74 out of 86 (86%) by PCR]. Similar results were obtained from recent studies.[25,26] According to previous epidemiological data regarding onychomycosis in Greece, a high prevalence of dermatophytes, with a predominance of *T. rubrum*, has been observed.[8-11] This is in accordance with other European countries, where this Dermatophyte PCR has been successfully introduced into routine practice[21-22] and its results have been very helpful for an evidence-based treatment. Fortunately, dermatophytes belonging to the less terbinafine-susceptible genus *Microsporum* are unanimously reported to be very rare agents of onychomycosis. So, the detection of DNA of dermatophytes and especially the only identification of *T. rubrum* by the pan-dermatophyte primers of Dermatophyte PCR assay could be useful information for the routine clinical practice in every setting.[21,25-26]

According to our calculations, the cost of Dermatophyte PCR kit was 5 fold higher than that of conventional diagnostics (16.2€ vs 3.5€, respectively), in contrast to other studies, where the cost of analysis per sample with conventional PCR is almost the same as with traditional methods.[32] It should be noted that in our study a commercial kit has been used, and, moreover, the exact difference between prices depends on the accounting system, the available laboratory equipment, as well as similar factors. For a precise cost-benefit analysis, it must been mentioned that a PCR approach reduces additional cost and inconvenience for patients requiring two or more visits to the physician, as well as additional diagnostic methods and laboratory work (e.g. subcultures and further phenotypic tests increase the cost of diagnosis). In our setting, turnaround time was reduced from weeks to hours (or several days, under realistic routine conditions). This was a valuable advantage, especially in cases when a rapid decision is needed for the application of systemic therapy. So, a quick final diagnosis could considerably reduce treatment costs. Moreover, Dermatophyte PCR assay had a higher positivity rate than any of other individual conventional test used alone or in combination (Fig2). Considering all the above, in our opinion, total cost could be reduced even further compared with the traditional approach.

The data of our study confirm that the introduction of Dermatophyte PCR assay is a useful diagnostic tool to rapidly detect and identify dermatophytes. The rapid detection and identification in few days by positive PCR result are very important for the clinician. As for the cases of negative result, the classic procedure should be continued. In this way, it is secured that, even in a negative result, the detection of infections by other fungi are not missed. New samples collection and repetition of the procedures can be useful in cases of positive microscopy and negative PCR and culture results. In this frame, taking into consideration the advantages of the method being easy and rapid, it appears that the Dermatophyte Multiplex PCR assay can represent an additional diagnostic tool to the conventional methods, adapted to the economic requirements of every country and fitted to the local epidemiologic interests.

Acknowledgements

This work was partly supported by the Hellenic Society of Medical Mycology. The authors wish to thank Mrs Ioanna Pournou for her excellent technical assistance.

Compliance with Ethical Standards

Funding: This work was supported by internal funding; two Dermatophyte PCR kits were offered by the Hellenic Society of Medical Mycology

Conflict of Interest: The authors declare no conflict of interest in relation to this study

Ethical approval: the study underwent ethics review and approval

Informed consent: All patients included in the study had voluntarily come to the hospital, and all diagnostic tests performed in the context of routine laboratory practice, so there was no need for informed consent.

Περίληψη

Αξιολόγηση ταχείας πολυπλεκτικής PCR στην ταυτόχρονη ανίχνευση και ταυτοποίηση δερματοφύτων σε δείγματα νυχιών από ασθενείς με διάγνωση πιθανής ονυχομυκητίασης

Γεωργία Βρυώνη[1], Μαρία-Βασιλική Καζάνη[2], Κωνσταντίνος Τσιάμης[1], Ελένη Παπαδογεωργάκη[2], Αθανάσιος Τσακρής[1]

[1]Εργαστήριο Μικροβιολογίας Ιατρικής Σχολής, Εθνικό και Καποδιστριακό Πανεπιστήμιο Αθηνών, Αθήνα
[2]Εργαστήριο Μικροβιολογίας, Νοσοκομείο Αφροδισίων και Δερματικών Νοσημάτων «Α. Συγγρός», Αθήνα

Η ονυχομυκητίαση αποτελεί συχνή πάθηση των νυχιών, η κλινική εικόνα της οποίας μπορεί να μιμηθεί άλλες καταστάσεις, όπως ονυχοδυστροφία και ψωρίαση. Η ακριβής διάγνωση είναι απαραίτητη γιατί η μυκητίαση των νυχιών απαιτεί μακροχρόνια συστηματική θεραπεία. Οι κλασικές μεθοδολογίες διάγνωσης, άμεση μικροσκοπική εξέταση των ρινισμάτων των νυχιών και καλλιέργεια, είναι ιδιαίτερα χρονοβόρες, με υψηλά ποσοστά ψευδώς αρνητικών αποτελεσμάτων. Η εφαρμογή μοριακών τεχνικών έχει βοηθήσει στη βελτίωση της ευαισθησίας και στη μείωση του χρόνου διάγνωσης σε περίπτωση ονυχομυκητίασης. Σκοπός της παρούσας εργασίας ήταν η εφαρμογή ταχείας πολυπλεκτικής (multiplex)-PCR μεθόδου στην ταυτόχρονη ανίχνευση και ταυτοποίηση δερματοφύτων σε δείγματα νυχιών ασθενών με κλινική διάγνωση πιθανής ονυχομυκητίασης και η σύγκρισή της με τις κλασικές μεθόδους διάγνωσης, την άμεση μικροσκοσκοπική εξέταση και καλλιέργεια. Υλικό αποτέλεσαν 252 ξέσματα νυχιών από ασθενείς με πιθανή ονυχομυκητίαση που προσήλθαν για εξέταση στα Εξωτερικά Ιατρεία του Νοσοκομείου «Α. Συγγρός». Σε αυτά εφαρμόστηκε, εκτός της άμεσης μικροσκοπικής εξέτασης με ΚΟΗ 20% και της καλλιέργειας σε Sabouraud Dextrose Agar 2%, η πολυπλεκτική-PCR μεθοδολογία (Dermatophyte PCR kit, Statens Serum Institut, SSI Diagnostica, Denmark). Αυτή επιτρέπει ταυτόχρονα την ανίχνευση των δερματοφύτων και την ταυτοποίηση του *T. rubrum*, που αποτελεί και το συχνότερο αίτιο ονυχομυκητίασης, με τη χρήση δύο ζευγών εκκινητών (primers). Η ανάγνωση των αποτελεσμάτων γίνεται με ηλεκτροφόρηση σε γέλη αγαρόζης. Η μέθοδος, μαζί με την απομόνωση του DNA (extraction), ολοκληρώνεται σε περίπου 5 ώρες. Συνολικά από τα 252 δείγματα από ισάριθμους ασθενείς που μελετήθηκαν 86 (34.1%) βρέθηκαν θετικά με την PCR, ενώ 79 (31.3%) με τις κλασικές μεθόδους διάγνωσης (55 με μικροσκοπική εξέταση και καλλιέργεια, 23 μόνο με μικροσκοπική και ένα μόνο με καλλιέργεια). Με τη χρήση της PCR αυξήθηκε τόσο ο αριθμός των θετικών δειγμάτων (αύξηση 7.1%), όσο και η ταυτοποίηση σε επίπεδο είδους (αύξηση 11.9%). Προηγηθείσα λήψη αντιμυκητικής αγωγής δεν επηρέασε το αποτέλεσμα της PCR [12 δείγματα (4 αρνητικά με κλασικές τεχνικές και 8 θετικά με την μικροσκοπική εξέταση) από ασθενείς που είχαν λάβει συστηματική ή τοπική αντιμυκητιακή αγωγή βρέθηκαν PCR-θετικά]. Συμπερασματικά, η εφαρμογή της πολυπλεκτικής PCR αποτελεί μία εύκολη και ταχεία μέθοδο για την άμεση ανίχνευση και ταυτοποίηση δερματοφύτων σε δείγματα νυχιών. Επομένως μπορεί να αποτελέσει συμπληρωματικό εργαλείο των κλασικών μεθοδολογιών στη διαγνωστική προσέγγιση ασθενών με πιθανή ονυχομυκητίαση καθώς και στη θεραπευτική τους αντιμετώπιση.

Λέξεις κλειδιά
Ονυχομυκητίαση, πολυπλεκτική PCR, μοριακή διάγνωση

References

1. Mentoza N, Arora A, Arias CA et al. Cutaneous and subcutaneous mycoses. In: Anaissie EJ, McGinnis MR, Pfaller MA, editors. Clinical Mycology, 2nd ed. Churchil Livingstone: Elsevier; 2009. pp 509-523
2. Richardson MD, Moore CB, Summerbell RC, Gupta AK. Superficial and subcutaneous fungal pathogens. In: Cohen J, Powderly WG, Opal SM et al, editors Infectious Diseases. 3rd ed. Mosby Elsevier; 2010. pp 1853-1867
3. Nenoff P, Krüger C, Schaller J et al. Mycology - an update part 2: dermatomycoses: clinical picture and diagnostics. *J Dtsch Dermatol Ges*. 2014;12:749-777
4. Burzykowski T, Molenberghs G, Abeck D, et al. High prevalence of foot diseases in Europe: results of the Achilles Project. *Mycoses* 2003;46:496-505
5. Piérard G (2001). Onychomycosis and other superficial fungal infections of the foot in the elderly: a pan-European survey. *Dermatology* (Basel) 2001;202:220-224
6. Ghannoum MA, Hajjeh RA, Scher R, et al. A large-scale North American study of fungal isolates from nails: the frequency of onychomycosis, fungal distribution, and antifungal susceptibility patterns. *J Am Acad Dermatol*. 2000;43:641-648.
7. Gupta AK, Jain HC, Lynde CW, et al. Prevalence and epidemiology of onychomycosis in patients visiting physicians' offices: a multicenter Canadian survey of 15,000 patients. *J Am Acad Dermatol.* 2000;43:244-248
8. Koussidou T, Devliotou-Panagiotidou D, Karakatsanis G et al. Onychomycosis in northern Greece during 1994–98. *Mycoses* 2002;45: 29–37
9. Rigopoulos D, Katsiboulas V, Koumantaki E et al. Epidemiology of onychomycosis in southern Greece. *Int J Dermatol.* 1998;37: 925–928
10. Ioannidou DJ, Maraki S, Krasagakis SK, et al. The epidemiology of onychomycoses in Crete, Greece, between 1992 and 2001. *J Eur Acad Dermatol Venereol.* 2006;20:170-174
11. Frangoulis E. Dermatophytosis of the nails: Aetiopathogenesis and clinical and laboratory aspects. *Acta Microbiol Hellenica* 2005;50:282-288
12. Summerbell RC. *Trichophyton, Microsporum, Epidermophyton*, and agents of superficial mycoses. In: Versalovic J, Carroll KC, Funke G et al, editors. Manual of Clinical Microbiology. 10th ed. ASM Press, Washington DC, 2011. pp 1919-1942
13. Nenoff P, Krüger C, Ginter-Hanselmayer G, Tietz HJ. Mycology - an update. Part 1: Dermatomycoses: causative agents, epidemiology and pathogenesis. *J Dtsch Dermatol Ges.* 2014;12:188-209
14. Tchernev G, Penev PK, Nenoff P et al. Onychomycosis: modern diagnostic and treatment approaches. *Wien Med Wochenschr* 2013;163:1-12
15. Córdoba-Fernández A, Távara-Vidalón P, Mandredi-Márquez MJ. Therapeutic approach for toenail onychomycosis: Literature review and cost-effectiveness analysis. *Foot Ankle Online* J. 2014;7:3
16. Ameen M, Lear JT, Madan V et al. British Association of Dermatologists' guidelines for the management of onychomycosis 2014. *Br J Dermatol.* 2014;171:937-958
17. Milobratović D, Janković S, Vukičević J et al. Quality of life in patients with toenail onychomycosis. *Mycoses* 2013;56:543–551
18. Szepietowski JC, Reich A. Stigmatisation in onychomycosis patients: a population-based study. *Mycoses* 2009;52:343–349
19. Belyayeva E, Gregoriou S, Chalikias J et al. The impact of nail disorders on quality of life. *Eur J Dermatol.* 2013;23:366-371
20. Gupta AK, Simpson FC. Diagnosing onychomycosis. *Clin Dermatol.* 2013;31:540–543
21. Brillowska-Dabrowska A, Saunte, DM. Arendrup, MC. Five-hour diagnosis of dermatophyte nail infections with specific detection of *Trichophyton rubrum*. *J Clin Microbiol.* 2007;45:1200–1204
22. Brillowska-Dabrowska A., Nielsen SS, Nielsen HV, Arendrup MC. Optimized 5-hour multiplex PCR test for the detection of tinea unguium: performance in a routine PCR laboratory. *Med Mycol.* 2010;48:828–831
23. Kondori N, Abrahamsson A-L, Ataollahy N, Wenneras C. Comparison of a new commercial test, Dermatophyte-PCR kit, with conventional methods for rapid detection and identification of *Trichophyton rubrum* in nail specimens. *Med Mycol.* 2010;48:1005–1008
24. Chandran NS, Pan JY, Pramono ZA, Tan HH, Seow CS. Complementary role of a polymerase chain reaction test in the diagnosis of onychomycosis. *Australas J Dermatol.* 2013;54:105–108
25. Dubljanin E, Colovic Calovski I, Vujcic I et al. Clinical evaluation of a *T. rubrum*-specific polymerase chain reaction and pandermatophyte polymerase chain reaction in the diagnosis of suspected onychomycosis in 183 Serbian patients *Br J Dermatol.* 2014;171:1555–1608
26. Spiliopoulou A, Bartzavali C, Jelastopulu E et al. Evaluation of a commercial PCR test for the diagnosis of dermatophyte nail infections. *J Med Microbiol.* 2015;64:25–31
27. Brasch J, Beck-Jendroschek V, Gläser R. Fast and sensitive detection of *Trichophyton rubrum* in superficial tinea and onychomycosis by use of a direct polymerase chain reaction assay. *Mycoses* 2011;54:e313–e317
28. Nenoff P, Krüger C, Paasch U, Ginter-Hanselmayer G Mycology – an update Part 3: Dermatomycoses: topical and systemic therapy. *J Dtsch Dermatol Ges* 2015;13:387-410

29. Bontems O, Hauser PM, Monod M. Evaluation of a polymerase chain reaction-restriction fragment length polymorphism assay for dermatophyte and nondermatophyte identification in onychomycosis. *Br J Dermatol* 2009;161:791–796
30. Mehlig L, Garve C, Ritschel A et al. Clinical evaluation of a novel commercial multiplex-based PCR diagnostic test for differential diagnosis of dermatomycoses. *Mycoses* 2014;57:27–34
31. Gupta AK, Drummond-Main C, Cooper EA et al. Systematic review of nondermatophyte mold onychomycosis: diagnosis, clinical types, epidemiology, and treatment. *J Am Acad Dermatol.* 2012;66:494–502
32. Kardjeva V, Summerbell R, Kantardjiev T et al. Forty-eight-hour diagnosis of onychomycosis with subtyping of *Trichophyton rubrum* strains. *J Clin Microbiol.* 2006;44:1419-1427