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Original article

Use of real time multiplex PCR for the diagnosis of dermatophytes onychomycosis in patients with empirical antifungal treatments

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ABSTRACT

Background: Onychomycosis is a nail fungal infection mainly caused by dermatophytes. Diagnostic confirmation is conventionally made by direct microscopy and culture, which suffer from low or moderate sensitivity. Several molecular methods have been used for dermatophytes detection and identification directly from nail samples. The aim of this study was the evaluation of the DermaGenius®(DG) multiplex kit in detecting and identifying dermatophytes from nail samples of untreated and treated patients with a clinical suspicion of onychomycosis.

Methods: All the patients underwent a nail scarification, performed with a sterile scalpel to collect small nail fragments from the suspected site of infection. All nail clippings were first analysed by microscopic and culture methods to define a diagnostic confirmation. DG PCR assays were retrospectively applied to the same samples.

Results: A total of 109 toenails were collected for the microscopic, culture and DG PCR assays. The sensitivity, specificity, positive and negative predictive values of DG in the onychomycosis diagnosis in all 109 patients were respectively 78.5%, 100%, 100%, and 75.9%. Only for cultural exams the rate of positive results was significantly different in the two groups of patients with a percentage of 73.7% in untreated patients versus a 40.7% value in treated patients ($P < 0.05$).

Conclusions: Our results suggest that the use of DG kit could be useful to confirm the diagnosis of onychomycosis, implementing sensitivity especially in patients who underwent antifungal treatments without any clinical improvement.

1. Introduction

Onychomycosis can be defined as a nail fungal infection characterized by clinical signs such as color change, subungual hyperkeratosis and subsequent onycholysis and onychoschisis. This infection represents more than 50% of the nail pathologies [1,2], even if it is infrequent in children. Onychomycosis incidence progressively increases with age, up to a prevalence of 20% between 40 and 60 years and exceeds 40% in the elderly [3–5]. Risk factors for the onset of onychomycosis are trauma [6], advancing age [5,7] and comorbidities, such as diabetes [8,9], obesity and immunosuppression [10,11]. The main etiological agents of onychomycosis are dermatophytes and particularly *Trichophyton rubrum*, which is responsible for most fungal nail infections. *T. rubrum* is followed by non-dermatophyte molds (NDMs) and yeasts [2,12–16]. The onychomycosis can arise in different ways. A pre-existing infection outbreak from another location, usually on the foot's surfaces, can be considered as a possible source. On the other hand, onychomycosis could start as a primary infection of the nail. The onset of such types of nail infections should be considered as a reservoir for dermatophytes, which can easily spread in domestic environments and community backgrounds. Con-

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cerning the increasing rate of fungal infections and their possible role in health complications in some patients (for example, diabetics suffering from foot pathologies and immunocompromised patients), a prompt laboratory diagnosis is advisable to correctly confirm fungal etiology. Negative results from laboratory tests are helpful to quickly plan a differential diagnosis from other onychopathies such as psoriasis, squamous cell carcinoma of the nail bed or yellow nail syndrome or chronic trauma [17–19]. The diagnosis of onychomycosis is conventionally made by direct microscopy through potassium hydroxide preparations and fungal cultures. These traditional methods suffer from a moderate sensitivity rate [3]. Moreover, culture-based detection methods may take associated with a long turnaround time (TAT) and remain negative in 20–30% of positive microscopy cases [20,21]. Several molecular assays have been produced for fungal etiology confirmation in clinical settings [22,23] and several commercial kits have been validated for dermatophytes detection in nail samples [24–26]. The introduction of these molecular methods, that allow detection and identification of dermatophytes directly from the nail plate, could be useful to confirm the diagnosis of onychomycosis, especially in those patients who started empirical antifungal treatments, without a mycological diagnosis or any clinical improvement. In these specific cases, the use of a molecular method would help to get a rapid and more sensitive diagnosis, favoring a more appropriate use of the antifungal drugs [27]. This study aimed to evaluate the diagnostic role of the DermaGenius®(DG) multiplex kit (PathoNostics, The Netherlands) in detecting and identifying dermatophytes from nail samples of patients with suspected onychomycosis. Involved patients have suffered from inadequate previous antifungal treatments or problematic conventional diagnosis outline.

2. Material and methods

A non-interventional, retrospective study was conducted at the mycology laboratory of the University hospital “Policlinico-Vittorio Emanuele”, Catania, Italy, over a 1-year period (January 2019–December 2019). According to the applicable relevant national legislation, prior formal approval of local Research Ethics Committee and informed consent were not mandatory, so they were not applied.

The intention was to involve patients who have suffered from difficulties in onychomycosis diagnostic confirmation and/or from problems in the consequent therapeutical management, to simplify or redirect diagnostic procedures and therapeutic resolution. According to this initial idea, two groups of patients with suspected onychomycosis were considered. The suspicion of onychomycosis was derived from the observations of one or more of the following signs: nail thinness, lifting of the nail plate, nail distortion, nail opacification and surrounding tissues inflammation. One group of patients involved untreated patients with suspected onychomycosis, while a second one was organized with patients suffering from a history of previous inefficient antifungal treatments. Inefficient antifungal treatments were topical and/or systemic therapies, applied for respectively 2–9 months and 4–10 months and followed by the absence of a clinical improvement. Systemic antifungal drugs frequently prescribed were fluconazole (52.9%), itraconazole (23.5%) and terbinafine (17.6%), while topical antifungals used were amorolfine (32.3%), ciclopiroxolamine (42.5%) and tioconazole (25.2%). All the enrolled patients underwent nail scarification, which was practised with a scalpel to collect small nail fragments from the site involved in the suspicion of the infection. Each specimen was divided into three pieces: one piece was reserved for microscopy, a second one was dedicated to culture, and the remaining nail pieces were stored at room temperature in sterile screwed vials for the subsequent DNA extraction and PCR assay. The microscopic examination was performed resuspending the nail clippings in 15% potassium hydroxide and 40% DMSO (Dimethyl Sulfoxide) buffer, to dissolve larger keratinocyte material, after clarification at room temperature for at least 30 min. Microscopic slides were observed with a light microscope to highlight the presence or absence of fungal elements. For the culture, each sample was inoculated on both Sabouraud chloramphenicol and Sabouraud chloramphenicol plus cycloheximide dextrose agar plates. Agar plates were incubated at 30 °C for 4 weeks and identification for the molds was performed using standard phenotypic methods, based on both macroscopic and microscopic morphological studies. Yeasts identification was performed using ID32C kit (bioMérieux, Marcy l′Étoile, France). Among all the cases, dermatophytic onychomycosis was finally defined as the combination of clinical evidence of nail disorder and positive results for dermatophytes from microscopy and/or cultures, which can be considered as gold standards for dermatophytic onychomycosis. The DermaGenius® complete multiplex kit (PathoNostics, The Netherlands) was used for the detection the most clinical prevalent dermatophytes species (12 targets: *T. rubrum*, *T. interdigitale*, *C. albicans*, *T. tonsurans*, *T. mentagrophytes*, *T. soudanense*, *T. violaceum*, *T. benhamiae*, *T. verrucosum*, *M. canis*, *M. audouinii* and *E. floccosum*). The DNA was extracted by using the PathoNostics Extraction Kit (PathoNostics, The Netherlands) following the manufacturer's instructions. The multiplex PCR was performed according to manufacturer’s instructions [28]: 5 µl of DNA extract were added to the PCR mix and a Rotor-Gene Q (Qiagen) was used for amplification and melting curve analysis. Positive control and negative template control (NTC) were included in each PCR protocol.

Positive molecular results were confirmed after the observation of melting curve analysis, which allowed differentiation of specific dermatophyte species.

3. Statistical analysis

Data were analysed using the MedCalc Statistical Software version 17.9.2 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2017). Medians with ranges were used to describe non-normally distributed continuous variables and compared using the Mann-Whitney U-test. Categorical variables are reported as percentages and compared using the two-tailed χ^2 test or Fisher’s exact test, as appropriate. The following parameters of diagnostic performance and their 95% confidence intervals (CIs) in a group of naïve patients and empirical treated patients were calculated: sensitivity, specificity, positive and negative predictive value (PPV, NPV). Results are significant at the 5% significance level ($P < 0.05$).

Table 1
Characteristics of patients included in the study.

Patients	Total (n = 109)	Onychomycosis (n = 65)	No Onychomycosis (n = 44)	*p value*
Male sex (%)	58 (53.2)	38 (58.5)	20 (45.4)	0.18
Age, median years (range)	60 (18–86)	69 (25–86)	53 (18–76)	< 0.001
Diabetes	17 (15.6)	14 (21.5)	3 (6.8)	< 0.05
Previous trauma	25 (22.9)	15 (23.1)	10 (22.7)	0.96
Empirical antifungal treatment (%) **No. of patients (%) with a:**	57 (52.3)	27 (41.5)	30 (68.2)	< 0.05
Positive Microscopy	57 (52.3)	57 (87.7)	0	< 0.0001
Positive Culture	42 (38.5)	39[a] (60)	3 (6.8)	< 0.0001
Positive DG	51 (46.8)	51 (78.5)	0	< 0.0001

[a] 10 case with culture positive for a non-dermatophyte molds and 2 case with culture positive for *Candida parapsilosis*.

4. Results

A number of 109 patients were included in the study, with a related total of 109 toenails collected for microscopic, culture and DG PCR assays. The combination between a clinical nail disorder and positive results from microscopy and/or culture allowed to confirm a total of 65 (59.6%) cases of onychomycosis, diagnosed only by conventional laboratory tests. Patients' characteristics are shown in Table 1. Age (P < 0.001) and diabetes (P < 0.05) were significantly higher in the group of patients with onychomycosis while the empirical antifungal treatment (P < 0.05) was higher in the group of patients without onychomycosis. The sensitivity, specificity, PPV, and NPV and their 95% confidence intervals (CIs) of microscopy for the diagnosis of onychomycosis in all 109 patients were respectively 87.7% (77.2–94.5%), 100% (91.9–100%), 100%, and 84.6% (74.2–91.3%). The microscopy was positive in 57/109 samples among which 31 specimens had a fungal culture positive for dermatophytes (19), yeasts (2) or NDMs (10).

The fungal cultures were positive in 42/109 samples and in particular 27 were dermatophytes, 2 were *Candida parapsilosis*, and 13 were positive for a NDMs including *Fusarium solani* (n = 2) and *Fusarium oxysporum* (n = 1), *Acremonium kiliense* (n = 2) and *Acremonium potronii* (n = 1), *Aspergillus flavus* (n = 4), *Aspergillus niger* (n = 2) and *Aspergillus sydowii* (n = 1). *Trichophyton rubrum* was the most frequently detected species with 15/27 (55.5%) followed by *T. mentagrophytes* with 12/27 (44.4%). The sensitivity, specificity, PPV, and NPV of the culture methods were respectively 60% (47.1–72%), 93.2% (81.3–98.6%), 92.8% (81.1–97.5%), and 61.2% (53.7–68.2%). The PCR was positive in 51/109 nail samples, among which 43 where microscopy positive (43/51, 84.3%) and 26 where culture positive for dermatophytes (23/51, 45.1%).

The DG PCR assay was positive in 8 samples which showed a negative microscopic result and a positive culture for a dermatophyte. Furthermore, the same assay was positive in 25 samples which had a positive microscopic result and a negative culture for a dermatophyte. These results showed the ability of the DG PCR assay in identifying non-growing fungal agents. Among the 51 DG PCR positive results, thirty samples were associated with a *T. rubrum* signal, while seventeen and three samples were respectively associated with *T. interdigitalis* and *T. mentagrophytes* signals. Only one positive result was associated to an *M. canis* signal. The DG PCR results obtained from the 109 nails specimens, compared with conventional laboratory test results, are reported in Table 2. The sensitivity, specificity, PPV, and NPV of DG PCR for the diagnosis of onychomycosis in all 109 patients were respectively 78.5% (66.5–87.7%), 100% (91.9–100%), 100%, and 75.9% (66.4–83.3%).

Empiric topical and/or systemic antifungal treatment was applied in 52.3% of patients included in the study. A difference in the administration of antifungal treatment between patients with and without onychomycosis (41.5% vs. 68.2%; P < 0.05) were observed. In patients with confirmed onychomycosis, the analysis of the results was performed to highlight possible differences between untreated and treated patients. Significant differences were not detected about the frequency of microscopic positive cases

Table 2
PCR results obtained from the 109 nails in comparison with microscopy and culture results.

Techniques	PCR positive			
	Dermatophyte	*Candida albicans*	Negative PCR	Total
Microscopy				
Positive	43 (39.4%)	0	14 (12.8%)	57 (52.3%)
Negative	8 (7.3%)	0	44 (40.4%)	52 (47.7%)
Culture				
Dermatophytes	26 (23.8%)	0	1 (0.9%)	27 (24.8%)
Candida albicans	0	0	0	0
NDM[a] + Yeast[b]	0	0	15 (13.8%)	15 (1.8%)
Negative	25 (22.9%)	0	42 (38.5%)	67 (61.5%)
Total	51 (46.8%)	0	58 (53.2%)	109 (100%)

% refer to the total number of samples (n = 109). PCR, polymerase chain reaction.
[a] Non-dermatophytes molds.
[b] Yeasts other than *Candida albicans*.

Table 3
Comparison of positive results of microscopic, cultural and DG PCR assays in untreated and treated patients.

Techniques	Patients with onychomycosis (65)		
	Antifungal treatment (27)	Untreated (38)	*p value*
Positive Microscopy	24 (88.9%)	33 (86.8%)	1.00
Positive Culture	11 (40.7%)	28 (73.7%)	< 0.05
Positive DG	25 (92.6%)	26 (68.4%)	< 0.05
	Patients with onychomycosis (53)[a]		
	Antifungal treatment (25)	**Untreated (28)**	***p value***
Positive Microscopy	22 (88%)	23 (82.1%)	0.70
Positive Culture	9 (36%)	18 (64.3%)	< 0.05
Positive DG	25 (100%)	26 (92.8%)	0.49

[a] Patients' number deprived by 12 cases of onychomycosis from non-dermatophytes molds or yeasts.

Table 4
Sensitivity, specificity, VPP and VPN in naïve patients and in those treated with previous empirical antifungal drugs.

Techniques	Sensitivity (%)		Specificity (%)		PPV[a]		NPV[b]	
	Untreated (52)	PAT[c] (57)	Untreated (52)	PATc (57)	Untreated (52)	PATc (57)	Untreated (52)	PATc (57)
Microscopy	86.8%	88.9%	100%	100%	100%	100%	73.7%	91%
Culture	73.7%	40.7%	100%	90%	100%	78.6%	58.3%	62.8%
DG	68.4%	92.6%	100%	100%	100%	100%	53.8%	93.7%

[a] Positive predictive value.
[b] Negative Positive predictive value.
[c] Previous empirical antifungal drugs.

between the untreated and treated patients' groups (86.8% versus 88.9%). On the other hand, the frequency of culture positive results in the group of untreated patients was significantly higher (73.7% versus 40.7%) than in treated patients with onychomycosis ($P < 0.05$). Positive results from the DG assay were 92,6% in the untreated patients' group and 68,4% in the treated patients' group ($P < 0.05$). Analyzing the frequency of positive cases from microscopy, culture and DG PCR assays, and excluding the 12 cases of onychomycosis from NDMs, only culture analysis showed a significant difference. Infact, the frequency of culture positive results in the group of untreated patients were significantly higher (64.3.% versus 36%) than in patients with onychomycosis treated with previous empirical antifungal drugs ($P < 0.05$) (Table 3). Sensitivity, specificity, PPV, and NPV of microscopic, culture, and DG PCR assays among treated and untreated patients for the diagnosis of onychomycosis are shown in Table 4.

5. Discussion

Diagnostic confirmation for dermatophytic onychomycosis can be considered an ongoing challenge, because of the possibility of alternative non-infectious nail disorders with a similar clinical impact (trauma, psoriasis, yellow-nail syndrome, etc.) [29]. Improvements in diagnostic flows are essential to avoid the unnecessary use of antifungal treatments and expressions of their side effects. Empiric treatment for onychomycosis is still largely prescribed by many general practitioners, podiatrists, and some dermatologists [30].

In our retrospective study, the diagnostic role of the DermaGenius®(DG) multiplex kit in confirming dermatophytes onychomycosis was evaluated. The aim was the investigation of the antifungal treatment impact on the possible molecular detections of dermatophytes from nail samples. For onychomycosis diagnosis, most clinical laboratories use a combination of direct microscopic and culture methods, despite a long TAT and a low sensitivity, especially in patients who previously underwent empirical antifungal treatments. According to our data, the sensitivity of the microscopy reached 87.7% with 100% specificity, while the culture sensitivity was only 60% with 93.2% specificity, regardless of the presence of antifungal treatment or not. In patients with onychomycosis treated with previous empirical antifungal drugs, the sensitivity of the culture was significantly lower (40.7%), while no significant difference between the two groups of patients in microscopy sensitivity was observed. The DG PCR assay has been specifically designed to detect the most clinical prevalent dermatophytes species affecting nails. There is currently no other commercial real-time PCR assay that allows the detection of dermatophytes at the species level and *Candida albicans* or NDM in nails. Sensitivity and specificity rates for DG PCR assay in all 109 patients were respectively 78.5% and 100%. Excluding 12 positive cases with an etiology related to NDMs and yeasts such as *C. parapsilosis*, the PCR sensitivity was 96.2%. Analyzing the data regardless of the empirical antifungal treatment, the results obtained by PCR were not significantly different from those obtained for microscopy and culture, although the PCR assay was positive in 25 samples with negative culture and positive microcopy for dermatophytes. Among these 25 samples, sixteen were from patients who had performed previous empirical antifungal treatment. Conversely, data differ in a significant way considering antifungal treatment. 11,11% of treated patients had a negative result from the microscopic exam, a positive result from cultural analysis and finally a positive molecular detection for dermatophytes. 3,70% of treated patients showed negative results both from cultural and microscopic exams but had a diagnostic confirmation of their clinical condition by molecular DG assay. According to other literature data about molecular diagnosis in dermatophytic onychomycosis confirmation [23,26,31], patients who had used oral or topical antifungal agents in the previous 3–6 months were excluded. This choice was applied to avoid false-positive related to the impossibility of PCR assays in distinguishing dead fungal genome fragments from alive ones. Our results show that eventual antifungal treatment has not a significant impact on the molecular detection of dermatophytes from nail samples: fungal DNA can result from a molecular analysis even if vital microorganisms are inhibited by antifungal drugs. This information can also be considered as a limitation for the DG PCR assay: some symptomatic treated patients with a positive DG test are difficult to manage because the presence of fungal DNA could be related to non-vital

microorganisms and clinical signs could derive from other onychopathies. Another possible limitation for the study is the impossibility to detect the fungal genome of microorganisms different from dermatophytes: NDMs may be involved in onychomycosis, but molecular tests are not equipped to detect them. Although these limitations, DG assay shows benefits related to the possibility to reduce inadequate antifungal treatments and subsequent side effects on the patients' health or resistance rate spread. The detention of dermatophytes in nails is highly associated with infection and very less with colonization. According to the obtained results (greater sensitivity than culture and 100% specificity), we believe that patients treated with previous antifungal drugs and without any clinical improvement should resolve diagnostic difficulties with molecular confirmation. DG PCR assay can be considered as a confirmation step in dermatophytic onychomycosis diagnosis also in urgent cases, considering the long turnaround time for microscopic and cultural exams and the false-negative results (20–30%) of these conventional methods.

In conclusion, we believe that the use of DG PCR assay for the identification of dermatophytes and *Candida albicans* directly from nail samples, could be useful to confirm onychomycosis diagnosis, especially in patients who started antifungal treatments without a mycological assessment and without any clinical improvement.

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Ethical approval

The authors confirm to adhere to the ethical policies of the journal. For the study, prior formal approval of the local Research Ethics Committee and informed consent are not mandatory. This information agrees with legislative degree 81/2008.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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