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EVALUATION OF REAL TIME POLYMERASE CHAIN REACTION ASSAY FOR IDETIFICATION OF COMMON DERMATOPHYTE SPECIES

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Abstract: *Traditionally, laboratory detection and identification of dermatophytes consists of culture and microscopy which yields results within approximately 2–6 weeks. A real-time PCR assay was developed which simultaneously detects and identifies the most prevalent dermatophytes directly in nail, skin and hair samples and has a turnaround time of less than two days. A real-time PCR assay was developed which simultaneously detects and identifies the most prevalent dermatophytes directly in nail, skin and hair samples and has a turnaround time of less than two days. For 681 clinical samples, we compared the results obtained from both culture and real-time PCR. This study showed that real-time PCR significantly increased the detection rate of dermatophytes compared to culture. Furthermore, excellent concordance between culture and real-time PCR identification was achieved.*

Key words: *dermatophyte, dermatophytosis, diagnosis, real time polymerase chain reaction.*

1. Introduction

Dermatophytosis is one of the most common infectious diseases in the world. Dermatophytes are keratinophylic fungi which comprise three genera: *Trichophyton, Microsporum* and *Epidermophyton,* that are responsible for the majority of superficial fungal infections of the skin, hair and nail plates.

The identification of dermatophyte species is essential for appropriate diagnosis and treatment of dermatophytosis. Currently, the diagnosis of dermatophytosis is based on the demonstration of fungal structures in direct microscopy of clinical specimens plus microscopic and macroscopic observation of *in vitro* cultures and identification of the causative species. This combination is referred to as the diagnostic gold standard [3]. With direct microscopic examination of samples, fungi can be detected, but it is impossible to identify the species present in the samples. Culturing and subsequent species identification is slow, usually requiring 2–4 weeks, and has a low sensitivity (c. 70%) especially for hair and nail infections [2]. Furthermore, both microscopy and identification of culture requires considerable training of personnel.

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In the past years several molecular methods for the detection and identification of dermatophytes from clinical samples have been tested. Conventional polymerase chain reaction (PCR)-based diagnostic methodologies have not offered a better alternative to the standard methods, mainly because the extraordinary genomic homogeneity characteristic of the dermatophyte species has not permitted successful design of specific primers for each dermatophytic species.

A potentially more sensitive approach is used in real-time PCR (rtPCR) techniques that are based on detection with SYBR green or with specific fluorophore-labeled oligonucleotide probes, assay which has revolutionized the way human pathogens are diagnosed.

The aim of this study was to evaluate the performances of a multiplex rtPCR assay, for rapid and sensitive detection and identification of the common pathogenic dermatophytes directly in clinical specimens.

2. Materials and Methods

2.1. Study population

The study population was selected from patients who attended the dermatology outpatient Dermamed clinic Braşov for lesions that were suggested dermatophytic infections:

- circinate erythematous plaques with vesicles, pustules and scales on the glabrous skin, or erythematous macerated lesions on the folders
- thickening and discoloration of the nail plate, subungual hyperkeratosis, transverse and longitudinal striae, nails pitting or onycholysis.
- erythemato-squamous alopecic plaques or discolored, lusterless, and brittle hairs, or inflammatory, purulent and crusted plaques on the scalp.

To evaluate the diagnostic performance of rtPCR were exanimate 681 clinical samples (351 samples of nail scraping, 307 samples of skin scales and 23 samples of hair) from 638 patients suspected for mycosis in the period May 2009 to April 2011.

All clinical specimens were separated into three parts: the first part was examined by direct microscopy with 40% KOH, the second part was cultured onto Sabouraud dextrose agar and the third part was stored at -20ºC for use in the rtPCR.

Culture was performed with Sabouraud dextrose agar supplemented with chloramphenicol, gentamicin and cycloheximide (Bio-Rad, France), at 37ºC for up to 4 weeks. Identification by morphological methods was performed based on the typical macroscopic and microscopic characteristics after 3 days, 14 days and respectively 4 weeks of incubation.

DNA from the clinical specimens was extracted with the QiaAmp DNA extraction kit (Qiagen, Hilden, Germany) after preceding enzymatic digestion with Proteinase K (Qiagen).

Briefly, finely cut clinical specimens were incubated overnight at 56°C in 200 µl lysis buffer containing: 10 mmol/l Tris–HCl pH 8.0, 10 mmol/l Na_2-EDTA (ethylenediamine tetraacetic acid), 100 mmol/l NaCl, 2% sodium dodecyl sulfate (Sigma, SUA) supplemented with 15 µl proteinase K (20 mg/ml) and 20 µl from solution 1 mol/ml dithiothreitol (Sigma). An additional 10 µl proteinase K was added and the samples were incubated for a further 3 h. Subsequently, nucleic acid extraction was performed from each specimen with the QiaAmp DNA extraction kit (Qiagen).

At the end of the procedure, DNA was eluted in 60 µl of AE buffer (10 mmol/l Tris-Cl, 0.5 mmol/l EDTA, pH 9.0) provided by the manufacturer. Negative controls (sterile distilled water) were included in each extraction run.

The target for rtPCR was the ITS1 region located between the genes coding for 18S and 5.8S rRNA. Primers and probes were designed using the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov), following guidelines specified by Applied Biosystems (table 1). Discrimination of targets is achieved by using species-specific and species-complex-specific *Taqman* and *minor groove binder (MGB)* probes carrying discrete fluorophores. Anchor probes were designed to match ITS1 sequences of two or three dermatophyte species, whereas sensor probes were designed to perfectly match only one of those species. Thus, this assay can detect up to three different species in one probe using melting curve analysis.

Table 1

Primers and probe sequences

Specia amplificată	Primerul sau secvența probă	Markerul 5′	Markerul 3’	Mărimea produsului PCR (bp)
T. mentagrophytes complex	5’-GAGGCAACCGAGTAA-3’	FAM	NFQMGB	287
T. rubrum	5′-CACCAAGAAAAAATTCTCTGAA- 3′	FAM	NFQMGB	229
T. violaceum	5’-CAAGGAAAATTCTCTGAAGGGCTG-3’	FAM	NFQMGB	301
T. interdigitalae	5′-ACGCTGGACCGCGC-3′	NED	NFQMGB	260
T. verrucosum	5’-GGAGGACAGACATCAAAAAATCTTGAAGA-3’	VIC	NFQMGB	268
T. tonsurans	5′-TTGAGCCGCTATAAAG-3′	VIC	NFQMGB	260
M. canis	5’-GGTGGGTGGTTACTG-3’	FAM	NFQMGB	245
M. audouinii	5’-GGTGGGTGGTTATTG-3’	VIC	NFQMGB	245
E. floccosum	5’- CTA CGA AAT CTC CAT AGG TGG TTC AGT CT-3’	LC705	FL	285
Microsporum spp.	5′-CCCGAAGCTCTTCCGT-3′	FAM	NFQMGB	250
Pandermatophyte	5′- GGT TGC CTC GGC GGG CC -3′			155

5 µl of DNA isolated from rtPCR reactions containing 4 µl of LC Multiplex Master Hyb Probes (Roche), 400 nM primer DERMF3 and 1300 nM primer DERMR2, 330 nM each of LC hybridization probe, 1.3 mM $MgCl_2$, 0.5 µl dimethylsulphoxide, and 0.2 units of uracil DNA glycosylase (Roche). Positive and negative extraction and PCR controls were included in each run.

The following PCR program was performed on a LightCycler 2.0 (Roche): initial denaturation for 10 min at 95°C, and 50 cycles of 20 s at 95°C, 20 s at 55°C, and 20 s at 72°C. This was followed by a melting curve analysis (1 min at 95°C, cooling for 1 min at 50°C, and ramp to 85°C, at a ramp rate of 0.1°C/s).

Table 2

Melting temperature (Tm) ranges determinated for different dermatophyte species on LightCycler 2.0 in four detection channels

Species	Channel	Tm (°C)
M. canis	610	65,00-67,50
M. audouinii	610	60,00-62,00
T. rubrum	640	66,30-68,00
T. verrucosum	640	62,30-64,00
T. violaceum	640	64,50-66,25
T. mentagrophytes	670	59,90-61,90
T. interdigitale	670	64,00-66,00
T. tonsurans	670	56,60-58,60
E. floccosum	705	67,50-69,50
Internal Control	705	63,80-65,80

The presence of an amplification curve in the respective channels (610, 640, 670 and 705 nm) of the LightCycler, in conjunction with a melting curve with the appropriate melting temperature (Tm) and a Tm peak height of $\geq$ 0.01, was considered to be a positive result for the given dermatophyte species (table 2).

2.2. Statistical analysis

Sensitivity, specificity, positive and negative predictive values, likelihood ratio were calculated for all testes with a 95% interval.

3. Results

194 samples (28.48%) out of 681 patient specimens analyzed by conventional “gold standard” diagnostics (microscopy and culture) yielded positive results while the rtPCR detected dermatophyte DNA in 198 samples (29.07%). Overall, direct microscopy was more sensitive than culture, as fungal filaments were observed in 188 specimens (27.6%), while culture was positive for an aetiological agent in only 161 specimens (23.64%). 6 culture positive specimens were negative in direct microscopy and also 7 specimens positive in direct microscopy were negative in culture.

The specificity of direct microscopy and culture were 94.35% and 99.58% respectively. Positive predictive values of direct microscopy were 87.44% and the culture one 98.7%. Negative predictive values of the “gold standard” tests were 96.78% for direct microscopy and 91.89% for culture.

The sensitivity of the rtPCR was 97.5% (198/203 samples) with a specificity of 100%, a positive predictive value of 100% and a negative predictive value of 98.96%. RtPCR showed a significant increase in detection rate for dermatophytes in clinical samples compared to culture (p=0.0000).

The isolates obtained in culture included 137 dermatophytes belonging to *Trichophyton* genera (117 *T. rubrum* isolates, 17 representatives of the *T. interdigitale*, one *T. mentagrophytes var. asteroides* isolate, two *T. mentagrophytes var. quinckeanum* isolates), 20 dermatophytes from *Microsporum* genera (19 isolates of *M. canis*, one *M. audouinii* isolate) and 4 dermatophytes of *Epidermophyton* genera (4 *E. floccosum* isolates) (figure 1). Also culture scored 43 samples positive for non-dermatophytes infection (41 *Candida albicans* isolates, two *Aspergillus* isolates) and 6 samples positive for an association of one dermatophyte and one levuric infection.

The rtPCR detected dermatophyte DNA in 198 samples being identified 170 dermatophytes of *Trichophyton* genera (*T. rubrum* in 146 specimens, *T. interdigitale* in 21 probes), 23 of *Microsporum* genera (*M. canis* in 22 probes and *M. audouinii* in one specimen) and 5 of *Epidermophyton* genera (*E. floccosum* in 5 specimens). No *T. tonsurans, T. violaceum* and *T. verrucosum* were detected both culture and rtPCR assays.

Concordance between culture and rtPCR was achieved to the species level in 95.65% and to the genus level in 100%. In only 4 samples rtPCR and culture identified different dermatophyte species. Two samples were identified by culture as *T. rubrum* and as *T. interdigitale* by rtPCR, and other two samples were identified by culture as *T. interdigitale* and as *T. rubrum* by rtPCR.

Three samples were identified by culture as *T. mentagrophytes var. quinckeanum* (n=2) and *T. mentagrophytes var. asteroides* (n=1) which remained *T. mentagrophytes spp.* positive using rtPCR.

In 8 of the 43 (18.6%) samples that yielded a non-dermatophyte in culture, real-time PCR was able to detect dermatophyte DNA.

Overall 23.9% of sample of scraping nail, 34.52% of skin scales and 34.78% of samples of hair were positive by rtPCR and only 18.8%, 28.66%, respectively 30.4% by culture.

RtPCR results indicate that nail infections in 84 samples were almost solely caused by *T. rubrum* (n=80) and *T. interdigitale* (n=4). The same two dermatophytes were identified by culture from nail scraping: *T. rubrum* (n=61), *T. var. interdigitale* (n=5), instead culture allowed us to identify the non-dermatophytes: *Aspergillus* in 2 samples, and *Candida albicans* in 31 specimens, too (figure 2).

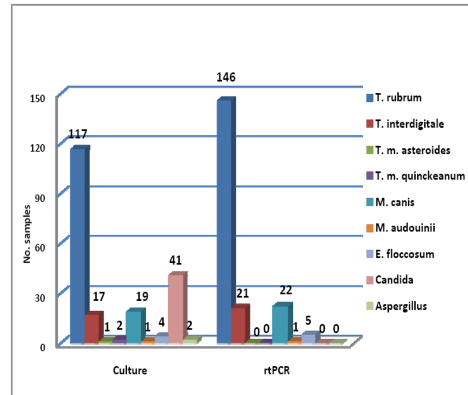

Fig. 1. *Culture and rtPCR results from 203 samples*

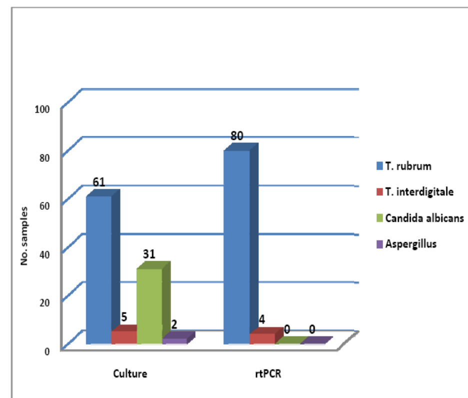

Fig. 2. *Comparative distribution of dermatophytes identified in nail scraping samples*

In skin samples were detected by rtPCR: *T. rubrum* in 64 of samples, *T. interdigitale* in 19 samples, *M. canis* in 18 and *E. floccosum* in 5 samples and by culture *T. rubrum* in 53 of samples, *T. interdigitale* in 15 samples, *M. canis* in 16 and *E. floccosum* in 4 samples. Also by culture could be isolated *Candida albicans* in 13 specimens (figure 3).

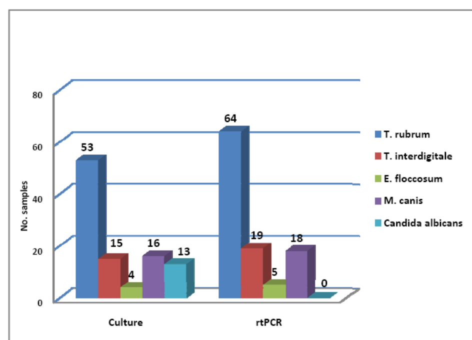

Fig. 3. *Comparative distribution of dermatophytes identified in skin scales samples*

In the hair specimens *M. canis* (n=3), *M. audouinii* (n=1), and 3 species of *Trichophyton* genus were identified by rtPCR. By culture were isolated: *T. asteroides* (n=1), *T. quinckeanum* (n=2), *M. canis* (n=3) and *M. audoiunii* (n=1) (figure 4).

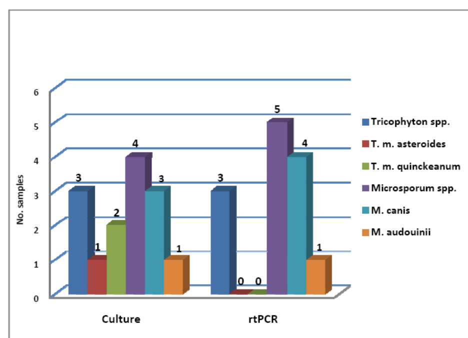

Fig. 4. *Comparative distribution of dermatophytes identified in hair samples*

4. Discussion

Molecular techniques are more beneficial for dermatophyte identification as they are rapid and more sensitive. Moreover, these methods rely on genetic makeup, which is more constant than phenotypic characterization, and they can identify atypical dermatophytes that could not be identified by culture-based techniques.

In the past few years several molecular methods for the detection and identification of dermatophytes from clinical samples have been developed. Some of these molecular methods require a culture step, like oligonucleotide arrays and DNA sequencing [4,5,7].

Other techniques like PCR RFLP require a final detection step after PCR has been performed. In 2007 the PCR-reverse line blot technique developed by Bergmans [2] was adapted to a real-time PCR assay that detects the most prevalent dermatophyte species in clinical samples.

The rtPCR assay consists of one multiplex PCR reaction and does not need a preculture or separate final detection step, reducing time to result from 2–6 weeks to 1-2 days.

In our study we applied a multiplex rtPCR assay for rapid direct detection and identification of the most frequently pathogenic dermatophytes (*T. rubrum*, *T. mentagrophytes* spp., *T. interdigitalae*, *T. violaceum*, *T. verrucosum*, *T. tonsurans*, *M. canis*, *M. audouinii*, *E. floccosum*) in clinical specimens.

Our study showed a great increase in detection rate of dermatophytes using rtPCR (31.03%) over the culture (25.23%) and over the direct microscopy (29.46%). Also, Bergmans et al. in their study obtained more positive results by rtPCR than classic methods (61.7% vs. 47.5%) [2].

The sensitivity of the rtPCR was 97.5%, much higher compared to conventional methods. The specificity, positive and negative predictive values of rtPCR assay were estimated as 100%, 100% and 98.86%, respectively.

Concordance between culture and rtPCR was achieved to the species level in 95.65% and to the genus level in 100%. Discrepancies between dermatophyte culture and rtPCR results were observed in 42 samples: in 37 of cases culture was

negative and rtPCR positive, and in 5 cases of positive cultures the rtPCR were negative, possibly due to sampling error. In only 4 samples rtPCR and culture identified different dermatophyte species.

Although culture did not identify any mixed dermatophytic infection, rtPCR co-detected *T. rubrum* and *T. interdigitale* in two nail samples, in both cases only *T. rubrum* dermatophyte yielded in culture. Sampling variation is a more likely explanation as the culture also needed multiple pieces of sample to yield growth for both dermatophytes. Another explanation is that if multiple dermatophyte species are present in a sample, using culture the predominant dermatophyte is likely to outperform the lesser abundant one.

Whatever type of clinical samples was used, rtPCR obtained the higher rate of detection compared to culture. RtPCR results indicated that nail infections were almost solely caused by *T. rubrum* and *T. interdigitale*. In skin and hair samples the diversity of dermatophyte species was higher with 8 different species, data that are in concordance with other studies performed in Europe [6,9,11].

5. Conclusion

Concluding, this rtPCR assay was demonstrated to be an assay with excellent performance characteristics, that increases detection rates of dermatophytes and drastically reduces time to result compared to culture.

Highly sensitive rtPCR assays could also contribute to elucidating whether subclinical dermatophyte infections are implicated in some eczematous, psoriatic and hyperkeratotic skin lesions, or whether positive PCR assays from nonlesional clinical specimens would substantiate asymptomatic transient dermatophyte colonization as a realistic clinical entity.

Although rtPCR assay seams more expensive than classical methods, into laboratory where molecular techniques can be implemented by routine, the price difference is not so high, and the rtPCR may replace both direct microscopy and culture, combining the speed of microscopy with the specific information generated by culture.

References

1. Arabatzis, M., Bruijnesteijn van Coppenraet, L.E., Kuijper, E.J., de Hoog, G.S., Lavrijsen, A.P., Templeton, K., van der Raaij-Helmer, E.M., Velegraki, A., Gräser, Y., Summerbell, R.C.: *Diagnosis of common dermatophyte infections by a novel multiplex real-time polymerase chain reaction detection/identification scheme.* In: British Journal of Dermatology (2007) No.157, Vol. 4, p. 681-689.
2. Bergmans, A.M., van der Ent, M., Klaassen, A., Böhm, N., Andriesse, G.I., Wintermans, R.G.: *Evaluation of a single-tube real-time PCR for detection and identification of 11 dermatophyte species in clinical material.* In: Clinical Microbiology and Infection (2010) No. 16, Vol. 6, p. 704-710. Epub 2009 Jul 20.
3. Gutzmer, R., Mommert, S., Kuttler, U., Werfel, T.H.: *Rapid identification and differentiation of fungal DNA in dermatological specimens by LightCycler PCR.* In: Journal of Medical Microbiology (2004), No. 53, p. 1207-1214.
4. Li, H.C., Bouchara, J.P., Hsu, M.M., Barton, R., Chang, T.C.: *Identification of dermatophytes by an oligonucleotide array.* Journal of Clinical Microbiology (2007), No. 45, p. 3160–3166.

5. Makimura, K., Tamura, Y., Mochizuki, T., Hasegawa, A., Tajiri, Y., Hanazawa, R., et al.: *Phylogenetic classification and species identification of dermatophyte strains based on DNA sequences of nuclear ribosomal internal transcribed spacer 1 regions*. In: Journal of Clinical Microbiology (1999), No. 37, p. 920-924.
6. Mugge, C., Haustein, U.F., Nenoff, P.: *Causative agents of onychomycosis - a retrospective study.* In: Journal of the German Society of Dermatology (2006), No. 4, p. 218-28.
7. Ninet, B., Jan, I., Bontems, O., Lechenne, B., Jousson, O., Panizzon, R.: *Identification of dermatophyte species by 28S ribosomal DNA sequencing with a commercial kit.* In: Journal of Clinical Microbiology (2003), No. 41, p. 826–830.
8. Onsberg, P.: *Dermatophyte species, microscopic and cultural examination.* In: Mycopathologia (1979), No. 67, p. 153–155.
9. Perea, S., Ramos, M.J., Garau, M., Gonzalez, A., Noriega, A.R., del Palacio, A.: *Prevalence and risk factors of tinea unguium and tinea pedis in the general population in Spain.* In: Journal of Clinical Microbiology (2000), No. 38, p. 3226–3230.
10. Summerbell, R.C., Cooper, E., Bunn, U. et al.: *Onychomycosis: a critical study of techniques and criteria for confirming the etiologic significance of nondermatophytes.* In: Medical Mycology (2005), No. 43, p. 39–59.
11. Saunte, D.M., Svejgaard, E.L., Haedersdal, M., Frimodt-Moller, N., Jensen, A.M., Arendrup, M.C.: *Laboratory-based survey of dermatophyte infections in Denmark over a 10-year period.* In: Acta Dermato-Venereologica (2008), No. 88, p. 614–616.