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Rapid detection of PAN-Dermatophytes by Real-time PCR

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Abstract

Dermatophytosis is one of the most common infectious diseases in the world and can be caused by several dermatophyte species. Routine procedures for dermatophyte species detection and identification rely on examination of the colony and microscopic morphology. In this study we have evaluated Real-time Polymerase chain reaction (qPCR) for the detection of Dermatophytes species directly from the clinical samples (skin scrapings, hair and nail). We have also evaluated rapid DNA purification from the clinical samples that compatible with Real-time PCR reaction. A total of 100 patients clinically suspected with dermatophytoses were included in the study. Of which 65 skin scrapings, 23 nail and 12 were hair. KOH microscopy, fungal culture and Real-time PCR were done on clinical specimens, and results compared. Real-time PCR for PAN-dermatophytes was positive in 86% specimens, followed by KOH microscopy (75%), and fungal culture (30%). Results indicate that Real-time PCR may be considered as high sensitivity gold standard for the diagnosis of Dermatophytosis and can aid the clinician in initiating prompt and appropriate antifungal therapy.

Keywords: Dermatophytosis, qPCR, KOH microscopy, diagnosis, antifungal.

INTRODUCTION

Dermatophytoses caused by the genus of dermatophytes taxonomically classified into three genera (*Epidermophyton*, *Microsporum* and *Trichophyton*) is thought to be one of the most significant public health problems yet not solved. Dermatophytes are highly specialized pathogenic fungi and the most common cause of superficial mycoses in humans and animals, affecting millions of individuals annually [1]. Dermatophytoses cause morbidity which poses a major public health problem especially in tropical countries like India due to the hot and humid climate.

Routine procedures for dermatophyte species identification rely on examination of the colony (pigmentation of the surface and reverse sides, topography, texture, and rate of growth) and microscopic morphology (size and shape of macroconidia and microconidia, spirals, nodular organs, and pectinate branches). Further identification characteristics include nutritional requirements (vitamins and amino acids) and temperature tolerance, as well as urease production, alkaline production of bromocresol purple medium, in vitro hair perforation, etc. [2,3]. Morphological and physiological characteristics can frequently vary; in fact, the phenotypic features can be easily influenced by outside factors such as temperature variation, medium, and chemotherapy [4] and therefore strain identification is often difficult. However, this system of identification is time-consuming and may pose difficulties for non-experts in differentiation of the morphology of cultured colonies. Furthermore, even the same strains may show morphologically diverse colonies, making the identification of the causative organism more difficult [4].

In the last few years genotypic approaches have proven to be useful for solving taxonomic problems regarding dermatophytes; in fact, genotypic differences are considered more stable and more precise than phenotypic characteristics [4,5]. Molecular methods, such as restriction fragment length polymorphism analysis of mitochondrial DNA [6, 7, 8], sequencing of the Internal Transcribed Spacer (ITS) region of the ribosomal DNA [9,10], sequencing of protein-encoding genes [11,12], and PCR-random amplification of polymorphic DNA (RAPD) [3], arbitrarily primed PCR (AP-PCR) [4,14], and PCR fingerprinting [5], have brought important progress in distinguishing between species and strains. However, most of these techniques (e.g., restriction fragment length polymorphism analysis, sequencing) are complex, laborious, time-consuming, and not easily employable for routine identification of dermatophytes; in contrast, PCR technology is simple, rapid, and, in the absence of specific nucleotide sequence information for the many dermatophyte species, able to generate species- specific or strain-specific DNA polymorphisms on the basis of characteristic band patterns detected by agarose gel electrophoresis [4,5]. From a clinical point of view, for definition of species or for performance of an epidemiological study, it is important to have a reliable method for the identification of dermatophyte species. The present research aimed to find out the feasibility of successful detection of members of dermatophytes targeting ITS conserved gene by Real-time PCR directly from the clinical samples.

MATERIALS AND METHODS

Sample selection

A total of 100 patients clinically suspected with Dermatophytosis were included in the study irrespective of their age or gender. In skin dermatophytoses the clinical specimens collected were epidermal scales. The scales were scrapped from near the advancing edges of the lesions after disinfecting the lesions with 70% alcohol.

Where the advancing edges were not evident, scrapings were collected from areas representing the whole infected area. In hair Dermatophytoses basal root portion of hair was collected by plucking the hair with sterile forceps. In cases with black dot, scalpel was used to scrape the scales and excavate small portions of the hair roots. In nail dermatophytoses, cleaned and disinfected with 70% ethanol. Nail was scrapped from near advancing edges.

The collected specimens were divided into three portions. The first portion of the specimens was examined microscopically using 20% potassium hydroxide (KOH) with 40% dimethyl sulfoxide. The second portion was cultured on Sabouraud's dextrose agar containing chloramphenicol (0.05%) with and without cycloheximide (0.5%) and incubated at 25°C for 4 to 6 weeks. Clinical isolates were identified based on phenotypic characteristics of the colonies, microscopic examination of lactophenol cotton blue wet mounts, and physiological tests such as urease production, in vitro hair perforation, and nutritional requirement tests.

DNA purification and qPCR

Two Rapid buffers 0.2 M NaOH (Buffer-A) and 0.0025% SDS (Buffer-B) were prepared. DNA extraction was performed using both Rapid buffers as well as Spin column method. 10 milligrams of skin scrapings nail and ten hair follicles were used for the DNA purification. In a fresh 1.5ml centrifuge tube, clinical samples were transferred and 250µl of Buffer-A and -B were added. Using micro pestle, samples were grinded well and incubated at 95ºC for 10min. The mixture was centrifuged at 10000rpm for 3min. The clear supernatant 100ul was transferred into fresh 1.5ml centrifuge tube and stored at -20°C for further analysis.

Spin column DNA purification performed as per the (Helini Biomolecules, Chennai) kit manufacturer protocol. Briefly, samples are mixed with Lysis buffer and 20µl of Proteinase K, 5µl of internal control template (to monitor the purification efficiency) were added and incubated at 56°C for 15min. To this, 220µl of 100% ethanol added and mixed. The mixture transferred into spin column and centrifuged at 10000 rpm for 1min. The flow through was discarded and 500µl of Wash Buffer-1 added and centrifuged at 10000rpm for 1min. The flow through discarded and 500µl of Wash Buffer-2 added and centrifuged at 10000rpm for 1min. The wash buffer-2 step was repeated once. Without adding any reagents, the spin column centrifuged at 12000rpm for 2min. A fresh 1.5ml centrifuge tube inserted into spin column and 100µl of elution buffer added. The spin column with collection tube was centrifuged at 10000rpm for 2min. The eluted DNA was stored at -20°C for further analysis. 10µl of DNA from both purification methods was used for the Real-time PCR.

Primer Probe designing

Available ITS gene sequence of Dermatophytes family was downloaded from NCBI nucleotide tool in NCBI website. The downloaded sequences were aligned by software CLUSTALW multiple alignment, a part of Bio-Edit software. Bio-Edit is a free software application available for genomics research. Conserved regions among the sequences are identified. The sequences are having following parameter considered for designed Primer Probe; GC content: 40 – 60%, Primers: Length of 18 – 24 nucleotide – Melting temperature between 56ºC to 62ºC, Probe: Length of 20 – 27 nucleotide – Melting temperature between 65ºC to 70ºC, Using Primer3 software [free online software], the primer probe designed for universal detection of Dermatophytes. Designed Primer Probe specificity verified using NCBI BLAST tool.

Forward Primer: 5'-TGCCTGTTCGAGCGTCATTT-3'
Reverse Primer: 5'-ACTGCTTTTCGGGCGCGT-3'
Probe: FAM-5'-TCAAGCCCGGCTTGTGTGATGGACGA-3'-BHQ1
Amplified gene length: 103bp

The Probe PCR Master Mix (25µl) for Real-time PCR [HELINI Biomolecules, Chennai] contained 10X buffer (100mM Tris-HCl, 500mM KCl, and 0.8% [vol/vol] Nonidet P40; 2.5mM MgCl2, 0.2mM dNTPs Mix, 2U Hot start DNA polymerase). The Primer probe added in the concentration of 10pmoles of each of primers and 2pmoles of probe, and 10µl of purified DNA template. We used internal control primer probe system (from HELINI Biomolecules, Chennai) to validate the DNA purification from both methods. Internal control is non-complimentary sequence targeting primer probes used to monitor the purification efficiency as well as PCR inhibition.

The Real-time PCR thermal profile was 95°C for 15 min, followed by 45 cycles of denaturation at 95°C for 20sec, annealing at 60°C for 20sec, and extension at 72°C for 20sec. The data collection was performed in the annealing step (at 60ºC). Along with clinical samples DNA, Negative and Positive controls were included. PCR graded water added in the Negative control and DNA purified from control dermatophytes cultures (obtained from Microbial Type Culture Collection, IMTECH, Chandigarh, India) were added in the positive control. The sensitivity, specificity, positive and negative predictive were calculated.

RESULTS AND DISCUSSION

Of the 65 clinically suspected cases of skin Dermatophytosis, 87.7% (n = 57/65) were positive for PAN-Real-time PCR, 66.1% (n=43/65) by KOH microscopy and 26.2% (n = 17/65) by culture method. Among 23 clinically suspected cases of hair Dermatophytosis, positivity by Real-time PCR was highest 86.9% (n = 20/23) followed by KOH microscopy 56.5% (n = 13/23) and fungal culture 30% (n = 7/23). Among 12 clinically suspected cases of nail Dermatophytosis, positivity by Real-time PCR was highest 83.3% (n = 10/12) followed by KOH microscopy 30% (n = 4/12) and fungal culture 26% (n = 3/12). On Statistical analysis, sensitivity and specificity of PAN-Real-time PCR for skin scrapings was 87.7%, 86.9% for hair and 83.3% for nail samples. The sensitivity and specificity of KOH microscopy for skin scrapings was 66.1%, for hair 56.5%, and for nail 30%. The sensitivity and specificity of fungal culture for skin scrapings was 26.2%, for hair samples 30% and for nail samples was 26%.

Table 1. Dermatophytes - Genus and species – ITS DNA sequence for Primer Design

Trichophyton	***Microsporum***	***Epidermatophyton***
Anthropophilic	*Microsporum amazonicum*	*Epidermophyton floccosum*
Trichophyton rubrum	*Microsporum audouinii*	
Trichophyton megnini	*Microsporum boullardii*	
Trichophyton mentagrophytes	*Microsporum canis*	
Trichophyton concentricum	*Microsporum distortum*	
Trichophyton schoenleinii	*Microsporum cookei*	
Trichophyton soudanense	*Microsporum distortum*	
Trichophyton tonsurans	*Microsporum duboisii*	
Trichophyton violaceum	*Microsporum equinum*	
Trichophyton yaoundei	*Microsporum ferrugineum*	
	Microsporum fulvum	
Zoophilic	*Microsporum gallinae*	
Trichophyton ajelloi	*Microsporum gypseum*	
Trichophyton equinum	*Microsporum langeronii*	
Trichophyton redellii	*Microsporum nanum*	
Trichophyton simii	*Microsporum persicolor*	
Trichophyton verrucosum	*Microsporum praecox*	
	Microsporum ripariae	
Geophilic	*Microsporum rivalieri*	
Trichophyton flavescens		
Trichophyton gloriae		
Trichophyton erinacei		
Trichophyton onychocola		
Trichophyton phaseoliforme		
Trichophyton terrestre		
Trichophyton vanbreuseghemii		

Table 2. Real-time PCR results

Samples	Real-time PCR Ct value		Classical Dermatophytes confirmation method	
	Internal control	PAN-dermatophytes	Culture	Microscopic
Control – Candida albicans	21.5	Nil	Negative	Negative
Healthy Human DNA	22.5	Nil	Negative	Nil
Trichophyton rubrum	18.5	21.0	Positive	Positive
Microsporum canis	19.2	21.5	Positive	Positive
Microsporum gypseum	18.2	21.0	Positive	Positive
Epidermophyton floccosum	19.5	22.5	Positive	Positive
Hair	21.6	31.6	Positive	Positive
Nail	22.2	29.5	Positive	Positive
Skin	21.3	28.6	Positive	Positive

Dermatophytosis cannot be easily diagnosed based on clinical manifestations as several other conditions mimic the clinical presentation. The differential diagnosis of Dermatophytoses includes seborrheic dermatitis, atopic dermatitis, contact dermatitis, psoriasis, candidal intertrigo, erythrasma, eczema etc [1, 15]. Further it is more difficult to diagnose Dermatophytosis in immunocompromised patients, as clinical presentation is often atypical [1]. It is essential that good laboratory methods are available for rapid and precise identification of the dermatophytes involved, in order to apply appropriate treatment and prevention measures. The conventional methods of fungal detection have their own drawbacks; for e.g. KOH microscopy has low specificity and fungal culture is associated with low sensitivity and takes long time. Further dermatophyte isolates from patients on antifungal treatment generally do not show characteristic morphology on culture, thus further compromising the results of culture isolation [16]. The changing profiles of human Dermatophytoses among countries have further necessitated the development of improved diagnostic methods for identification of dermatophytes [16]. Thus, newer fungal diagnostic methods are need of the hour as identification of the etiological agent is required not only for accurate diagnosis, but also but also for post-therapeutic strategies [17, 18].

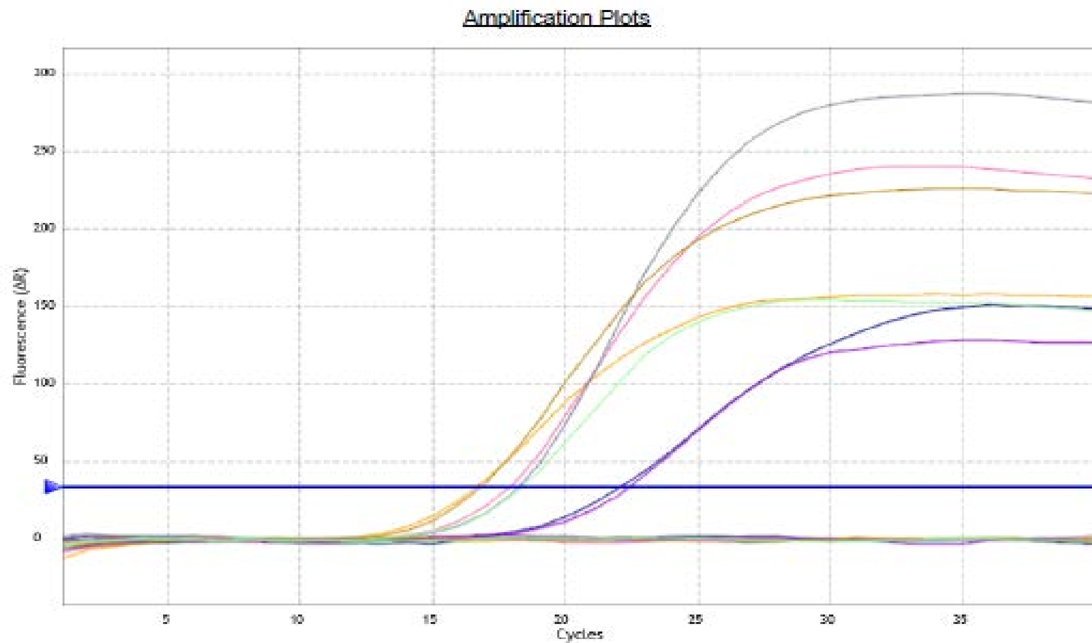

Figure 1. Real-time PCR graph of PAN-Dermatophytes

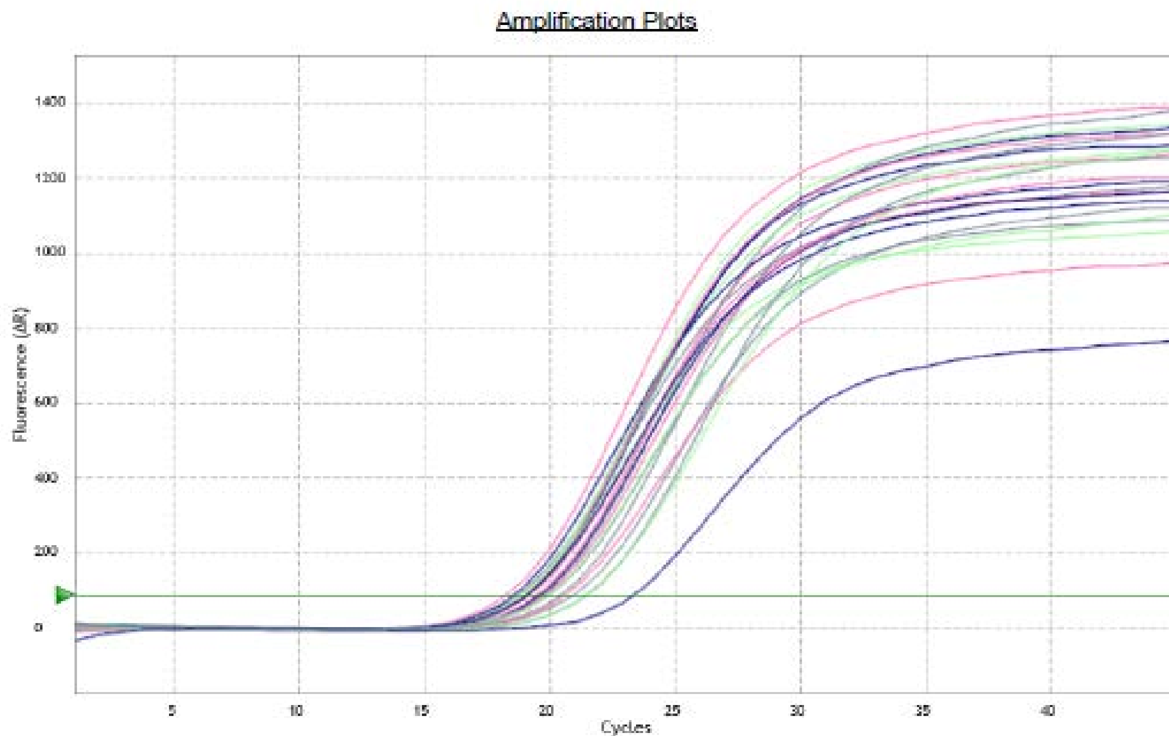

Figure 2. Real-time PCR graph of Internal Control

CONCLUSION

In the present study, Real-time PCR for both skin and hair dermatophytoses was observed to be more sensitive for the detection of dermatophytes than culture isolation and KOH microscopy. It may therefore be concluded that Real-time PCR detection may be considered the gold standard for detection of dermatophytes in patients with dermatophytoses and can aid the clinician in initiating prompt and appropriate antifungal therapy. This technique is not only rapid but also simple and cheap in comparison to other molecular methods for detection of dermatophytes.

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