

Rapid identification of dermatophyte species by 28S rDNA Polymerase Chain Reaction (PCR)

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Abstract: Dermatophytes are the main cause of onychomycoses, but various nondermatophyte filamentous fungi are often isolated from abnormal nails. The correct identification of the aetiological agent of nail infections is necessary in order to recommend appropriate treatment. To evaluate a rapid polymerase chain reaction (PCR) assay based on 28S rDNA for fungal identification in nails on a large number of samples in comparison with cultures. Infectious fungi were analyzed using PCR in 22 samples of swabs and nail samples in which fungal elements were observed in situ by direct mycological examination (positive samples). The results were compared with those previously obtained by culture of fungi on Sabouraud agar from the same samples. PCR identification of fungi in nails allowed validation of the results obtained in culture when three *Trichophyton* spp. and one *Microsporum* spp grew from infected samples. Improved sensitivity for the detection of fungi in nails was obtained using the PCR assay. Rapid and reliable molecular identification of the infectious fungus can be used routinely and presents several important advantages compared with culture in expediting the choice of appropriate antifungal therapy.

Keywords: Dermatophytes, *Trichophyton* spp., *Microsporum* spp., PCR, 28S rDNA

I. Introduction

Molecular techniques are increasingly being employed in the clinical microbiological laboratory for identification and direct detection of microbes in clinical specimens because of the high sensitivity, specificity and speed (1). Classical diagnosis of dermatophytosis consists of direct microscopy and culture with a subsequent species identification mainly based on macroscopic and microscopic features of the culture (2, 3, 4).

Dermatophytes commonly infect keratinaceous tissue such as hair, skin, and nails. This characteristic is thought to be due to an inhibitory agent in blood or serum that precludes establishment of infections at other body sites. While these organisms are pathogenic in man, they freely exist as soil saprophytes or zoopathogens.

Microsporum and *Trichophyton* are human and animal pathogens. *Epidermophyton* is a human pathogen. The dermatophytes can be geophilic, zoophilic, or anthropophilic (5). The term geophilic is used for fungi whose natural habitat is in soil. Zoophilic refers to fungi that infect humans as well as lower animals. Anthropophilic means man-loving. Organisms in this category prefer to infect man (6). It is important to underscore the distinction between molecular diagnostics, which relates to the direct detection of dermatophyte DNA in the clinical specimen (nail, skin or hair), and molecular identification in the sense of application of molecular tools for species identification of fungal isolates (7, 8). Numerous targets within the fungal genome have been evaluated, with much of the current work using sequence areas within the ribosomal DNA (rDNA) gene complex (9). This section of the genome includes the 18S, 5.8S and 28S genes which code for ribosomal RNA (rRNA) and which have a relatively conserved nucleotide sequence among fungi (10).

II. Research Methodology

2.1. Samples collection

22 Samples were collected from Dept. of Dermatology, King George Hospital, Visakhapatnam in the period of March 2011 to August 2013. Infected nail clippings and swabs were collected from patients.

2.2. Culturing and lacto phenol blue staining

Samples were inoculated on sabourand dextrose agar and incubated for 7-10 days. Cultured specimens were stained with cotton blue and observed under microscope. Cotton blue (China blue) stains chitin and cellulose. Since cell walls of fungi are primarily chitin, this stain is an excellent choice for observing fungi in clinical specimens (11). Placed a small drop of lactophenol cotton blue (LPCB) in the center of a clean glass slide. Removed a fragment of fungus culture with a teasing needle and placed in the LPCB and gently tease apart. Gently placed a cover slip onto the preparation. Examined the slide using the low power (10x) objective of a microscope (12).

2.3 DNA Isolation

To evaluate a rapid polymerase chain reaction (PCR) assay based on 28S rDNA for fungal identification in samples (nails and swabs) on a large number of samples in comparison with cultures (9, 10). Infectious fungi were analyzed using PCR in 22 samples of swabs and nail samples in which fungal elements were observed in situ by direct mycological examination (positive samples). Scrap the fungal 1-2 colonies into a 1.5mL tube which is containing phosphate buffer or distilled water. Centrifuge at 1100g for 3 min. Make the pellet homogenous by hand flicking the tube. Added 5% Chelax (Resin) which is just vortexed and add 0.2µg Proteinase K and mix well. Incubated the samples at 65°C for 20 min followed by 100°C for 10 min. Centrifuge at 1100g for 3 min. Supernatant was used for subsequent PCR reactions.

2.4. Amplification

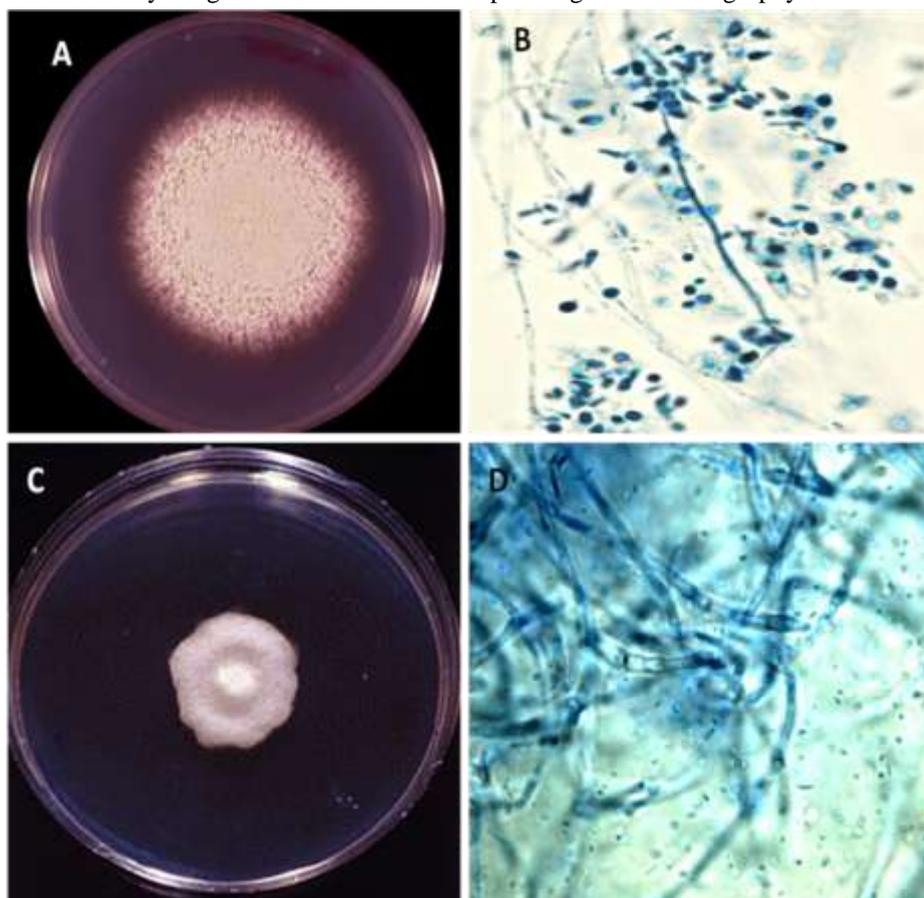
A polymerase chain reaction (PCR) was then carried out to amplify the DNA sequences of interest by denaturing the DNA molecule and replicating it by utilizing primers, free nucleotides, and a polymerase designed to help the DNA withstand the high temperatures involved in PCR. Because PCR can only be applied when the nucleotide sequence of at least one DNA segment is known, primers were used. 28S rDNA forward primer was 5'- GGTTGGTTTCTTTTCCT -3' and its reverse primer was 5'- AAGTAAAAGTCGTAACAAGG -3'. PCR conditions as follows, 5 minute denaturing at 95°C, denaturing at 95°C for 30 seconds, annealing at 53°C, and 30 seconds extension at 72°C for 40 cycles (13-15).

2.5. Electrophoresis and Analysis

Agarose gel electrophoresis was then performed to analyze the DNA samples using a 2% agarose gel at 100 V for 30 minutes. Ethidium bromide was used to colour each band under ultraviolet light. Band sizes were subsequently compared to the molecular weight band markers for 100-1000 base pairs for confirmation.

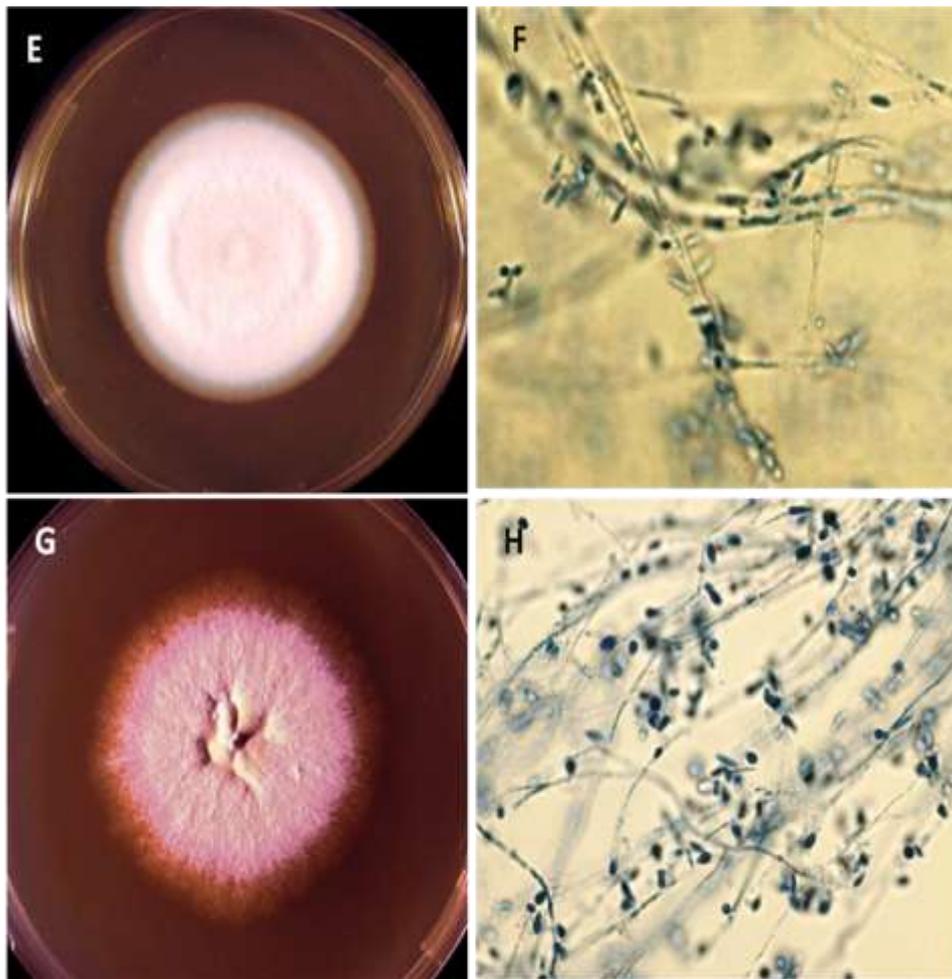
III. Results

Figure-1 shows the mycological culture and microscopic images of *T. mentagrophytes* and *M. audouinii*.



A. *Trichophyton mentagrophytes* culture B. *Trichophyton mentagrophytes* microscopic picture
C. *Microsporum audouinii* culture D. *Microsporum audouinii* microscopic picture

Figure-2 shows the mycological culture and microscopic images of *T. rubrum* and *T.tonsurans*.



E. *Trichophyton rubrum* culture F. *Trichophyton rubrum* microscopic picture
G. *Trichophyton tonsurans* culture H. *Trichophyton tonsurans* microscopic picture

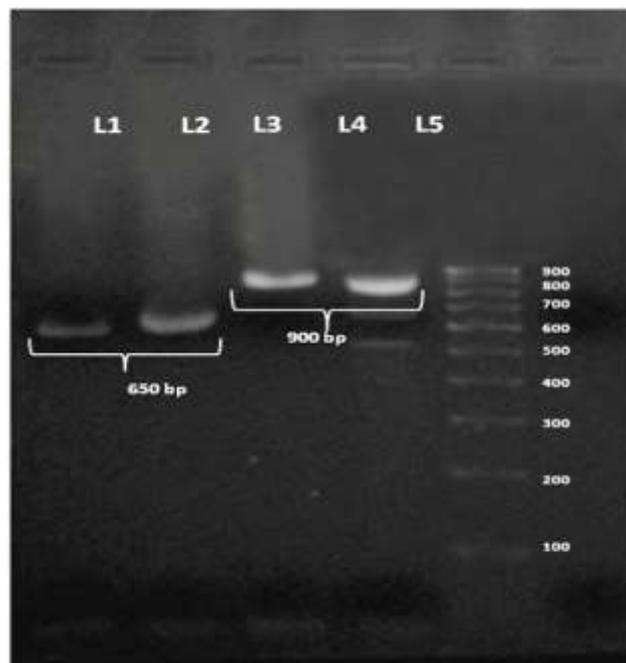


Figure -3 shows the PCR bands for 28SrDNA

Among 22 samples, successfully we isolated 4 species, 3 belonged to Trichophyton, and one belonged to the Microsporum. L1 - Trichophyton rubrum, L2- Trichophyton tonsurans gave band size 650bp. L3- Trichophyton mentagrophytes and L4- Microsporum audouinii gave band at 900bp. L5- 100bp DNA ladder.

IV. Discussion

Dermatophytes are fungi that belong to three genera: Epidermophyton, Microsporum, and Trichophyton. Identification of dermatophyte species is essential for appropriate diagnosis and treatment of dermatophytosis (16). Routine identification depends on macroscopic and microscopic morphology, which is time-consuming and does not identify dermatophyte strains (17, 18). In this study, PCR-based method used for their abilities to identify 22 dermatophyte isolates obtained from KGH hospital patients to the species and strain levels. Here in the present study we employed a method: PCR amplification, using 28S rDNA primers.

Dermatophyte strains were also identified using a conventional culture method (19). Out of 22 samples, successfully we isolated 4 species, 3 belonged to Trichophyton, and one belonged to the Microsporum. Our results showed that the conventional culture method identified four species: Microsporum audouinii, Trichophyton mentagrophytes Trichophyton rubrum, and Trichophyton tonsurans. Trichophyton rubrum, Trichophyton tonsurans gave band size 650bp. Trichophyton mentagrophytes and Microsporum audouinii gave band at 900bp. Moreover, both PCR methods agreed with the diagnosis made using the conventional approach.

This report describes the application of PCR fingerprinting for the identification of species and varieties of common dermatophytes and related fungi utilizing 28S rDNA primer (20). The primer was able to amplify all the strains, producing species-specific profiles for Microsporum audouinii, Trichophyton rubrum, Trichophyton mentagrophytes, and Trichophyton tonsurans.

V. Conclusion

Improved sensitivity for the detection of fungi in clinical samples were obtained using the PCR assay. The spectrum of fungi detected depends on the test designs and requires careful evaluation with the local epidemiology in mind. Challenges associated with DNA extraction from clinical specimens seem to be resolved. Rapid and reliable molecular identification of the infectious fungus can be used routinely and presents several important advantages compared with culture in expediting the choice of appropriate antifungal therapy. However, very few assays have been externally evaluated, and thus future studies are needed for nonbiased validations.

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