Genotyping of Dermatophytes isolated from Dermatophytoses Sudanese Patients

Mujeeb A. Kabbashi¹, Al Fadhil A. Omer¹, and Khalid A. Enan²

¹ Department of Medical Microbiology, Faculty of Medical Laboratory Sciences, University of Sciences & Technology, Khartoum, Sudan
² Department of Virology, Central Laboratory, Khartoum, Sudan

Abstract

Background: Dermatophytes are prevalent causes of cutaneous mycoses and are able to cause disease in immunocompetent individuals. They infect keratinized tissue such as skin, hair, and nails, resulting in tinea infections, including ringworm.

Objectives: To study the phenotyping and genotyping characterization of dermatophytes isolated from dermatophytoses Sudanese patients.

Materials and Methods: A total of 200 patients clinically suspected with dermatophytosis were included in this study. The clinical specimens collected were epidermal scales. Conventional methods were applied to make an early diagnosis. Specific identification was made by first round and nested PCR on positive fungal culture specimens.

Results: The commonest conditions investigated were tinea pedis, tinea corporis, tinea cruris, tinea capitis, tinea barbae, and tinea unguium. Nested PCR for dermatophytes was positive in 100% of the fungal culture specimens, followed by first round PCR (86.5%). Phylogenetic tree findings elucidated that isolates from Sudan are grouped in two. The first group isolates were closely related to Trichophyton violaccum and Arthroderma benhamiae. The second group isolates were very close to Epidermophyton floccosum.

Conclusion: Nested PCR was found to be a gold standard for the diagnosis of dermatophytoses and can aid the clinician in initiating prompt and appropriate antifungal therapy.

Key words: Dermatophytes, Genotyping, Molecular sequencing.

Kabbashi, et al., 2016: Vol 1(2)
Introduction

Superficial fungal infections are common skin diseases, affecting millions of people worldwide\(^1\). These infections occur in both healthy and immunocompromised patients and etiologic agents consist of dermatophytes, yeasts and non dermatophyte molds\(^2\). The estimated lifetime risk of acquiring dermatophytes infection is between 10–20\(^\%\)\(^3\). Dermatophytes consist of three genera *Trichophyton*, *Microsporum*, and *Epidermophyton* (in their anamorphic state) and *Arthroderma* (in their telomorphic state). The teleomorphs for all dermatophyte species have not yet been identified, but the generic name for both *Trichophyton* and *Microsporum* is *Arthroderma*\(^4\).

The laboratory diagnosis of dermatophytoses routinely involves direct microscopic examination of clinical specimen followed by *in vitro* culture techniques. Microscopic identification of fungal elements directly from clinical specimen is a rapid diagnostic method but it lacks specificity and sensitivity, with false negative results in up to 15\(^\%\) cases\(^5\). *In vitro* culture is a specific diagnostic test but it is slow technique, and may take up to 8 weeks to give the results\(^6\).

The advent of molecular technology has enabled the development of techniques like polymerase chain reaction, which is a highly sensitive and specific test and can be used for diagnosis of various microorganisms including fungal pathogens. In our study we have evaluated nested PCR targeting the *chitin synthase 1* (*CHS1*) gene (DDBJ accession no.-AB003558) shared by three genera, i.e., *Trichophyton*, *Epidermophyton*, and *Microsporum*, in patients with clinically suspected cases of onychomycosis\(^3\).

Recently, the molecular biology-based techniques used are restriction fragment length polymorphism (RFLP)\(^3\), real time PCR\(^7\), and multiplex PCR assay\(^8\). These molecular methods have a good potential to directly detect dermatophytes in clinical specimens. However these methods are yet to be standardized for routine clinical laboratories. Since PCR-RFLP is a complex technique with poor discriminative power, real time PCR appears to be promising but is not practical enough for a large number of laboratories that are either small scale or very tightly budgeted.

Materials and methods

A total of 200 patients clinically suspected with dermatophytosis were included in the study irrespective of their age or gender. The most common clinical presentation among skin dermatophytosis (n = 80) was tinea pedis (n = 18), followed by tinea corporis (n = 42), and tinea cruris (n = 20). Among hair dermatophytosis (n = 100), tinea capitis (n = 85) was most frequent followed by tinea barbae (n = 15). Among nail dermatophytosis (n=20), was tinea unguim. The study participants included 50 control patients without clinical dermatophytosis.

Kabbashi, *et al.*, 2016: Vol 1(2)
Epidermal scales were collected from skin dermatophytosis. The scales were scraped 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 scales and excavate small portions of hair roots.

The collected specimens were examined microscopically using 20% potassium hydroxide (KOH) with 40% dimethyl sulfoxide, 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 on the basis of phenotypic characteristics of the colonies, microscopic examination of lacto phenol cotton blue wet mounts, and physiological tests such as urease production, in vitro hair perforation, and nutritional requirement tests.

DNA extraction:
DNA extraction was performed on fungal culture isolates by Quick CTAB extraction BYPstyle. 2 ml screw cap tubes were filled with 490 µl CTAB-buffer (2x1). 6-10 acid washed glass beads (1.5-2 mm) were added, and then fungal materials (1-10 mm³) were added and 10 µl (Proteinase K)¹ stock solution was added and mixed thoroughly on a MoBio vortex for 10 min. Then the tubes were incubated for 60 min. at 60° C (or until the tissue is completely dissolved). 500 µl SEVAG chloroform/isooamylalcohol (24:1) was added and shaken for 2 min. to form an emulsion and the emulsion was maintained by periodic shaking. The tubes were spun in a micro-centrifuge at 14,000 rpm for 10 min. (or long enough to produce a clear supernatant). The aqueous (upper) layer was collected in a clean, labeled tube. The volume of the DNA sample was estimated (e.g. 400 µl) and 2/3 volume (≈ 270 µl) of ice cold iso-propanol was added and mixed. The tubes was kept at -20° C overnight for a better yield and the tubes was spun in a micro-centrifuge at 14,000 rpm for 10 min then the alcohol supernatant was poured off. 1 ml of ice-cold 70% ethanol was add to each tube and mixed gently. The tubes were again centrifuged at 14,000 rpm for 2 min. and alcohol was poured off, and the samples were dried by air, and re-suspended in 50 µl TE-buffer, then stored frozen at -20° C. Quality of DNA was checked by running 2-3 µl on a 0.8% agarose gel.

DNA amplification:
For each sample, two sets of single PCR analyses were performed.
a) First-round PCR was performed using the following primer pairs:
CHS1 1S (5'-CAT CGA GTA CAT GTG CTC GC-3'; NT (70-89)
CHS1 1R (5'-CTC GAG GTC AAA AGC ACG CC-3'; NT (485-504)
Such primers may amplify a 435-bp DNA fragment of the dermatophyte-specific genes.
b) Nested PCR was performed using the following primer pairs:
JF2 (5'-GCA AAG CCT GGA AGA AG-3'; NT (111-130)
JR2 (5'-GGA GAC CAT CTG TGA GAG TTG-3'; NT (378-398).
Such primers may amplify a 288 bp DNA fragment of the dermatophyte-specific genes⁹.

Kabbashi, et al., 2016: Vol 1(2)
Procedure for first-round PCR amplification was performed according to (Maxime PCR premix kit REF) technique. 5ul of DNA extract was added to PCR premix (Maxime PCR premix kit i-Tag), containing i-Tag™ DNA polymerase, dNTP mixture, reaction buffer. Then 2 ul of primer (sense and antisense) was added and 13 ul of nuclease-free water were added. The reaction mixture was initially denatured at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 60 sec, annealing at 60°C for 75 sec, and extension at 72°C for 120 sec. This was followed by a final extension step for 7 min at 72°C in a thermal cycler (Biometra Goettingen, Germany).

The PCR mixture for nested PCR consisted of 2 μl primers JF2 and JR2, 3μl diluted product of the primary cycle as the DNA template and 13μl nuclease-free water; the rest of the constituents were the same as those described above. The running conditions of nested PCR were similar to the first-round PCR except that an annealing temperature of 63° C and 40 cycles were used. Triple-distilled water and DNA of Trichophyton mentagrophytes were used as the negative and positive controls, respectively.

To document the amplified products, 5 μl of product from first-round PCR and nested PCR was electrophoresed on a 1.5% agarose gel (containing 1.5μg/100 ml ethidium bromide) in Tris-borate-EDTA buffer, along with the tracking dye bromophenol blue, initially at 120 V and A 35 for 60 min. Thereafter, bands were visualized under UV light and amplicon of 288 bp was considered positive for dermatophytes (Fig. 1).

![Image of gel showing bands](image_url)

Fig. 1: Plot of nested PCR of clinical dermatophytoses specimens
M: Molecular Marker. 1: Positive control. 2: Negative control. 3, 4: 5, 6 Positive cases

Kabbashi, et al., 2016: Vol 1(2)
DNA Sequencing:
This was carried out to confirm identification of the pathogen. Commercial DNA sequencing was conducted using Macrogen (Korea) technique. The DNA fragment (288 bp) from internal sequence of the amplicons of first round PCR were obtained using the specific primers. Mutations were located at positions NT 111-130 and NT 378-398 of the amplified fragment. Two sequence fragments were generated for 10 samples. They were edited manually to correct possible base errors using BIOEDIT 7.0. They were then subsequently joined to reconstruct a fragment of 222 bp spanning gene for chitin synthase 1 of *Arthroderma*. The reconstructed sequences were aligned using CLUSTAL-X 2.1 against the reference sequence of GenBank accession number AB003558. An un-rooted neighbor joining tree of all the sequences was reconstructed using MEGA 6.0. The best substitution model for the sequences (HKY+G) was determined using the model test algorithm implemented in MEGA 6.0. All the generated sequences had been deposited in the GenBank under the accession number GI: AB003558.

**Results**

In this study, 200 patients were investigated for dermatophytes. 85 specimens were found positive (42.5%) by culture method. 73 samples (85.9%) of the positive culture specimens were found positive by first round PCR and all of them (100%) were found positive by nested PCR technique (Table 1).

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>Culture</th>
<th>First round PCR</th>
<th>Nested PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hair (100)</td>
<td>45</td>
<td>40</td>
<td>45</td>
</tr>
<tr>
<td>Skin (80)</td>
<td>34</td>
<td>31</td>
<td>34</td>
</tr>
<tr>
<td>Nail (20)</td>
<td>6</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>85</strong></td>
<td><strong>73</strong></td>
<td><strong>85</strong></td>
</tr>
</tbody>
</table>

In our study both nucleotide and protein maximum likelihood phylogenetic trees elucidated that isolates from Sudan are grouped in two. The first group that contained 7 species was closely related to *Trichophyton violaccum* that is reported to cause > 90% of tinea capitis cases across Africa, and *Arthroderma benhamiae* is a zoophilic dermatophyte species belonging to the *Trichophyton mentagrophytes* complex, which produces highly inflammatory tinea corporis and tinea capitis in humans.

Kabbashi, *et al.*, 2016: Vol 1(2)
The second group which contained 3 species was very close to *Epidermophyton floccosum* which is anthropophilic dermatophyte with a world-wide distribution in the tropics and subtropics, and frequently causes tinea cruris, tinea pedis, tinea corporis and onychomycosis.

The results of the DNA sequencing were based on the wild-type gene obtained from the NCBI gene bank (Accession number GI: AB 003558). Genotyping of dermatophytes species was performed using chitin synthesis primers. The positive results in nested PCR gene sequencing are as follows:

Sequence ID: adj |AB003558.1| Length: 615 Number of Matches: 1
Related Information:
Range 1: 190 to 504 GenBankGraphics Next Match Previous Match
Alignment statistics for match #1

<table>
<thead>
<tr>
<th>Score</th>
<th>Expect</th>
<th>Identities</th>
<th>Gaps</th>
<th>Strand</th>
</tr>
</thead>
<tbody>
<tr>
<td>403 bits</td>
<td>2e-108</td>
<td>291/325 (90%)</td>
<td>10/325(3%)</td>
<td>Plus/Plus</td>
</tr>
</tbody>
</table>

Query 1
CCTTGCCGGTCTAGGGTTTTACCAAGATGGCATTGCTAAGCAGCAGGTTAACG
AAAGA 60

Subject 190
CCTTGCCGGTCTTGGTGTTTACCAGGACGGCATTGCCAAACAGCAGGTTAACGG
CAAGA 249

Query 61
CGTCACCTGCTCACATCTACTAATATACACCCAGAGAGGATGGAGGTCAAGGG
CACCCA 120

Subject 250
CATCACTGCTCACATCTACGAATATACCGCCCAGATAGGCATGGAGGTCAAGGG-
ACCCA 308

Query 121
GGTTATCCTCAAGCAGGCAGCCAGGATGCCAGTCCAGTTCTCTCTGTCTT
GAGAA 180

Subject 309
GGTTATCCTCAAGCAGGCAGCCAGGATGCCAGTCCAGTTCTCTCTGTCT
CT-CAAGAG-A 364

Query 181
AAATCATAAAAGATCAGCTCAGACAGATGGTTCCCTACAGCCTTTGGCCG
CTCGA 240

Subject 365
GAA-C-CAGAGAGATCAACTCTACAGGATGGTTCCCTACAGCCTTTGGCCG
-CTCCTCGA 419

Kabbashi, et al., 2016: Vol 1(2)
Query 241
CCCCGATATCTGTGTTCCTCCTCAGATGTGGAAACACCCAGCGGCGCAAGTAT
ATACCA 300 |||| |||| || |||| ||||||||||||||||||||
Subject 420
CCCCCATTATCGTGTGTTATCCTCAGCTGGAAACACCCAGCGGCGCAAGTAT
ATACCA 479
Query 301
GCTCTGGCGTGCTTTTGACCTCGAG 325 |||| |||| ||||
Subject 480
GCTCTGGCGTGCTTTTGACCTCGAG 504

Discussion

Molecular techniques are more advantageous 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\(^4\).

These genotypic approaches can identify the dermatophytes to the species as well as the strain levels\(^4\). In this study, we used nested PCR-based methods for identification of dermatophyte species isolated from patients with dermatophytosis.

In this study the results also indicate that nested PCR may be considered as gold standard for the diagnosis of dermatophytosis and can aid the clinician in initiating prompt and appropriate antifungal therapy and this also supports our finding.

The nested PCR was considered a gold standard for the diagnosis of dermatophytosis. In our study both nucleotide and protein maximum likelihood trees elucidated that isolates from Sudan are grouped in two groups.

The first group (n = 7) is closely related to *Trichophyton violaceum* that is reported to cause >90% of tinea capitis cases across Africa, and *Arthroderma benhamiae* is a zoophilic dermatophyte species belonging to the *Trichophyton mentagrophytes* complex, which produces highly inflammatory tinea corporis and tinea capitis infections in humans.

The second group (n = 3) is very close to *Epidermophyton floccosum* which is anthropophilic dermatophyte with a world-wide distribution in the tropics and subtropics and frequently causes tinea cruris, tinea pedis, tinea corporis and onychomycosis.

Conclusion: The nested-PCR technique is a valuable tool for the laboratory diagnosis of dermatophytes. It was found to be a gold standard for the diagnosis of dermatophytoses and can aid the clinician in initiating prompt and appropriate antifungal therapy.

Kabbashi, *et al.*, 2016: Vol 1(2)
References


Kabbashi, et al., 2016: Vol 1(2)