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Original Research Article

Molecular Identification of Dermatophytes by Sequencing ITS Region of Ribosomal RNA

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Abstract:

Background: Dermatophytes are a group of fungi that cause tinea or dermatophytosis. In tropical and subtropical countries like India, prevalence of these infections is much more because of high heat and humidity for most part of the year, over population and poor hygienic living conditions.

Materials and Methods: Skin, nail and hair samples of 150 patients with suspected tinea lesions were subjected to direct microscopy and culture for phenotypic identification, followed by genotypic confirmation by PCR and strain identification by sequencing of ITS regions.

Results: Tinea corporis was the most common clinical presentation (44.12%) followed by T. manuum and T. pedis (14.71% each) .A total of 44 (29.33%) cases showed fungal growths, 34(22.67%) isolates were pure dermatophytes and 10(6.67%) isolates were non-dermatophytic molds. Most common isolate was Trichophyton rubrum (50%) followed by T. interdigitale (32.35%). Phenotypic identification of the isolates in our study showed 5 different species viz T. mentagrophytes, T. rubrum, T. verrucosum, T. interdigitale and M. gypseum. However, molecular method identified only 4 different species viz. T. mentagrophytes, T. interdigitale, T. rubrum and M. gypseum.

Conclusion: In the present study, some of the dermatophyte species misidentified by conventional methods were identified accurately by molecular method. Hence, to conclude, we can say that molecular method of species identification helps in accurate identification of species which helps to choose proper antifungal therapy for patient care.

Keywords: Dermatophytes, Trichophyton rubrum, Trichophyton interdigitale, PCR, Sequencing.

Introduction

Dermatophytes are a distinct group of fungi that infect the skin, hair and nails of human and animals producing a variety of cutaneous infections, colloquially known as “ring worm” or tinea or dermatophytoses. Infections of the keratinized tissues of the body caused by dermatophytes are a common global problem as evident by high prevalence of superficial mycotic infections which shows that 20-25% of the world’s population has skin mycoses.[1]

In tropical and subtropical countries like India, prevalence of these infections is much more because of high heat and humidity for most part of the year, over population and poor hygienic living conditions.[2]

Though not fatal, the dermatophyte infections cause high morbidity, thereby becoming a costly disease in terms of loss of working days and prolonged duration of treatment, imposing a considerable economic burden. Moreover, injudicious intake of steroids both in immunocompetent & immunosuppressed individuals in recent years, has made diagnosis and management of dermatophyte infections a definite challenge to mankind in the years to come.[3]Above all, it has been seen that various species of dermatophytes show varying susceptibility to antifungal agents. [4] In view of all the above reasons, a correct diagnosis is essential not only to initiate appropriate treatment but also for epidemiological purposes. Hence, for an accurate and rapid diagnosis of dermatophyte infections in addition to epidemiological relationship, distinction among the isolates to the species level is a first step and can easily be performed by molecular methods like Polymerase Chain Reaction (PCR) and sequencing.[5]

Materials and Methods

Study setting:

The present study was conducted from June 2016 to July 2017(1yr), in the department of Microbiology of a tertiary care centre. It was a hospital based cross sectional study and total of 150 patients with clinically suspected tinea lesions were included in the study. The ethical clearance was obtained from the Institutional Ethics Committee (Human). Written informed consent was obtained from parents or legal guardians of patients. Demographic, socioeconomic and clinical data were documented in case report forms.

Inclusion Criteria: Every consecutive clinically suspected patients with tinea lesions.

Exclusion Criteria:

1. Infant and children were excluded from the study.
2. Isolates other than dermatophytes were excluded from the study.
3. Patients taking anti- fungal drug treatment during last 1 month.

Specimen collection-

Skin, nail scrapings and hair samples: Collected after cleaning thoroughly with 70% alcohol. All the samples were transported in paper envelope to the laboratory within 2 hours of sample collection to ensure optimum culture yield. [6]

Sample processing: Each sample was divided in two parts –one for direct microscopy by KOH mount and the other part for culture.

Direct microscopy: KOH mount:- 10% KOH was used for skin scrapings and 40% KOH was used for nails and hairs.

Culture:

Done in Sabouraud Dextrose agar (SDA) (Himedia), SDA with chloramphenicol and Cycloheximide (Himedia) & Dermatophyte test medium (DTM) (Himedia). They were incubated at room temperature (24ºc-28ºc) and examined twice weekly for upto 4 weeks before being discarded as negative. Culture isolates were identified by macroscopic appearances of the colonies and microscopically using the stain Lactophenol cotton blue (LPCB).

Molecular methods of detection:

All the clinical isolates which were identified based on phenotypic characteristics were subjected to genotypic confirmation by Polymerase Chain Reaction (PCR) and strain identification by sequencing of Internal Transcribed Spacer (ITS) regions. Genomic DNA extraction was done by phenol chloroform method [7]. Conventional Polymerase chain reaction (PCR) was carried out using Thermal Cycler (Thermo Scientific) following Elavarashiet al [8], with slight modification. The final volume of the reaction mixture was 50 µl. Amplification of ITS1, 5.8S r-RNA and ITS2 were carried out using primers ITS1 and ITS4, sequences of the primers being TCCGTAGGTGAACCTGCGG(5^1 – 3^1) and TCCTCCGCTTATTGATATGC(5^1 – 3^1) respectively.

Each amplified PCR product was electrophoresed in 1% agarose gel in TAE buffer and visualized by UV fluorescence after ethidium bromide staining (0.5mg/dl). PCR amplicons were purified and sent for ITS sequencing to Eurofins Genomics Bangalore, India. Forward and reverse DNA sequencing reactions of PCR amplicons were carried out with forward and reverse primers using Big Dye Terminator version 3.1 Cycle sequencing kit(Applied Biosystems) on ABI 3730 xl Genetic Analyzer (Applied Biosystems). Consensus sequence of ITS1, 5.8S r RNA and ITS2 region was generated from forward and reverse sequence data using Codon Code aligner software (Codon Code Corporation).

Trace files were also checked manually and ambiguous bases were corrected depending on forward and reverse sequences with the PHRED scores received with the sequence trace files. Species were identified by searching databases using the BLAST sequence analysis tool (http://www.ncbi.nlm.nih.gov/BLAST/) and comparative ITS sequence identification was done using reference sequences available at Genbank and http://its.mycologylab.org/.If the query sequence of ITS1-5.8S r RNA-ITS2 matched more than 98.5%,it was taken as correct identification. Any query sequence matching less than 98.5% with database were taken as uncertain identification. For Phylogenetic analysis, sequences were aligned using CLUSTAL W that is the part of the software MEGA version 7.0. Phylogenetic tree was constructed after selecting the best fit model after model test. [9,10]

Table1: showing sequences of NCBI database used as reference in the study

Species	Reference strain
T.rubrum	CBS 392.58
T.rubrum	CBS 118892
T.rubrum	ATCC 52013
T.rubrum	ATCC 28188
T.interdigitale	ATCC 24957

T.interdigitale	ATCC 42896
T.interdigitale	ATCC 200101
T.interdigitale	ATCC 18613
T.mentagrophytes	ATCC 34551
M.gypseum	ATCC MYA-4604
M.gypseum	WCH-MG001
Aspergillus niger(outgroup)	KU847849.1

Data Analysis: Data analysis was done using the Epi Info 7 software and Microsoft office excels 2007. Significance testing was done using both the Chi square test of goodness of fit and Fisher's exact test.

Results

Out of a total of 150 patients suspected with tinea or dermatophytosis, 97(64.7%) affected were males while 53 (34.3%) were females, with a ratio M: F=1.83:1.

Out of the 34 confirmed cases, 15 (44.12%) cases were involved in agricultural work with 9 being farmers and 6 tea garden workers. Majority of the cases 23(67.7%) belonged to the age group of 21-40 yrs followed by 7 (20.6%) cases in the 41-60 yrs bracket. Tinea corporis was the most common clinical presentation (44.12%) followed by Tinea manuum and Tinea pedis (14.71% each). (Table 2)

Table 2: Showing distribution of different clinical forms of tinea.

Clinical forms	Number (%)
T.corporis	15(44.12)
T.manuum	5(14.71)
T.pedis	5(14.71)
T.cruris	4(11.16)
T..faciei	2(5.88)
T.unguium	2(5.88)
T.capitis	1(2.94)

A total of 44 (29.33%) cases showed fungal growths, 34(22.67%) isolates were pure dermatophytes and rest 10(6.67%) isolates were non-dermatophytic molds.

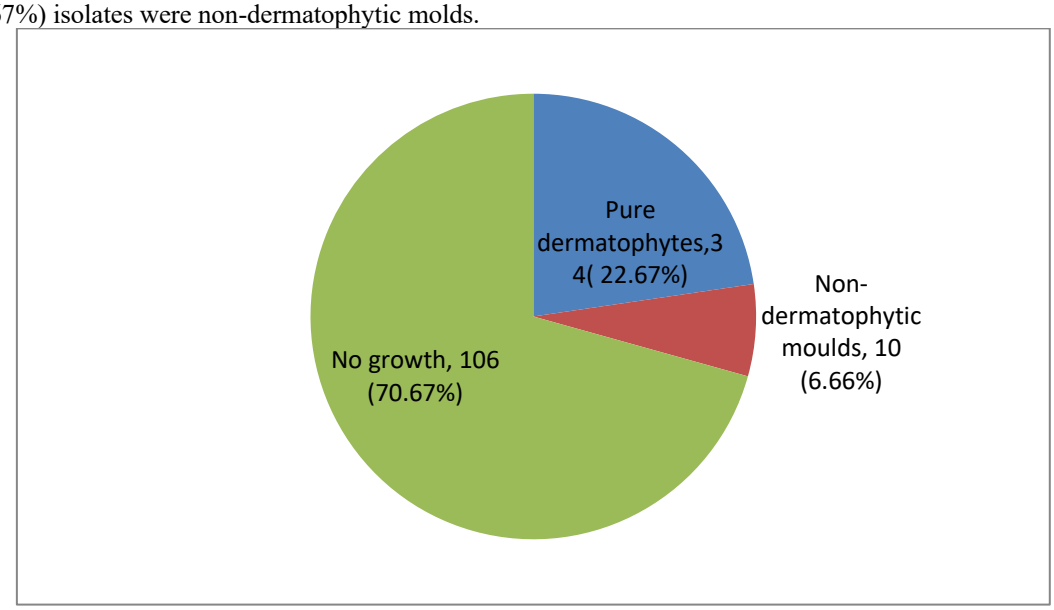


Figure 1: Culture findings in the 150 cases

56 out of 150 cases included in this study were KOH positive by direct microscopy and 34(60.71%) of these KOH positive cases were culture positive with growth of pure dermatophytes. KOH negative samples did not yield growth of dermatophyte.

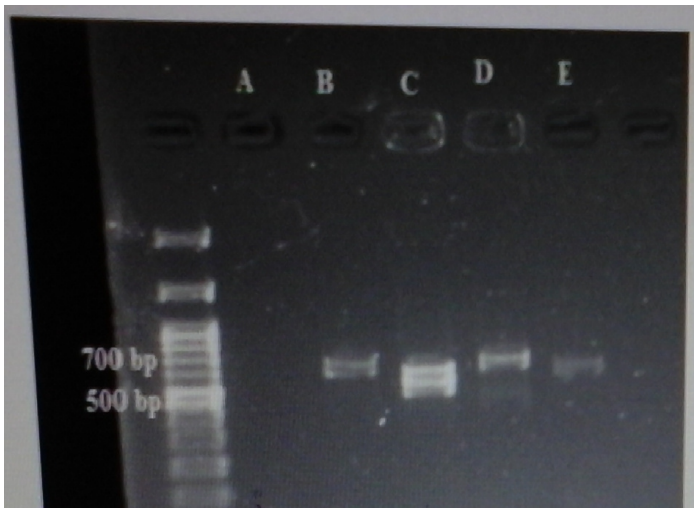
Table 3: showing the results of direct microscopy by KOH and cultures of the isolate.

Results of KOH mount and fungal culture	Number (%)
KOH positive, culture positive	34 (22.67)
KOH positive, culture negative	22 (14.67)
KOH negative, culture positive	0 (0)
KOH negative, culture negative	94 (62.67)

Trichophyton rubrum was the most common isolate 17(50%) followed by Trichpphyton interdigitale 11(32.35%).

Table 4: Showing distribution of species of dermatophytes isolate

Species	Number (%)
T.rubrum	17(50)
T.interdigitale	11(32.35)
T.mentagrophytes	3(8.82)
M.gypseum	3(8.82)



Lane A-Negative control
Lane B-T.rubrum
Lane C-T.interdigitale
Lane D-T.mentagrophytes
Lane E-M.gypseum
100 bp Ladder was used and 500bp and 700 bp are marked in the Ladder.

Figure 2: PCR amplified ITS-1 and ITS-2 regions of all 34 dermatophyte isolates using universal fungal primers, ITS-1 and ITS-4 showed amplicons of size between 500-700 bp.

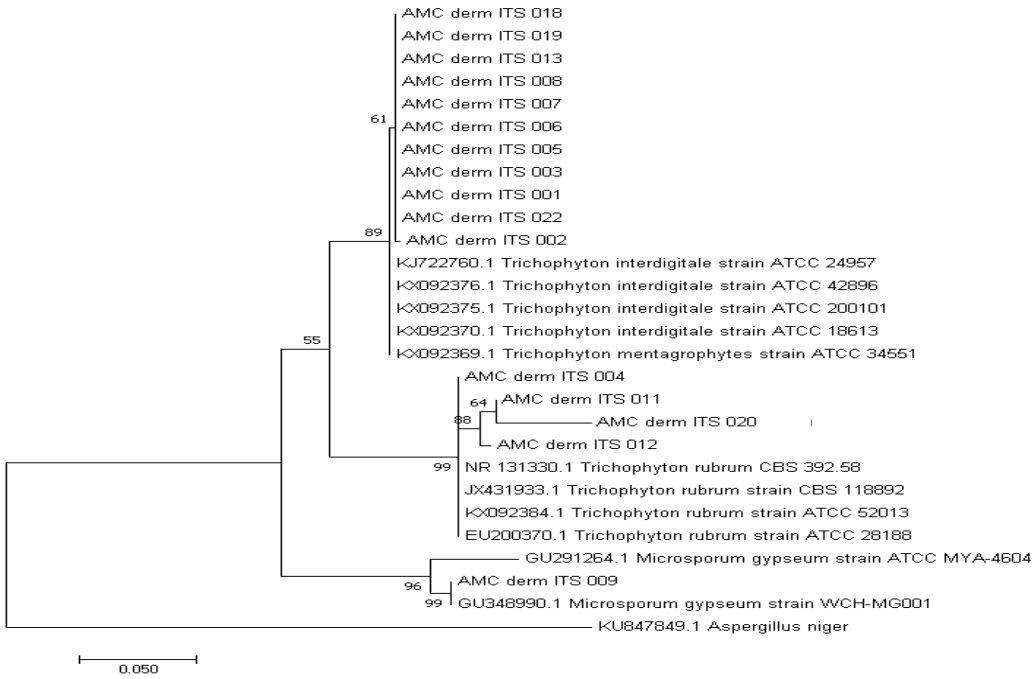


Figure 3: Phylogenetic tree constructed by the maximum likelihood method using 28 whole ITS sequences with 1000 bootstrap replications (Kimura 2 parameter method).

Table 5: Initial phenotypic identification confirmed by ITS sequencing

Strain	Phenotypic identification	Molecular identification
AMC/MICRO/001	T.mentagrophytes	T.interdigitale
AMC/MICRO/002	T.mentagrophytes	T.interdigitale
AMC/MICRO/003	T.interdigitale	T.interdigitale
AMC/MICRO/004	T.rubrum	T.rubrum

AMC/MICRO/005	T.mentagrophytes	T.interdigitale
AMC/MICRO/006	T.interdigitale	T.interdigitale
AMC/MICRO/007	T.interdigitale	T.interdigitale
AMC/MICRO/008	T.verrucosum	T.interdigitale
AMC/MICRO/009	M.gypseum	M.gypseum
AMC/MICRO/010	T.rubrum	T.rubrum
AMC/MICRO/011	T.rubrum	T.rubrum
AMC/MICRO/012	T. species	T.interdigitale
AMC/MICRO/013	T.rubrum	T.rubrum
AMC/MICRO/014	T.verrucosum	T.interdigitale
AMC/MICRO/015	T.rubrum	T.rubrum
AMC/MICRO/016	T.rubrum	T.rubrum
AMC/MICRO/017	T.rubrum	T.rubrum
AMC/MICRO/018	T.mentagrophytes	T.mentagrophytes
AMC/MICRO/019	T.mentagrophytes	T.interdigitale
AMC/MICRO/020	M.gypseum	M.gypseum
AMC/MICRO/021	T.rubrum	T.rubrum
AMC/MICRO/022	T.rubrum	T.rubrum
AMC/MICRO/023	T.rubrum	T.rubrum
AMC/MICRO/024	T.rubrum	T.rubrum
AMC/MICRO/025	T. mentagrophytes	T.mentagrophytes
AMC/MICRO/026	T.rubrum	T.rubrum
AMC/MICRO/027	T.rubrum	T.rubrum
AMC/MICRO/028	T.mentagrophytes	T.mentagrophytes
AMC/MICRO/029	M.gypseum	M.gypseum
AMC/MICRO/030	T.mentagrophytes	T.interdigitale
AMC/MICRO/031	T.rubrum	T.rubrum
AMC/MICRO/032	T.rubrum	T.rubrum
AMC/MICRO/033	T.rubrum	T.rubrum
AMC/MICRO/034	T.rubrum	T.rubrum

Table 6: Showing designated strain number and GenBank accession numbers of the submitted sequences

Species	Strain number	GenBank accession no
T.interdigitale	AMC/MICRO/001	MF800863
T.interdigitale	AMC/MICRO/002	MF800864
T.interdigitale	AMC/MICRO/003	MF800865
T.rubrum	AMC/MICRO/004	MF800866
T.interdigitale	AMC/MICRO/005	MF800867
T.interdigitale	AMC/MICRO/006	MF800868
T.interdigitale	AMC/MICRO/007	MF800869
T.interdigitale	AMC/MICRO/008	MF800870
M.gypseum	AMC/MICRO/009	MF800871
T.rubrum	AMC/MICRO/010	MF800872
T.rubrum	AMC/MICRO/011	MF800873
T.interdigitale	AMC/MICRO/012	MF800874
T.rubrum	AMC/MICRO/016	MF800875
T.rubrum	AMC/MICRO/017	MF800876
T.interdigitale	AMC/MICRO/019	MF800877

Discussion

In the present study, out of a total of 150 patients suspected with tinea lesions, males were 64.67% and females were 34.33 %(M: F=1.83:1), showing male preponderance. This is consistent with the findings of studies from others parts of India [11,12]. Male preponderance of dermatophytoses is due to the fact that they are involved more in the physical activities as well as social interactions for a living [11]. It may also be due to the fact that such skin disorders are considered to be one of the social stigmas, so females approach the medical facilities less often. Many of them rely upon home remedies and seek medical advice only in cases of chronic conditions and for cosmetic purposes [13].

As regards to occupational exposure, majority of the patients (44.12%) were found to be associated with agricultural work. This type of association was

also found in previous studies by various studies [14,15,16]. The probable factors accounting for this association in our study includes increased sweating while being engaged in outdoor activities, constant contact with plants , leaves and soil, poor personal hygiene and low socio-economic status of these patients.

In the context of overall age incidence, our study showed that the dermatophyte infections were predominant in the age group of 21 – 40 yrs. This finding is in conformity with the previous findings by different studies in India [20, 22]. The reason for this may be due to increased level of physical activity in this particular age group leading to excessive sweating which favors the growth of dermatophytes. Socialization with different people is also high compared to other age groups which eventually help in spreading the infection [22].

Among the different clinical forms of tinea infections encountered in our study, Tinea corporis was found to be the most common presentation accounting for 44.12% of cases. This is in corroboration to the findings of previous publications by different national and international studies [18,19,20], while a study in Northeast India found Tinea pedis to be most common [21].

In this study, 56 (37.33%) samples were positive for fungal hyphae in KOH mount while 34(22.67%) were positive in culture. All the culture positive cases were also KOH positive, which showed that direct KOH mount was a good screening test for dermatophyte identification. This correlates with the Indian study in the rate of positivity [17].

In our study, among 150 cases clinically suspected with tinea lesions, Trichophyton species was found to be the predominant etiological agent with 31 isolates out of 34 isolates whereas only 3 isolates were of Microsporum species. This finding is similar to that of the studies conducted in India [22,23]. Trichophyton rubrum was the predominant isolate in our study which was a similar finding to other studies done earlier in India and abroad [7,22]. The isolation rate of Trichophyton rubrum in this study was 50%, which corroborate with the study conducted by Poluri et al(58.06%)[18], Sudha et al(56.92%) [17] and Malik et al(58.5%)[24] but varies considerably from that of Balakumar et al(32.8%) [12] and Kumar et al(67.5%)[11].However, all these studies were based on phenotypic identification in contrast to our study which was based on phenotypic as well as molecular identification. Phenotypic identification of the isolates in our study showed 5 different species viz T.mentagrophytes, T.rubrum, T.verrucosum, T.interdigitale and M.gypseum. However, molecular method identified only 4 different species viz. T.mentagrophytes, T.interdigitale, T,rubrum and M.gypseum.

Preliminary phenotypic identification of genotypically confirmed T interdigitale in our study was T mentagrophytes in 5 isolates, Trichophyton species in one, T. interdigitale in 3 and T. verrucosum in two isolates. Accurate phenotypic identification of this species was found only in 27.27% (n=3/11) of the isolates. As most of the earlier reported studies were based on phenotypic identification methods it is possible that this cryptic species which was earlier considered to be a member of T. mentagrophytes complex was underreported.

Traditional methods of identification of dermatophytes such as macroscopic and microscopic appearances of cultures of fungi seem to be erroneous due to the polymorphic feature of these characters, besides increased by differences in media compounds, temperature variations, and other variables of cultivation. So currently, molecular studies have become crucial and necessary for identification of dermatophytes [24, 25]. In this regard, the internal transcribed spacer (ITS) region sequencing had been used as one of the sophisticated techniques for species identification as it is faster, helps in accurate species determination, specific, and are less feasible to be affected by exterior effects such as temperature changes and chemotherapy [26].

If we compare conventional and molecular methods of identification of dermatophytes, we found that all the T.rubrum and M.gypseum isolates by phenotypic methods revealed same identification in ITS sequencing also. One isolate of genus Trichophyton that could not be identified to the species level by culture and microscopy was detected as T.interdigitale by ITS sequencing and blast analysis. By standard conventional methods of dermatophyte identification, two isolates were misidentified as T.verrucosum which were further confirmed as T.interdigitale by molecular detection. Similarly, 5 isolates misidentified as T.mentagrophytes by conventional methods were further confirmed as T.interdigitale by molecular detection.

Conclusion

Infections caused by dermatophytes are of major concern now a days owing to their worldwide distribution with subsequent alarming rise in the number of infected patients. Favourable climatic conditions, demography and socioeconomic status of the people have made Assam, a part of Norh-east India, a suitable place for the growth of such fungi leading to increased prevalence of infections. However, the common species responsible for this infection may vary from region to region.

Emerging antifungal drug resistance is also a problem in recent times.

In the present study, some of the dermatophyte species misidentified by conventional methods were identified accurately by molecular method. In searching published literatures in the related field, it was found that in most of the Indian studies, dermatophyte identification was done on the basis of conventional methods only. Hence, to conclude, we can say that molecular method of species identification helps in accurate identification of species which help to choose proper antifungal therapy for patient care.

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