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Molecular strain typing of *Trichophyton mentagrophytes* (*T. mentagrophytes* var. *interdigitale*) using non-transcribed spacer region as a molecular marker

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***Background & objectives*: Dermatophytes are keratinophilic fungi that infect keratinized tissues of human and animal origin. *Trichophyton mentagrophytes* is considered to be a species complex composed of several strains, which include both anthropophiles and zoophiles. Accurate discrimination is critical for comprehensive understanding of the clinical and epidemiological implications of the genetic heterogeneity of this complex. Molecular strain typing renders an effective way to discriminate each strain. The objective of the study was to characterize *T. mentagrophytes* clinical isolates to sub-species level using molecular techniques and non-transcribed spacer (NTS) region as marker.**

***Methods*: Sixty four *T. mentagrophytes* clinical isolates were identified by phenotypic methods. These were subjected to polymerase chain reaction targeting three sub-repeat elements (SREs), TmiS0, TmiS1 and TmiS2 of the NTS region. Sequence analysis of internal transcribed spacer (ITS) region of different types was also done.**

***Results*: Strain-specific polymorphism was observed in all three loci. Totally, 13 different PCR types were obtained on combining all the three SREs loci. No variation was observed in the ITS region.**

***Interpretation & conclusions*: The study described the usefulness of molecular strain typing technique for the discrimination of the *T. mentagrophytes* isolates. This will help for the future explorations into the epidemiology of *T. mentagrophytes* and its complex.**

Key words Dermatophytes - molecular strain typing - non-transcribed spacer region - polymerase chain reaction - sub-repeat elements - *Trichophyton mentagrophytes* var. *interdigitale*

Dermatophytes are the most common agents of superficial mycoses. Dermatophytes are adapted to utilize keratin as a major nutritional source; hence, infection is generally cutaneous and restricted to the non-living cornified layers such as skin, stratum corneum, hair and nails of humans and animals[1]. In India, *Trichophyton mentagrophytes* is next to *Trichophyton rubrum* in causing dermatophytosis[2,3]. *T. mentagrophytes* is both anthropophilic and zoophilic. The downy form is associated with chronic human

infections, whereas the granular form causes animal and acute human infections[4].

Although *T. rubrum* and *T. mentagrophytes* are well separated in phylogeny, the clinical conditions manifested are similar. These show considerable variation in microscopic and cultural characteristics, but these cannot be taken as strain markers as the phenotypic characters of *T. mentagrophytes* can change notably on routine sub-culture.

Genotypic approaches have been proven to be useful for solving problems in dermatophyte taxonomy, as well as enhancing the reliability and speed of dermatophytosis diagnosis and strain differentiation[5]. Genotyping using variable tandem sub-repeat elements (SREs) of non-transcribed spacer (NTS) region in the ribosomal DNA (*rDNA*) gene cluster has been demonstrated in *T. rubrum*[6]. *T. mentagrophytes* var. *interdigitale* has also been shown to possess genetic polymorphisms that map to the rDNA[7-9]. The NTS region in *T. mentagrophytes* var. *interdigitale* consists of three highly repetitive SREs, namely TmiS0, TmiS1 and TmiS2. These regions are more prone to mutations due to unequal crossing over between strains and have been used to determine the intra-species variations[8,9]. Identifying the isolates to the strain level along with clinical details will aid in tracking the source of infection. Only a few studies have been conducted on strain typing of *T. mentagrophytes*[8,9] in other countries, which includes representative Indian clinical isolates. The present study was therefore, conducted to characterize *T. mentagrophytes* isolates to sub-species level using molecular strain typing techniques and using NTS region as a molecular marker.

Material & Methods

Sixty four *T. mentagrophytes* isolates received from April 2012 to March 2014 in the Mycology division, department of Microbiology, Sri Ramachandra Medical College & Research Institute, Chennai, India, were used in this study. All these isolates were characterized based on macroscopic and microscopic appearance from the primary culture.

Phenotypic characterization of T. mentagrophytes: All isolates were grown in Sabouraud dextrose agar (SDA) (Hi-Media, Mumbai) with actidione. The culture characteristics were observed on SDA and dermatophyte test medium (Hi-Media). Microscopic characteristics were observed in lactophenol cotton blue (LPCB) (Hi-Media) mount. Those isolates which did not demonstrate proper microscopic characters in LPCB mount were subjected to slide culture technique with oatmeal agar (Hi-Media) and observed. The isolates which were confirmed as *T. mentagrophytes* were taken for molecular characterization.

Molecular characterization of T. mentagrophytes

DNA isolation: DNA was extracted from all the 64 *T. mentagrophytes* isolates by boiling method[10], with certain modifications. Briefly, the culture was suspended in 400 µl lysis buffer [10 mM TRIS, (*p*H 8), 1 mM EDTA (*p*H 8), 3% SDS and 100 mM NaCl] in a 1.5 ml microfuge tube; 20 µl of proteinase K (1 mg/ml) (Merck GeNei, India) was added and incubated at 56°C for 30 min. It was boiled for one minute and 400 µl of phenol:chloroform (Sigma, USA) (1:1) mixture was added, mixed well and centrifuged at 8600 g for 10 min. The aqueous layer was transferred to a new microfuge tube; equal volume of chloroform was added, mixed well and centrifuged at 8600 g for 10 min. The aqueous layer was transferred to a new microfuge tube. DNA was precipitated using equal volume of ice cold isopropyl alcohol and washed twice with 70 per cent ethanol. The pellet was suspended in 40 µl sterile nuclease-free water and stored at −20°C until use.

Amplification of non-transcribed spacer (NTS) region: PCR amplification of all the three SREs was performed individually in an Eppendorf Gradient Mastercycler (Eppendorf, Germany). Primer sequences used for all three regions (sub-repeat regions, S0, S1 and S2) are listed in Table I. Each reaction mix contained 25 µl PCR master mix (Merck GeNei), 50 pmol forward (Sigma) and reverse primers (Sigma) each, and 1 µl DNA template. The volume was made up to 50 µl with nuclease-free water (Merck GeNei).

PCR was carried out for all three sub-repeat regions under the same reaction conditions with denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 90 sec. The cycle was repeated 35 times. Further, an initial denaturation at 95°C for five minutes, and final extension at 72°C for 10 min were added.

Amplification of internal transcribed spacer (ITS) region: The reaction mix contained 25 µl PCR master mix (Merck GeNei), 50 pmol universal fungal primers, ITS-1 (Sigma) and ITS-4 (Sigma) each, 1 µl of template DNA and the volume made up to 50 µl with nuclease-free water. Amplification was carried out under the following conditions: initial denaturation at

Table I. Primers for amplification of internal transcribed spacer region and sub-repeat elements

Primer name	Sequence
ITS region	
Forward primer ITS 1	5'-TCC GTA GGT GAA CCT GCG G-3'
Reverse primer ITS 4	5'-TCC TCC GCT TAT TGA TAT GC-3'
S0 sub-repeat element	
Forward primer TmiS0F	5'-CGA AGG ATA CTG TGG AAG ATG-3'
Reverse primer TmiS0R	5'-GCA ACA TAA GTG TAC AGC TG-3'
S1 sub-repeat element	
Forward primer TmiS1F	5'-CAG CTG TAC ACT TAT GTT GC-3'
Reverse primer TmiS1R	5'-TCG TTC GCC TCG AAG ACG CAC-3'
S2 sub-repeat element	
Forward primer TmiS2F	5'-GAC CTT CAT TCT AGC TAT G-3'
Reverse primer TmiS2R	5'-CCT ATC GTA CGA GAA CGT TAG-3'

Source: Ref 8. ITS, internal transcribed spacer

95°C for five minutes, denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 30 sec and final extension at 72°C for five minutes.

Agarose gel electrophoresis: All three SREs PCR products and ITS products were electrophoresed in 1.5 per cent agarose gel in tris-acetate-EDTA buffer, stained with ethidium bromide (0.5 µg/ml), visualized under ultraviolet light and photographed.

Gene sequencing: The ITS region was sequenced for representative isolates of each NTS type (SciGenom Labs, Cochin, Kerala, India). The sequences were then used for nucleotide-nucleotide search using the BLAST algorithm at the NCBI website (*http://www.ncbi.nlm.nih.gov/BLAST/*). BLAST hits more than 98 per cent were considered.

Results

Phenotypic characterization of T. mentagrophytes: The colonies were powdery (39/64) to floccose (25/64), cream to yellowish buff coloured. Microconidia were spherical, sessile, arranged in dense, grape-like clusters or along the hyphae. The spiral hyphae were present in 52 of 64 isolates (81%). Macroconidia were 3-8 celled, smooth and thin-walled, clavate to cigar-shaped.

Molecular characterization of T. mentagrophytes: All three SREs, TmiS0, TmiS1 and TmiS2 were amplified and each region produced different banding patterns. As banding patterns were not identical with the previous studies[8,9], all three SREs types were designated numerically and the previous study's nomenclature was not followed.

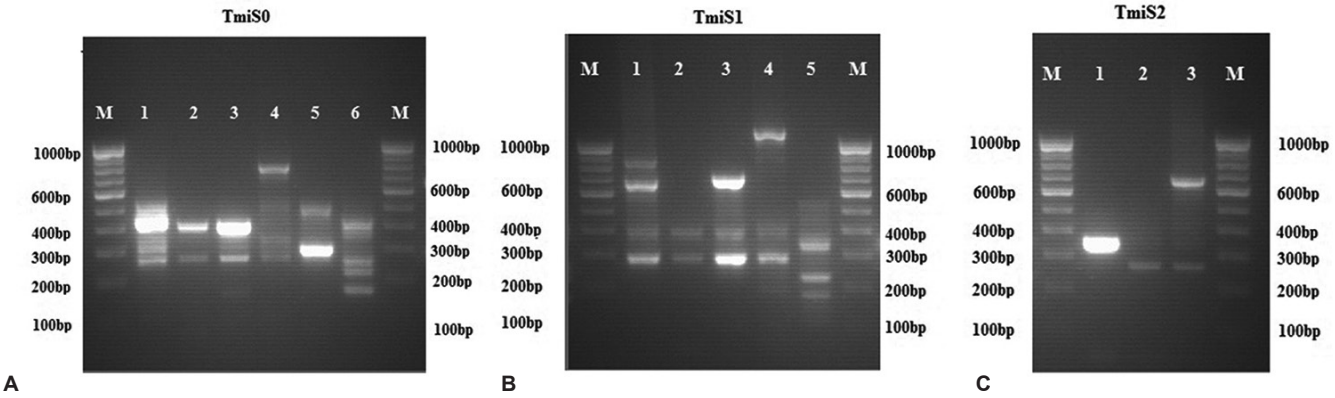

Figure. Polymerase chain reaction fingerprint of representative types of *Trichophyton mentagrophytes* TmiS0 (**A**), TmiS1 (**B**) and TmiS2 (**C**) loci.

Table II. Sub-repeat element (TmiS0, TmiS1 & TmiS2) fingerprint type of each isolate

Clinical isolates	Type of infection	PCR types		
		TmiS0	TmiS1	TmiS2
TM 01	Tinea corporis	1	1	1
TM 02	Tinea corporis	2	2	2
TM 03	Tinea corporis	3	3	3
TM 04	Tinea corporis	2	3	3
TM 05	Tinea cruris	4	4	3
TM 06	Tinea corporis	2	2	1
TM 07	Tinea corporis	3	3	3
TM 08	Tinea capitis	5	1	1
TM 09	Tinea corporis	3	3	3
TM 10	Tinea cruris	3	3	3
TM 11	Tinea corporis	2	3	3
TM 12	Tinea cruris	2	3	3
TM 13	Tinea corporis	4	2	3
TM 14	Tinea cruris	3	3	3
TM 15	Tinea cruris	6	5	3
TM 16	Tinea pedis	1	4	1
TM 17	Tinea manuum	1	3	3
TM 18	Tinea corporis	2	2	3
TM 19	Tinea cruris	1	3	3
TM 20	Tinea corporis	2	3	3
TM 21	Tinea unguium	2	3	3
TM 22	Tinea cruris	2	3	1
TM 23	Tinea corporis	2	3	3
TM 24	Tinea cruris	2	3	3
TM 25	Tinea corporis	2	3	3
TM 26	Tinea unguium	1	3	3
TM 27	Tinea manuum	1	3	3
TM 28	Tinea corporis	2	3	3
TM 29	Tinea corporis	2	2	3
TM 30	Tinea corporis	2	3	3
TM 31	Tinea corporis	2	3	3
TM 32	Tinea corporis	2	3	3
TM 33	Tinea unguium	2	3	3
TM 34	Tinea unguium	2	3	3
TM 35	Tinea corporis	2	3	3
TM 36	Tinea corporis	2	2	3
TM 37	Tinea corporis	2	3	3
TM 38	Tinea unguium	2	3	3
TM 39	Tinea unguium	2	3	3
TM 40	Tinea corporis	2	2	1
TM 41	Tinea corporis	2	3	3
TM 42	Tinea corporis	2	3	3
TM 43	Tinea corporis	3	3	3
TM 44	Tinea unguium	2	3	3
TM 45	Tinea unguium	2	2	3
TM 46	Tinea corporis	2	3	3
TM 47	Tinea corporis	2	3	3
TM 48	Tinea cruris	2	3	3
TM 49	Tinea corporis	2	3	3
TM 50	Tinea corporis	1	3	3
TM 51	Tinea unguium	2	3	3
TM 52	Tinea manuum	1	3	3
TM 53	Tinea corporis	2	3	3
TM 54	Tinea unguium	2	2	3
TM 55	Tinea corporis	2	3	3
TM 56	Tinea corporis	2	3	3
TM 57	Tinea corporis	2	3	3
TM 58	Tinea corporis	2	3	3
TM 59	Tinea cruris	2	3	3
TM 60	Tinea corporis	2	3	3
TM 61	Tinea corporis	2	3	3
TM 62	Tinea corporis	2	3	3
TM 63	Tinea cruris	2	3	3
TM 64	Tinea corporis	2	3	3

The PCR which targeted TmiS0 loci produced six different banding patterns with varying product size of approximately 180-800 bp (Fig. A). Two to four bands were observed in each type. The isolates were sorted based on the banding patterns and tabulated (Table II). Among them, type 2 was predominant accounting for a total of 46 of 64 isolates (71.8%). PCR which amplified TmiS1 loci produced five patterns with two to four bands in each type. The product size ranged approximately 180-1100 bp (Fig. B). Type 2 dominated with a total of 50 of the 64 isolates (78.1%) and the types are described in Table II. PCR amplification of TmiS2 loci produced three banding patterns with maximum of two bands. The types are tabulated in Table II. The product size ranged from 280 to 680 bp (Fig. C). Among them, type 3 was predominant with a total of 57 of the 64 isolates (89%).

Table III. Polymerase chain reaction (PCR) fingerprint profile of whole non-transcribed spacer region (TmiS0, TmiS1 and TmiS2)

Combined PCR types	SREs PCR types			Number of isolates (%)
	TMiS0	TMiS1	TMiS2	
1	1	1	1	1 (1.6)
2	2	2	2	1 (1.6)
3	3	3	3	6 (9.3)
4	2	3	3	37 (57.8)
5	4	4	3	1 (1.6)
6	2	2	1	2 (3.1)
7	5	1	1	1 (1.6)
8	4	2	3	1 (1.6)
9	6	5	3	1 (1.6)
10	1	4	1	1 (1.6)
11	1	3	3	6 (9.3)
12	2	3	1	1 (1.6)
13	2	2	3	5 (7.7)
Total				64 (100)

SREs, sub-repeat elements

The variation between the three SREs (TmiS0, TmiS1 and TmiS2) was determined and grouped based on the differences in the banding pattern (Table II). The variation in the whole NTS region was studied by combining the banding patterns of TmiS0, TmiS1 and TmiS2 loci (Table III). The variability in banding pattern produced by individual locus appeared to occur independently. When all the three PCR patterns were combined, a total of 13 types were observed from the 64 isolates.

Internal transcribed spacer (ITS) sequence: All the 64 isolates were phenotypically confirmed as *T. mentagrophytes*. Of the 13 different NTS types observed in this study, ITS region of one representative isolate from each type was amplified and sequenced to confirm the isolates genotypically. BLAST hits showed more than 99 per cent identity to *T. mentagrophytes* var. *interdigitale*. The sequences were deposited in Genbank database (Accession numbers KP099590-KP099602).

Discussion

Large and small unit ribosomal RNA genes are arranged in clusters as tandem repeats in multiple copies. The tandem repeats are flanked both sides by NTS region which is more prone to mutation than the transcribed regions. Hence, the NTS region can be utilized for typing of dermatophytes to sub-species level. Earlier studies on *T. rubrum*[6,11-13], *T. tonsurans*[14-18] and *T. mentagrophytes* var. *interdigitale*[8,9] have used NTS region for investigating polymorphism and sub-species level discernments. In the current study, the primers for NTS used to type *T. mentagrophytes* var. *interdigitale* in the UK and Japan[8,9], were utilized. As there was no similarity in banding patterns with previous studies, all three PCR types were named numerically to avoid overlap in pattern names. The TmiS0 locus had the greatest polymorphism by producing most number of PCR types among all three loci. Of the six types observed, band size 400 bp was shared by four types which accounted for almost 61 of 64 isolates (94%) but none of the isolate produced single band.

The TmiS1 locus also had five PCR types with multiple bands. Bands sized 300 and 400 bp were shared by type 1-4 which accounted for 63 of 64 isolates (98%). The TmiS1 locus was the longest and produced band of size 1100 bp. As demonstrated in earlier studies[8,9], TmiS2 locus was the least polymorphic region which produced only three types in which 280 bp sized band was shared by two types accounting 58 of 64 isolates (91%).

Conjoining all three loci in NTS region produced a total of 13 types among 64 isolates, of which 37 (57.8%) were type 2-3-3, six (9.3%) of each type 3-3-3 and 1-3-3, five (7.7%) were 2-2-3, two (3.1%) were 2-2-1 and other eight types were produced by single isolates. The study performed in the UK failed to amplify TmiS0 and TmiS1 loci[8], whereas in our study all three loci were amplified. None of the isolates produced patterns identical to those described in the UK or Japanese study[8,9]. The size of the PCR products from all three SREs was also very smaller than that observed in the earlier studies[8,9]. This could be due to degeneration in NTS region in Indian isolates compared to the UK and Japan strains[8,9]. Further investigations need to be done to examine the differences. The ITS region of one isolate from each of 13 NTS types was sequenced and analyzed for polymorphism. There was no difference observed in the ITS 1 or ITS 2 region. This showed that ITS region had less polymorphism compared to NTS region, making the latter one to be highly sensitive.

In conclusion, our study confirmed the molecular strain typing method as a reliable and suitable for

sub-species level discernment of *T. mentagrophytes* var. *interdigitale*. This method can be adopted as a valuable tool for future explorations into the epidemiology of *T. mentagrophytes* complex and to track the source of infection.

***Conflicts of Interest*:** None.

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