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Transcriptional Analysis of Metalloprotease, Metallocarboxypeptidase, Isocitrate Lyase, Citrate Synthase, Malate Synthase and Dipeptidylpeptidase V of Trichophyton Rubrum Isolates from Dermatophytosis Patients

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Abstract

Dermatophytes are pathogenic fungi that cause cutaneous infection of human and animal and grow exclusively on the stratum corneum, nail and hair. In a soy protein culture, a substantial proteolytic activity was seen, which was secreted by *Trichophyton rubrum, Trichophyton mentagrophytes* and *Microsporum canis*. This proteolytic activity was 55-75% inhibited by O-phenanthroline, which authenticate that metalloproteases were secreted by all three species. A consensus probe was constructed on previously characterized genes, which encodes metalloproteases (MEP) of the M36 fungalysin family in *Aspergillus fumigatus, Aspergillus oryzae* and *M. canis*. From genomic libraries of *T. rubrum, T. mentagrophytes* and *M. canis, T. rubrum* & *T. mentagrophytes* a five-member MEP family was isolated and also secretes MCP A & MCP B of the M14 family according to the MEROPS proteolytic enzyme databases. Study role of virulence genes of Trichophyton in dermatophytosis patients.

Introduction

Dermatophytes are pathogenic fungi which cause cutaneous infections in human and animal [1]. *Trichophyton rubrum, Trichophyton mentagrophytes* and *Microsporum canis* are the most frequent human pathogenic species, accounting for 72-95% of the species isolated in hospitals and private practices [2]. Dermatophytes are characterized by their capability to grow exclusively in the stratum corneum, nails or hair, and able to digest components of the cornified cell envelope. The characterization of this disease is discoloration and thickening of the nail, frequenty with onycholysis, and may also affect the adjacent skin. Clinically onychomycosis is characterized by nail discoloration (yellow, white or, brown where the fungus is dense), nail dissociation from the nail bed (onycholysis), brittleness, nail thickening and subungual accumulation of scale. In more severe cases ridging and onychocryptosis (in grown nail) apears. All researched dermatophytes produce proteolytic activity in vitro [3]. From a single dermatophytic species, there are several reports of the isolation and characterization of one or two proteases [4-6]. These proteases are generally described as keratinases and plays an important role in the provision of nutrients [7], host tissue invasion and control of host defence mechanisms [8-10]

Multiple endoproteases are secreted by dermatophytes that are classified into two broad protein families, i.e., subtilisins (serine proteases), and fungalysins (metalloproteases) [11].

Various studies suggested that metalloproteases produced by *M. canis* and *T. rubrum* may be virulence-related factors involved in dermatophytosis, and dominate the two dermatophyte keratinase families [12]. There is a very high degree of similarity between five MEP genes with the MEP genes of *A. fumigatus* and the neutral protease I gene of *A. oryzae,* as all of which contain the HEXXH sequence motif. Researcher confirmed that the MEP genes of *T. mentagrophytes, T. rubrum,* and *M. canis* are highly homogeneous with 72-97% similarity between the gene sequences of various species [13].

Moreover to this dermatophytes also secretes two leucine aminopeptidases (Lap), Lap1 and Lap2, and two dipeptidyl-peptidases (DPP), DPP IV and DPP V. Lap1 and Lap2 are belongs to metalloproteases while DPP IV and DPP V are classified as serine proteases with a Ser, Asp, His catalytic triad [14]. Dermatophytes also secrete a metallocarboxypeptidase A (MCP A) and two serine carboxypeptidases (Scp), ScpA and ScpB. In a medium containing protein as a sole nitrogen and carbon source, *T. rubrum* & *T. mentagrophytes* secretes MCP A & MCP B of the M14 family according to the MEROPS proteolytic enzyme database. MCP A & MCP B is homologous to human pancreatic carboxypeptidase A, and is synthesized as a precursor in a preproprotein form [15].

Amongst the all virulent genes, the role of enzyme involved in glyoxylate cycle are also co-related with the pathogenicity of *T. rubrum*, although their functions and mechanisms remain undetermined. The primary response to phagocytosis in *S. cerevisiae* was the introduction of gene products associated with the Glyoxylate cycle. The key enzymes of the Glyoxylate cycle, Isocitrate Lyase (IL) and Malate Synthase (MS) were highly induced in macrophages. Other transcripts, most notably those of the Tri carboxylic acid (TCA) cycle, were not induced under these conditions [16].

Real-time PCR analysis impart high levels of sub7 expression following growth on human nail, whereas all other virulence genes expression analysis were elevated following growth on human stratum corneum. So, in this study, we lookout at the m-RNA expression patterns and dynamics of genes encoding two major families of endoproteases, Exoproteases: Metallo-Protease (MEP) 3, MEP 4, Metallocarboxypeptidase (MCP) A, MCP B, and key enzyme of Glyoxylate cycle like Isocitrate lyase (IL), Citrate synthase (CS), Malate Synthase (MS) and Dipeptidyl-Peptidases (DPP V) in *T. rubrum isolates* by real-time PCR from patients suffering from dermatophytosis in nail [17].

Materials and Methods

The present study was conducted on 160 samples from clinically diagnosed onycomycosis patients, further subjected to culture from nail samples of patients attending dermatology OPD of a tertiary care hospital, Delhi from January 2016 to December 2018.

The mean age of patients was 29.8±11.41 (with the range from 16 to 60 years). The duration of dermatophytic infection ranged from 3 months to 15 years (9.74±6.81 months). Out of 160 samples, 100 were found to be KOH positive, of which 70 samples were culture positive for NDM & dermatophytes. Of the 70 isolates, 20 isolates were identified as *T. rubrum* and 50 isolates as NDM on phenotypic mycological assessment. *T. rubrum* was the predominant pathogen isolated from nail samples.

A portion of each clinical specimen was suspended in a drop of 40% potassium hydroxide (KOH) for processing of the nail respectively. KOH wet mount slides were viewed under 40X magnification. A portion of the sample was cultured on Sabouraud's dextrose agar (Hi-media, Mumbai) with antibiotics with chloramphenicol (0.05 g/l), gentamicin (20 mg/l) and cyclohexamide (0.5 g/l). All inoculated tubes were then incubated at 25ºC for 3-4 weeks optimal growth. After growth, the etiological agent was confirmed by the characteristic morphology of the colony and by studying the microscopic appearance of the fungus on Lacto Phenol Cotton Blue (LPCB) mount and Urease test [18]. The molecular confirmation of isolates was done by PCR and sequencing using species-specific primers of *T. rubrum*.

DNA extraction and PCR

DNA was extracted from the cultures grown on SDA by using the commercially available DNA extraction kit (HiYield Genomic DNA Kit, RBC, Taiwan). PCR was performed with species specific primer of *T. rubrum*, forward GACCGACGTTCCATCAGGGGT and reverse TCAGACTGACAGCTCTTCAGAG (203bp) for amplification of the desired gene segment [19]. Each PCR tube contained a total volume of 25 µl which included 2.5 µl buffer (10X), 5 µl of Q-buffer, 0.5 µldNTPs (200 µM), $MgCl_2$ 0.5 µl (1.5 mM), 0.15 µl Taq polymerase, 1 µl of each primer, forward and reverse (10 µM) (Taq PCR Core Kit, Fisher Scientific-Qiagen, Germany), 5 µl of DNA template and nuclease-free water to make up the volume. Amplification was performed in a Master cycler personal (Eppendorf, Hamburg, Germany). Initial denaturation was performed at 94°C for 10 min which was followed by 35 amplification cycles of 30 s at 95°C and 45 s at 65°C and 30 s at 72°C, and final extension of 10 min at 72°C. The amplified PCR products were analyzed by electrophoresis on 1.5% agarose gel, stained with Ethidium bromide and electrophoresed at 125 V and 15 mAh current in a 10 slot apparatus for 30 min. Molecular marker of 100 bp was used to determine the size of the amplicons.

Purification of PCR products was done by Sodium acetate method and DNA sequenc analysis was performed by comparison of the nucleotides with dermatophytes reference nucleotide sequence obtained from gene bank database (site http://www.ncbi.nih.gov/gene bank). On the basis of alignment of sequences of internal transcribed spacer region ITS 1 and 2 in the NCBI nucleotide database, the isolates were identified as *T. rubrum* with 99% similarity with reference strains. The representative sequences so obtained were submitted to gene bank database and accession numbers obtained were MH497367, MH497368 & MH497369.

RNA extraction and complimentary DNA synthesis

Total RNA was extracted from 20 culture isolates of *Trichophyton rubrum* using TRIzol™ Reagent (Invitrogen, USA). Briefly, 1 ml of TRIzol™ Reagent was added in the sample and mixed gently by micropipette to form a homogeneous cell lysate and incubated for 5 min at room temperature. 200µl of chloroform per ml of TRIzol™ reagent was added and centrifuged at 12,000 rpm for 15 min at 4°C to obtain a colorless upper aqueous phase. RNA containing aqueous phase was taken in a fresh 1.5 ml tube and washed with 500 µl of isopropanol. The eppendorf tube was centrifuged at 12,000 rpm for 10 min at 4°C and supernatant was discarded. 1 ml of 75% ethanol was added to the pellet and mixed by vortex. This was followed by centrifugation at 10,000 rpm for 5 min at 4°C. The supernatant was discarded

and the pellet was resuspended in RNase free water, and incubated at 55-60°C for 15 min. The isolated RNA was stored at -80°C immediately. The integrity of the RNA samples was confirmed by agarose gel electrophoresis. Concentration and purity of the samples were assessed by Nano-drop (Eppendorf, USA). Extracted RNA was used as a template for c-DNA synthesis by Superscript reverse transcriptase II (Invitrogen, USA). Total RNA (2-5 µg) from each sample was reverse transcribed into c-DNA under thermal conditions, 25ºC for 10 min., 37ºC for 120 min., 85ºC for 5 min. and 4ºC for infinite time.

Expression study of virulence genes by Quantitative Real-Time PCR (qRT-PCR)

To quantify the expression of virulence genes, Metalloprotease 3 (MEP 3), Metalloprotease 4 (MEP 4), Metallocarboxypeptidase A (MCP A), Metallocarboxypeptidase B (MCP B), Isocitratelyase (IL), Citrate synthase (CS), Malate synthase (MS) and Dipeptidylpeptidase V (DPP V), qRT-PCR was performed on Light Cycler® 480 Instrument (Roche Diagnostics, Germany). Twenty confirmed isolates of *T. rubrum* and a reference strain of *T. mentagrophytes* (ATCC no. 28185) were used to estimate the expression of virulence gene. The final Real-Time PCR reaction of 20µl contained 2 µl of c-DNA, 10 µl of SYBER Green Universal Master Mix, and 60M of random hexamer primers shown in table -1.a. The Real Time PCR conditions were standardized according to the melting temperature (Tm) of the primers as shown in **Table 1.b**.

Table 1a: Measurement of leaf length.

Gene Name	Primer
MEP 3	F 5'-AGCAGCACGCCAGCAACG-3' R 5'-GCAGACGGAAGGACTCGATGT-3'
MEP 4	F 5'-AGTCGGGACACCATTCTTCAG-3' R 5'-ATTTGGGCTTCTATGCTCTACG-3'
MCP A	F 5'-GCATTGAAGGCGGTGCAT-3' R 5'-GTCAACACTGTCTCCATTAACTTGGT-3'
MCP B	F 5'-GTTCTCGAGTGCAGTATGGCTACAACCAG-3' R 5'-GTTAGATCTTATTTTAACCTGAAAATAGGAT-3'
ISOCITRATE LYASE	F 5'-TGGAAAGATTCAAGATGGCGATA-3' R 5'-TCTGTGCATTTGATGGGTAATCA-3'
MALATE SYNTHASE	F 5'-TCTTTCTCTCCTCTCTTTCTTTCCTTCCAACCAC-3' R 5'-CAGAAGACGATTGACTTGGAATTTCTCAAGTGCTGTT-3'
CITRATE SYNTHASE	F 5'-GAACATGGTAAATGGTCAGGTGAA-3' R 5'-CGCCGAGGGTGAAGTCAA-3'
DPP V	F 5'-CTTAGATCTGTTCCTCCTCGTGAGCCCCG-3' R 5'-CTTGCGGCCGCTCATTCCTCTGCCCTCTCACC-3'
GAPDH	F-5'-CTTAGCACCCCTGGCCAAG-3' R-5'-TGGTCATGAGTCCTTCCACG-3'

Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) served as an endogenous expression control for normalization of data and the threshold cycle (Ct) values were calculated by the software. The dissociation curve analysis was performed to verify that a single product was amplified. Amplification curve and melt peaks for each virulence gene were shown in Figure-2. The fold change of the expression of virulence genes in clinical isolates was compared to ATCC strain of *T. rubrum* complex was calculated by $2^{-\Delta\Delta}$Ct method (R) [19-22].

Result

The distribution of *T. rubrum* isolates confirmed by genotypic method shown in Figure 1, obtained from infected nail specimen. All samples phenotypically confirmed were reconfirmed genotypically by PCR & sequencing. The mean age and duration of disease of these 20 patients were 29.8±11.41 years and 9.74±6.81 months, respectively. Figure-1 shows the infected nail in onycomycosis patients.

Figure-1: The culture of *T. rubrum* on SDA plate and figure-1f shows the *T. rubrum* band at 203 bp.

These 20 *T. rubrum* culture were confirmed phenotypic (LPCB) and genotypic (PCR) and reconfirmed by sequencing.

The real-time PCR analysis of virulence genes has shown that the expression of all the genes i.e. MEP 3, MEP 4, MCP A, MCP B, IL, CS, MS and DPPV were significantly up-regulated in all the clinical isolates as compared to reference strain of *T. mentagrophyte* ATCC no. 28185. The amplification curve and melt peak of the 8 virulence genes are shown in **Figure-2.** The expression levels and the qualitative status of the virulence genes represented as Ct values in the 20 clinical isolates versus ATCC strain is shown in **Table 2.**

Figure 2.a, 2.b: Amplification curve and melting peak of MEP 3, MEP 4, MCP A, MCP B, Isocitrate Lyase, Citrate Synthase, Malate Synthase and DPP V genes.

Table 1b: Thermal profile of Metalloprotease 3 & 4 (Mep3 & 4); Metallocarboxypeptidase A & B (Mcp A & B); Dipeptidyl-Peptidase V (DPP V); Isocitrate lyase; Citrate synthase; Malate synthase and GAPDH.

Temperature	Time	Cycles	Phase
95°c	10 min	1	Initial Denaturation
95°c	20 sec		Denaturation
60°c	30 sec	35	Annealing
72°c	20 sec		Extension
95°c	10 sec	1	Melting temperature

Table 2: Transcriptional analysis of differential expression level of virulence genes of T. rubrum calculated by fold change.

T. R.	MEP 3	MEP 4	MCP A	MCP B	IL	CS	MS	DPP V
Avg. ΔCT of isolates	-2.61	-3.75	-3.13	-4.57	-1.36	-3.22	-2.48	-1.43
Avg. ΔCT of ATCC Strains	1.85	-1.76	2.29	-0.95	3.24	3.38	0.06	-0.88
Fold change(2^- $^{\Delta\Delta CT}$ of isolates)	**23.89**	**27.43**	**30.15**	**64.07**	**9.83**	**35.31**	**50.57**	**21.90**

Among three non- protease virulence genes, we found Malate Synthase as highly expressed (50.57 fold high), followed by Citrate Synthase (35.31) and Isocitrate Lyase (9.83), in clinical isolates of *T. rubrum* as compared to ATCC strain. Furthermore, among secreted protease gene encoding the major keratinases, Metallocarboxypeptidase B was strongly up regulated (64.07 fold high) followed by Metallocarboxypeptidase A (30.15) and DPP V (21.90), in dermatophytic patients as compared to ATCC strain. Furthermore, among Endoprotease gene encoding the major keratinases, Metallocarboxypeptidase B was strongly up regulated (64.07 fold high) followed by Metalloprotease 4 (27.43) and Metalloprotease 3 (23.89), compared to *T. mentagrophytes* ATCC no. 28185 strain. Shown in (Figure 1a, 1b & 1c).

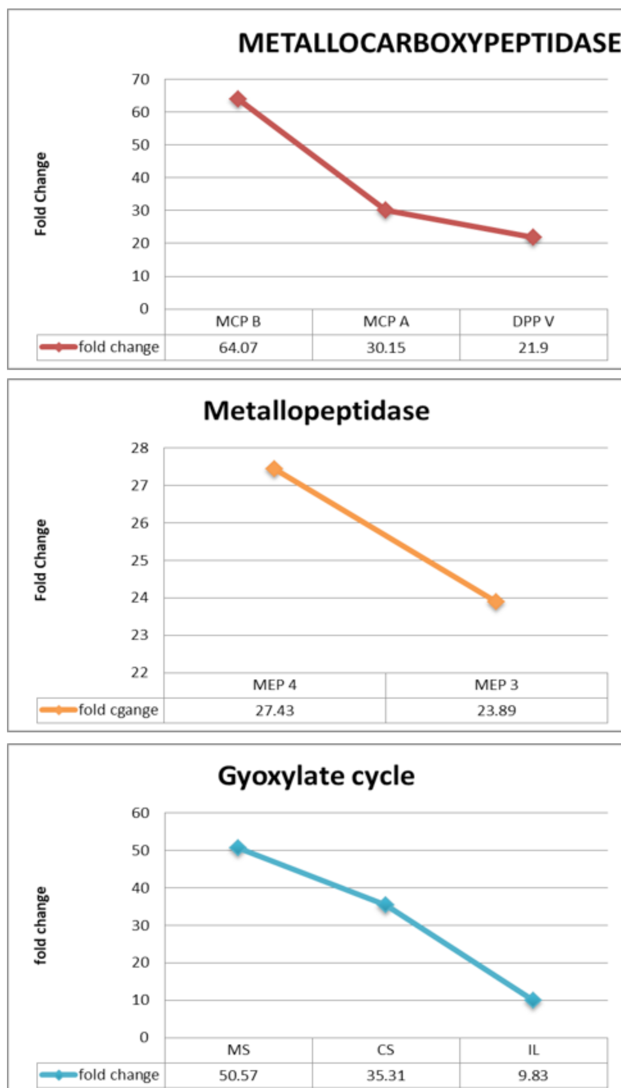

Figure 1a: Fold change of exoproteases, endoproteases and non-protease for transcriptional study.

Discussion

The dermatophytes species of Trichophyton and Microsporum are multiplication of an ancestral gene which encodes secreted fungalysin. These are the genes which invade keratinized tissues of an ancestral evolutionary gene of belongs to the family that encode secreted proteases.

Dermatophytes exclusively grow in the stratum corneum and utilized the keratin and structrally cross linked proteins of the cornified nail cell enclose as substrate. In the course of infection, dermatophytes produce various endo- and exo- proteases to degrade keratinized structure into short peptides and free amino acids, which is utilized as nutrients by the fungus.

The appearance of at least four gene duplication events in the putative ancestor of dermatophytes is required to explain the tree topology obtained (Figure 3). A primary duplication produced the ancestral types of MEP 1 and MEP 5 on the one hand, and of MEP 2, MEP 3 and MEP 4 on the other hand. Subsequent duplications produced MEP 1 and MEP 5, MEP 4, and the ancestral type of MEP 2 and MEP 3. The duplication of the latter produced MEP 2 and MEP 3 along with the loss of three introns in MEP 2. Ancient gene duplications are known as one of the main forces in the generation of gene families and the creation of new functional capabilities [23].

In soy proteins culture medium, *T. rubrum, T. mentagrophytes* and *M. canis* secrets two major MEPs (MEP 3 and MEP 4) encoded by orthologous genes, although full length c-DNA of all MEP types was found to be present in the *T. rubrum* c-DNA library. In the MEPs, several putative glycosylation sites were identified (Table 2). The multiplicity of MEP 3 and MEP 4 protein bands can be explained by different levels of glycosylation. So in the present study, we have also done transcriptional study in MEP 3 & MEP 4 by real time PCR, to check their expression level, though in our study MEP 4 is more up-regulated (27.43 fold) in compare to MEP 3 (23.89 fold), which is concordant to the study done by Maranho et.al 2007 & Leng W et.al 2009. A comparison of the potential pathogenicity of five metalloprotease genes from *T. mentagrophytes* led to the proposal that MEP 4 and MEP 5 were possibly affect pathogenicity, which is determined in a guinea pig model and a keratin degradation test, whereas expression of only MEP 4 was significantly upregulated after growth in vitro on keratin, collagen, elastin or human skin sections [24,25].

Among approximately 10 human pathogenic species dermatophytes isolated in Europe, *T.* rubrum, *T. mentagrophytes* and *M. canis* are most commonly observed, accounting for 72-95% of the species isolated in hospital and private practices [12]. All investigated dermatophytes produce proteolytic activity in vitro [13]. There is a report of the genome with perfect (or near-perfect) identity, the loss of one or various copies, or the acquisition of functional novelty through the accumulation of random mutations, also known as 'subfunctionalization' [24].

The isolation and characterization of five MEP and seven SUB genes from *T. rubrum* and *T. tonsurans* was demonstrated. The proteins (most of them being proteases) which are secreted in a medium containing proteins as the sole carbon and nitrogen source, likely represent the spectrum of enzymes that permit the degradation of keratinized tissues into assimilable compounds during the course of infection.

Burmester et.al 2011 identified DPP V as the *T. tonsurans* allergen Tri t 4, Tri r 4 of *T. rubrum* and Tri m 4 of *T. mentagrophytes.* Strikingly, when *T. benhamiae* was co-cultured with keratinocytes, expression of DPP V was up-regulated, but there is no change in the expression of the other exoproteases described above, but in our study exoprotease, such as MCP B is more up-regulated compared to MCP A & DPP V which is discordant from study of Burmester et.al 2011.

T. rubrum secretes two zinc-dependent metallocarboxypeptidases, viz. MCP A and MCP B, (M14A family), when grown on protein medium. Analysis of the dermatophytes revealed the presence of four M14 metallocarboxypeptidase genes in the genome of all isolates, except *T. benhamiae*, which possessed five such genes.

Metalloprotease genes of *T. rubrum* are most predominant genes for pathogenicity and the ability of dermatophytes to invade keratinized tissues and to be essentially confined to keratinize structures. It can be presumed that keratinolytic proteases (keratinases) might be significant virulence factors. Therefore, the characterization of keratinase appears to be a major step for a better understanding of dermatophytic infection, pathogenesis and subsequently the host-fungus relationship. Some keratinases have been isolated from *T. rubrum* [24-27].

Although keratinases are supposed to be involved in dermatophytic pathogenicity, only a few studies have evaluated there in vivo production [22]. Moreover to this, only one protease with keratinolytic activity, a recombinant *T. rubrum* protease was characterized at the gene level. In fact, most of the authors have only reported enzymatic activities of dermatophyte proteases on various macromolecular substrates and co-related there in vitro keratinolytic activity to the ability of dermatophytes to invade keratinized structures in vivo [26].

The MEP 4 and MEP 5 defective strains were the least pathogenic, while the MEP 3 mutant showed pathogenicity levels that were similar to the wild-type strain. The data suggested that the proteases coded for, by the MEP 4 and MEP 5 genes were more virulent than the other MEP genes, and were also the predominant proteases in the host invasion process of *T. mentagrophytes*. Similar test results for the MEP 3 mutant and the wild-type strain suggested that MEP 3 metalloprotease expression in *T. mentagrophytes* may not be the same as in *M. canis* or *T. rubrum* [27,28]. The MEP 1 and MEP 2 mutations had some effects on the pathogenicity of *T. mentagrophytes*; however, further research is needed to investigate their influence as inconsistent test results were observed. Furthermore research focusing on exocrine protein analysis of the transformants, variation in the protein composition, and transformation of other dermatophytes need further investigation of the functionality, mechanism, and specification of the MEP genes [29,30].

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