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Original Article

Subtilisin Gene Activity in Dermatophytes: A study on the Presence of the *Subtilisin* Gene in *Trichophyton verrucosum* and *Microsporum gypseum* in Clinical and Nonclinical Samples in Tehran, Iran

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Abstract

The keratinolytic activities of dermatophyte species are accompanied by the secretion of enzymes, such as serine proteases, which are coded by the *Subtilisin* (*SUB*) genes. This study aimed to determine the presence of the *SUB* genes in the clinical and nonclinical samples of *Trichophyton verrucosum* and *Microsporum gypseum*. Isolation was carried out by direct and laboratory examination. Following that, for the determination of the presence of the *SUB* gene, polymerase chain reaction with specific primers was conducted. The frequencies of the *SUB* gene were observed in almost 66% of the isolates. Statistical analysis showed a significant relationship between the presence of the *SUB* gene and the samples collected from human, animals, and soil ($P<0.005$). The current investigation has been the first study of the presence/absence of the *SUB* gene in the clinical and nonclinical isolates of *T. verrucosum* and *M. gypseum* in Iran which may be a new step to perform further studies.

Keywords: *Dermatophyte, Microsporum gypseum, Pathogenicity, SUB gene, Trichophyton verrucosum*

Activité du gène de la *Subtilisine* (SUB) chez les Dermatophytes: une Étude sur la Présence du Gène SUB dans *Trichophyton verrucosum* et *Microsporum gypseum* dans des Échantillons Cliniques et Non Cliniques à Téhéran, Iran

Résumé: Les activités kératinolytiques des espèces dermatophytes s'accompagnent de la sécrétion d'enzymes, telles que les sérines protéases, codées par les gènes de *subtilisine* (SUB). Le but de cette étude était de déterminer la présence de gènes SUB dans les échantillons cliniques et non cliniques de *Trichophyton verrucosum* et *Microsporum gypseum*. L'isolement a été effectué par examen direct et en laboratoire. Ensuite, pour la détermination de la présence du gène SUB, une réaction en chaîne par polymérase avec des amorces spécifiques a été réalisée. Les fréquences du gène SUB ont été observées dans près de 66% des isolats. L'analyse statistique a montré la relation significative entre la présence du gène SUB et chaque dermatophyte, l'origine des isolats (c'est-à-dire les humains, les animaux ou le sol) (P=0.005). L'enquête actuelle a été la première étude de la présence/absence du gène SUB dans les isolats cliniques et non cliniques de *T. verrucosum* et *M. gypseum* en Iran, ce qui pourrait être une nouvelle étape pour effectuer d'autres études.

Mots-clés: Gène SUB, Pathogénicité, Dermatophyte, *T. verrucosum*, *M. gypseum*

1. Introduction

The investigation of virulence genes of dermatophytes, causing diseases, has been one of the research issues in recent years. That is why the gene library of this group of microorganisms is not so rich (Jousson et al., 2004a; Tarabees et al., 2013). Dermatophytes can exclusively grow in the skin, hair, and nail of humans and animals to digest components of the cornified cell envelope (Aljabre et al., 1993; Jousson et al., 2004b; Oborilova and Rybnikar, 2005). Keratinolytic enzymes are among the most important factors which are effective in the development and spread of infection (Moallaei et al., 2009; Chinnapun et al., 2016; Robati et al., 2018). Although the role of *SUB* gene in the adherence and inflammation steps of infection should be more studied (Baldo et al., 2012), pathogenic fungi, belonging to the S8A *SUB*family, can produce serine proteases as their virulence factors (Monod et al., 2002).

Despite the fact that *M. gypseum* is endemic in different regions of the world, there has been a limited number of studies conducted on its molecular analysis (Muhsin et al., 1997; Lemsaddek et al., 2010). Some studies demonstrated low enzyme activity of *Subtilisin*-like protease in *M. gypseum* isolates (Moallaei et al., 2009). Muhsin et al. (1997) revealed the elastinolytic activity in *M. gypseum* and *T. verrucosum*. The low activities of *SUB*2-3 in *M. gypseum* and high activities of all *SUB* family (*SUB*1-7) in *T. verrucosum* were also reported (Lemsaddek et al., 2010).

Studies on the presence of two families, metalloproteases and *Subtilisins*, performed by polymerase chain reaction (PCR) using specific primers in Portugal indicated the presence of these two genomic families in the genus *Trichophyton* and the absence of some genomic sequences in some clinical isolates as well as a genus of microsporum (Lemsaddek et al., 2010). The investigation of the enzymatic activity and molecular characterization of *Subtilisin* protein in *Microsporum* and *Trichophyton* species in Iran has been performed for pathogenic and other practical purposes (Moallaei et al., 2009). Various studies have demonstrated that the secretions of proteinases (e.g., the *SUB* family) can not only break down proteins (e.g., creatine, elastin, and collagen) to provide fungal nutrients but also can control host defense mechanisms and cause delayed type sensitivity (Hadadi et al., 2014). The Tri r2 allergen investigation (*SUB*6) has been introduced as a diagnostic marker using the protein analysis (proteomics) method produced by *Trichophyton* in clinical and nonclinical specimens (Mehul et al., 2016).

The aim of the current study was the identification of the presence/absence of *SUB* gene in *T. verrucosum* isolated from human hair and cattle skin and in the clinical (i.e., dog and horse hair) and nonclinical (i.e., soil) isolates of *M. gypseum*, acting as three different *Sub*strates for the photolytic activities of dermatophytes. The knowledge of the virulence genes can help the researchers to diagnose the infection and enable them to develop new drugs and vaccines which may be very effective treatments for dermatophytosis.

2. Material and Methods

2.1. Sample Collection

Within 22 February 2018 and 21 September 2018, 8 human isolates with dermatophytosis of *T. verrucosum* and *M. gypseum* referred to Mycology and Parasitology Laboratory, Tehran University of Medical Sciences, Tehran, Iran, 15 animal isolates with dermatophytosis of the two dermatophytes referred to the Mycology and Parasitology Laboratory, Tehran University of Medical Sciences, Tehran, Iran, and 7 soil isolates of *M. gypseum* were collected. The samples were kept in distilled water until examination.

2.2. Direct Examination and Culture of Dermatophytes

Direct microscopic examination was carried out by a drop of 10% potassium hydroxide solution on a clean glass slide of the samples and investigated at a magnification of 40×10. All the samples were

cultured in Sabouraud dextrose agar with cycloheximide and chloramphenicol, enriched by thiamine and inositol added to the media under aseptic conditions, and then incubated at 37°C for 3-4 weeks. Macro- and micromorphological features, such as the texture of colonies, color, and rate of growth, were studied. For microscopic examination, a small smear of the colony was put on the glass slide, stained with lactophenol cotton blue, and covered with a cover slide; then, it was observed by an optical microscope.

2.3. Design Specific Primers for *T. verrucosum* and *M. gypseum*

Two new sets of oligonucleotide primers with the access codes (National Centre for Biotechnology Information reference sequence: XM_003025488.1 for *T. verrucosum* and DQ923809.1: Gene Bank for *M. gypseum*) were designed. The primers were synthesized at SINA CLON Company (Iran) (Table 1).

2.4. DNA Extraction.

After growing the samples, the mycelium was collected and washed with distilled water and frozen at -80°C. The protocol of i-genomic Plant DNA Extraction Mini Kit (INtRON) (Lot. No. 13110450, Korea) was followed according to the manufacturer's instructions.

2.5. PCR Amplification

Amplification was performed in a thermal cycler using a total volume of 25 μl consisting of Tag DNA polymerase, buffer, Master Mix of SINA COLON. Co., magnesium chloride, and deoxyribonucleotide triphosphates. Furthermore, primers, dermatophyte DNA, and distilled water were added to it.

For PCR reactions, the guidelines in Table 2 were used, and the PCR products were run on 2% agarose gel visualized on ultraviolet-transilluminator, and photographed by the use of gel documentation system and 1000 kb ladder as molecular weight marker. In addition, the proven clinical isolates of *T. verrucosum* and *M. gypseum* were used as positive controls.

2.6. Statistical Analysis

The Chi-square and student t-test were employed for analyzing the data in order to show statistical independence between the presence of the virulent gene (*SUB*) in the two dermatophytes (i.e., *T. verrucosum* and *M. gypseum*) and origin of the samples (i.e., human and animal isolates and soil samples).

3. Results

Based on micro- and macroscopic examination, out of 30 samples, 14 isolates were identified as *T. verrucosum* and 4 clinical isolates and 7 soil samples were identified as *M. gypseum* (3/14 human and 11/14 cattle isolates).

3.1. Molecular Identification by PCR

The positive results of PCR products on the 2% gel showed that among all the isolates, 64% (16/25) of them carried *SUB* gen; however, the gene could not be amplified for 36% (9/25) of the isolates (Figures 1 and 2). In addition, 44% (11/14) of the *T. verrucosum* clinical (i.e., human and animal) isolates were positive for the presence of *SUB* gene, among which the human samples completely indicated the gene activity (3/3; 100%).

In case of *M. gypseum*, only about 20% (5/11) of the isolates demonstrated the presence of *SUB* gene in which the proportion of the soil samples was 2/7; nevertheless, 3/4 clinical samples carried this gene (Table 3). Statistical analysis showed a significant relationship between the presence of the *SUB* gene and the samples collected from human, animals, and soil ($P<0.005$).

Table 1. Information of specific primers designed for this study

Primer name	Primer sequence (bp)	Length
Mg-*SUB*ti -S1	5' GCAGCA GGA CAA CGT TCC AT 3'	420
Mg-*SUB*ti -As1	5' TGG GAG AAG GCA ACA CGA TG 3'	420
Tv-*SUB*ti -S1	5' TGT CCA GAC CCT CGC TGA TA 3'	461
Tv-*SUB*ti -As1	5' CAA CGA AGT TTG CAC CCC AG 3'	461

Table 2. Polymerase chain reactions

Step	Time	Temperature (°C)	Cycle
Initial denaturation	5 min	94	
Denaturation	30 sec	94	36
Primer annealing	45 sec	63	
Extension	1 min per kb	72	
Final extension	5 min	72	

Note: Anticipated annealing temperature considered at 51°C

Table 3. Demographic characteristics

Frequency					
M. gypseum	*M. gypseum*	*T. verrucosum*	*T. verrucosum*	Sample number	Sample type
Lack of genes	Presence of genes	Lack of genes	Presence of genes		
0	1	0	3	4	Human
0	0	3	8	11	Cow
0	1	0	0	1	Horse
1	1	0	0	2	Dog
5	2	0	0	7	Soil
6	5	3	11	25	Total
24	20	12	44		Frequency (%)

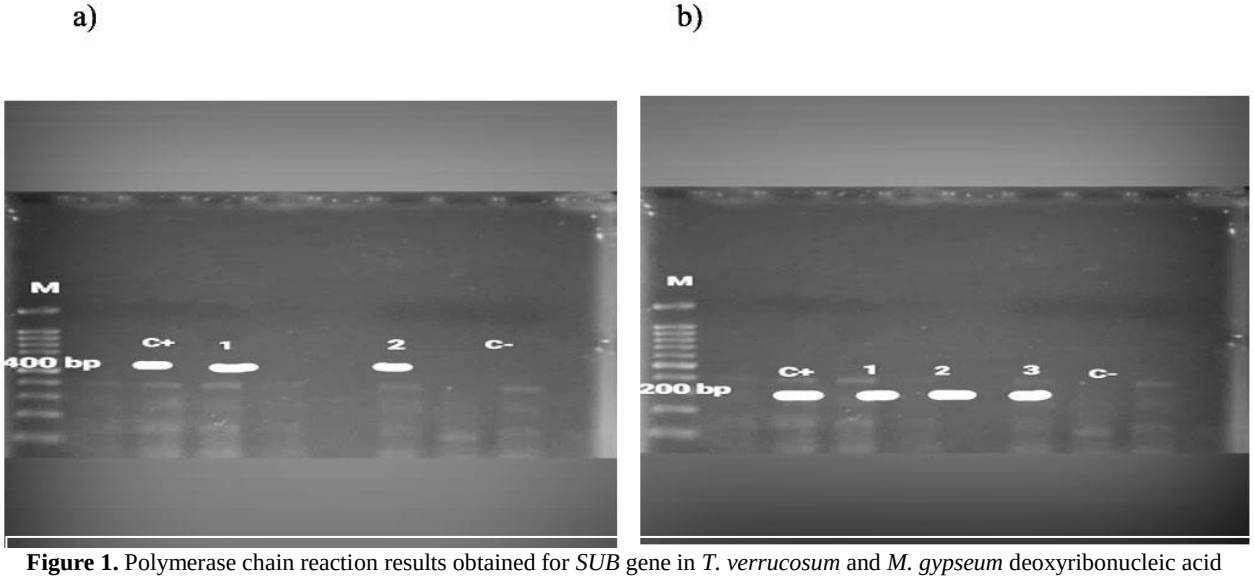

Figure 1. Polymerase chain reaction results obtained for *SUB* gene in *T. verrucosum* and *M. gypseum* deoxyribonucleic acid (DNA); lane M 100-1000-bp DNA molecular weight markers; C+ proved clinical isolates of *T. verrucosum* and *M. gypseum* used as positive controls; **a)** positive results of 400 bp fragment of *SUB* gene observed in *T. verrucosum* (11/14); lane 1: 3/11 isolates of human hair and face; lane 2: 8/11 isolates of cattle skin; **b)** positive results of 200 kb fragment observed in *M. gypseum* (5/11); lane 1: 1/5 isolate of human hair; lane 2: 2/5 dog and horse hair; lane 3: 2/5 isolates of soil

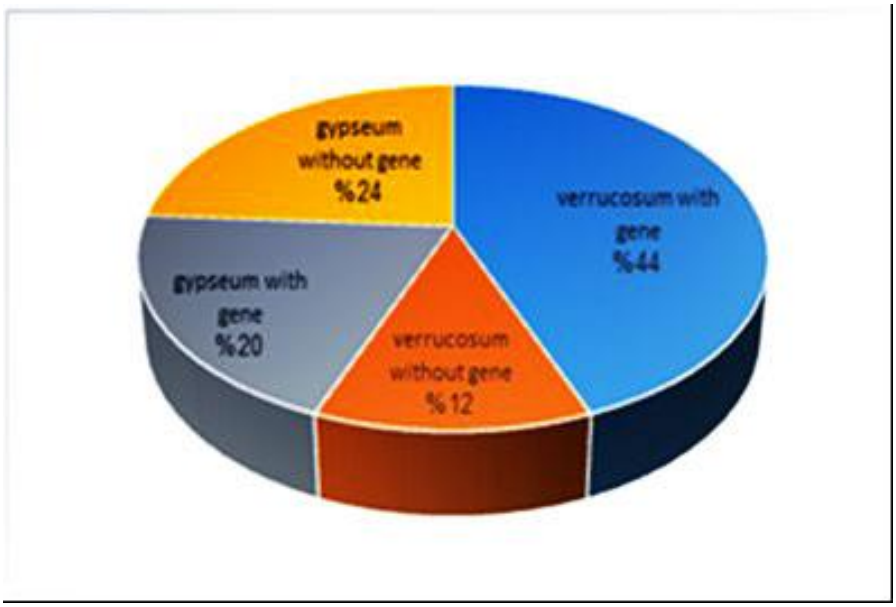

Figure 2. Frequencies of the *SUB* gene in *T. verrucosum* and *M. gypseum*

4. Discussion

The production of various proteolytic secondary metabolites, such as enzymes, and lytic activity of dermatophytes have already been described (Moallaei et al., 2009; Lemsaddek et al., 2010; Achterman and White, 2012; Tarabees et al., 2013), which have proven keratinase as a considerable virulent factor, concerned with clinical dermatophytosis (Achterman and White, 2012).

Despite the high prevalence of dermatophyte infections, there has been limited information about the pathogenicity mechanisms (i.e., the production of *SUB* gene) of *T. verrucosum* and *M. gypseum* (Grumbt et al., 2011). This finding shows that these two dermatophytes have been poorly studied at the molecular level. In the present study, 14 clinical isolates of *T. verrucosum* were investigated for the determination of the *SUB* gene activity.

Out of the total isolates (n=14), 11 isolates showed the presence of the gene (3 human and 8 cattle isolates), and confirmed the results of the study conducted by Lemsaddek et al. (2010).

Addressing a potential function of the *SUB* gene in the clinical isolates of *M. gypseum* (Tarabees et al., 2013), it was tried to identify the presence of the gene in its clinical (i.e., human and animal) and nonclinical (i.e., soil) isolates which can act as three different *SUB*strates for enzyme activity. In the present study, only three samples (human [n=1], dog [n=1], and horse [n=1]), showed the presence of the gene, which seemed to be consistent with the results of a study by Lemsaddek et al. (2010). Only, two (2/11) soil samples showed *SUB* gene activity that supported the investigation of the presence of the gene in the two dermatophytes in a study in Iran (Moallaei et al., 2009) and another study by Tarabees et al. (2013). Staib et al. (2010) by studying the role of unique secreted proteases in severe inflammatory skin infections in humans and rodents using microarray on the expression of at least 23 protease genes in vivo in dermatophytes. The results of their study indicated the expression of the *SUB*6 gene among the protease genes as an

important allergen gene that was strongly increased during infection.

Giudice et al. (2012) reported no activity of *SUB* genes in the clinical isolates of *M. gypseum*. They claimed that none of the designed primers was amplified in *M. gypseum* and only 1 kb band was produced with ERF3R3 primers indicating 92%, 88%, 78%, and 78% similarity with *Arthroderma gypseum*, *T. rubrum*, *T. tosorance*, and *T. verrucosum*, respectively. What they reported completely differed from the results of the present study and study by Lemsaddek et al. (2010).

Due to different reports of the presence/absence of the *SUB* gene (Muhsin et al., 1997; Lemsaddek et al., 2010; Giudice et al., 2012), it is necessary to collect and study more samples and perform gene sequencing and gene expression.

Lemsaddek et al. (2010) manifested the relative risk of the *SUB* 4-7 genes in clinical and nonclinical samples with 95% confidence level. The obtained findings from their study are in line with the results of the present study that showed the role of the *SUB* genes family in the prevalence of the two dermatophytes infection

At least two *SUB*-lysine family gene sequences were reported in the clinical and nonclinical collected samples; nevertheless, none of the seven genomic sequences in four clinical and nonclinical *Trichophyton* isolates were detected in a similar study (Lemsaddek et al., 2010).

Since the present study has been the first report of *SUB* gene activity in the clinical and nonclinical isolates of *T. verrucosum* and *M. gypseum* in Iran, it can be used as a platform for performing further studies in this regard. The obtained results also showed that there was a significant difference in the presence of *SUB* gene in the clinical and nonclinical samples.

Authors' Contribution

Study concept and design: F. N.
Acquisition of data: S. J. H. and F. N.
Analysis and interpretation of data: S. J. H. and M. B.
Drafting of the manuscript: F. N. and S. J. H.
Critical revision of the manuscript for important intellectual content: J. H. and S. R.
Statistical analysis: S. J. H. and F. N.
Administrative, technical, and material support: S. J. H. and F. N.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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References

Achterman, R.R., White, T.C., 2012. Dermatophyte virulence factors: identifying and analyzing genes that may contribute to chronic or acute skin infections. Int J Microbiol 2012, 358305.

Aljabre, S.H., Richardson, M.D., Scott, E.M., Rashid, A., Shankland, G.S., 1993. Adherence of arthroconidia and germlings of anthropophilic and zoophilic varieties of Trichophyton mentagrophytes to human corneocytes as an early event in the pathogenesis of dermatophytosis. Clin Exp Dermatol 18, 231-235.

Baldo, A., Monod, M., Mathy, A., Cambier, L., Bagut, E.T., Defaweux, V., *et al.*, 2012. Mechanisms of skin adherence and invasion by dermatophytes. Mycoses 55, 218-223.

Chinnapun, D., Palipoch, S., Hongphruk, H., 2016. Cloning and characterization of a putative gene encoding serine protease inhibitor (251Hbpi) with antifungal activity against Trichophyton rubrum from Hevea brasiliensis leaves. Plant Omics 9, 142-148.

Giudice, M.C., Reis-Menezes, A.A., Rittner, G.M., Mota, A.J., Gambale, W., 2012. Isolation of Microsporum gypseum in soil samples from different geographical regions of Brazil, evaluation of the extracellular proteolytic enzymes activities (keratinase and elastase) and molecular sequencing of selected strains. Braz J Microbiol 43, 895-902.

Grumbt, M., Monod, M., Staib, P., 2011. Genetic advances in dermatophytes. FEMS Microbiol Lett 320, 79-86.

Hadadi, F., Sabokbar, A., Dezfulian, M., 2014. Study of Relationship between Genetic Pattern and Susceptibility to Terbinafine in Clinical Isolated of Trichophyton rubrum. J Ardabil Uni Med Sci 14, 133-146.

Jousson, O., Lechenne, B., Bontems, O., Capoccia, S., Mignon, B., Barblan, J., *et al.*, 2004a. Multiplication of an ancestral gene encoding secreted fungalysin preceded species differentiation in the dermatophytes Trichophyton and Microsporum. Microbiology (Reading) 150, 301-310.

Jousson, O., Lechenne, B., Bontems, O., Mignon, B., Reichard, U., Barblan, J., *et al.*, 2004b. Secreted *Subtilisin* gene family in Trichophyton rubrum. Gene 339, 79-88.

Lemsaddek, A., Chambel, L., Tenreiro, R., 2010. Incidence of fungalysin and *Subtilisin* virulence genes in dermatophytes. 61, 56-64.

Mehul, B., Gu, Z., Jomard, A., Laffet, G., Feuilhade, M., Monod, M., 2016. *SUB*6 (Tri r 2), an Onychomycosis Marker Revealed by Proteomics Analysis of Trichophyton rubrum Secreted Proteins in Patient Nail Samples. J Invest Dermatol 136, 331-333.

Moallaei, H., Zaini, F., Rezaie, S., Nourbakhsh, F., Larcher, G.J.I.J.o.P.H., 2009. The Enzymatic Activity and Molecular Characterization of a Secreted *Subtilisin*-Like Protease in Microsporum gypseum and Trichophyton vanbreuseghemii. Iran J Public Health 38, 25-33.

Monod, M., Jaccoud, S., Zaugg, C., Lechenne, B., Baudraz, F., Panizzon, R., 2002. Survey of dermatophyte infections in the Lausanne area Switzerland. Dermatology 205, 201-203.

Muhsin, T.M., Aubaid, A.H., al-Duboon, A.H., 1997. Extracellular enzyme activities of dermatophytes and yeast isolates on solid media. Mycoses 40, 465-469.

Oborilova, E., Rybnikar, A., 2005. Experimental dermatophytosis in calves caused by Trichophyton verrucosum culture. Mycoses 48, 187-191.

Robati, A., Khalili, M., Hazaveh, S., Bayat, M., 2018. Assessment of the *Sub*til

isin genes in Trichophyton rubrum and Microsporum canis from dermatophytosis. Comp Clin Path 27, 1343–1347.

Staib, P., Zaugg, C., Mignon, B., Weber, J., Grumbt, M., Pradervand, S., *et al.*, 2010. Differential gene expression in the pathogenic dermatophyte Arthroderma benhamiae in vitro versus during infection. Microbiology (Reading) 156, 884-895.

Tarabees, R., Elsayed, M., Abdeen, E., 2013. Incidence of Fungalysins Virulence Genes (MEP1-5) in Dermatophytes Isolated Form Infected Cases in Egypt. Int J Microbiol Res 4, 180-187.