

## Proteolytic activity and cooperative hemolytic effect of dermatophytes with different species of bacteria

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### Abstract

**Background and Purpose:** Globally, dermatophytes are the most common filamentous group of fungi causing cutaneous mycoses. Dermatophytes were shown to secrete a multitude of enzymes that play a role in their pathogenesis. There is limited data on co-hemolytic (CAMP-like) effect of different bacterial species on dermatophyte species. In this study, we sought to evaluate exoenzyme activity and co-hemolytic effect of four bacteria on clinical dermatophytes isolated from patients in Shiraz, Iran.

**Materials and Methods:** A total of 84 clinical dermatophyte species were isolated from patients suffering from dermatophytosis and identified by conventional methods. Hemolytic activity was evaluated with Columbia 5% sheep blood agar. Proteolytic activity was determined by plate clearance assay method, using gelatin 8% agar. CAMP-like factor was evaluated with four bacteria, namely, *S. aureus*, *S. saprophyticus*, *S. pyogenes*, and *S. agalactiae*. Fisher's exact test was run for statistical analysis.

**Results:** *T. mentagrophytes* was the most predominant agent (27 [32.1%]) followed by *T. verrucosum* (20 [23.8%]), *T. tonsurans* (10 [11.9%]), *Microsporum canis* (7 [8.3%]), *T. rubrum* (6 [7.1%]), *E. floccosum* (6 [7.1%]), *M. gypseum* (5 [6%]), and *T. violaceum* (3 [3.6%]). The most common clinical area of dermatophytosis was the skin. All the isolates expressed the zone of incomplete alpha hemolysis. All the isolates had CAMP-positive reaction with *S. aureus* and the other bacteria were CAMP-negative. All the isolates expressed proteolytic activity and no significant differences were noted among diverse genera of dermatophytes and severities of proteolytic activity.

**Conclusion:** This study indicated that hemolysin and proteolytic enzymes potentially play a role in dermatophyte pathogenesis and *S. aureus* could be considered as a main bacterium for creation of co-hemolytic effect in association with dermatophyte species.

**Keywords:** CAMP-like, Dermatophyte, Hemolysin, Proteolytic, *Trichophyton mentagrophytes*

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### Introduction

Dermatophytes include three genera of *Trichophyton*, *Microsporum*, and *Epi-dermophyton* and are grouped as anthropophilic (human associated), zoophilic (animal associated), or geophilic (soil dwelling) according to their habitat [1]. It is believed that in addition to the mechanical penetration of the fungal elements, proteolytic enzymes degrade components of the cutaneous tissue. The hydrolysis of keratin by proteinases is an important aspect of fungal pathogenesis, providing a source of nourishment on stratum corneum, which constitutes an obstacle to

pathogens [2].

Dermatophytes are capable of infecting keratinized structures in stratum corneum, nails, or hair and have the ability to degrade keratin, which is considered a major virulence factor for invasion [1]. Dermatophytes were shown to secrete endopeptidases, exopeptidases, and more than 20 proteases when cultured in a medium containing nitrogen as the source of protein [3].

Identification and characterization of microbial proteases are prerequisites for understanding their role in the pathogenesis of infectious diseases. For this reason, expedient and sensitive techniques for

the detection and characterization of microbial proteases are highly desirable [4].

Hemolysins play an important role in bacterial infections; they were reported to have cytotoxic effects on the membranes of erythrocytes and phagocytic cells and cause pore-formation and lysis in other eukaryotic cells and cellular structures [5, 6]. Hemolytic activity of *Trichophyton* species was first described by Schaufuss et al. [7]. Their complementary study on *Trichophyton* hemolysin using erythrocytes from different mammalian species revealed that sensitivity was the highest to those of equines, followed by erythrocytes from the sheep, cattle, swines, canines, and humans [8].

The co-haemolytic effect that was named the 'CAMP factor' was first characterized by Christie, Atkins, and Munch-Peterson in 1944. Cooperative (CAMP-like) lytic process is the result of the interaction of at least two membrane-active factors of bacteria with biological membranes. *Streptococcus agalactiae* (Group B *Streptococcus*) produces a thermostable, extracellular, diffusible protein that acts synergistically with the beta-lysin of *S. aureus* to produce an improved lysis zone in ovine and bovine erythrocyte cultures [9, 10].

This reaction was examined with all species of dermatophyte colonies using various types of bacteria such as *S. aureus*, *S. intermedius*, and *Listeria ivanovii* in several studies and CAMP-like effects in the form a detectable half-moon-shaped zone of complete hemolysis was observed below the mycelium of the colonies [9].

There is a shortage of data on the co-haemolytic effect of dermatophyte species and bacterial species that could be commonly found as the normal flora of the skin or bacteria associated with an area infected by a dermatophyte invasion. The aim of this study was to evaluate hemolysin and proteolytic activities of different species of dermatophytes causing dermatophytosis; as human skin contains various bacterial species as normal flora that contribute to an enzymatic activity, the second objective of this study was to determine co-hemolytic effect (CAMP-like factor) with different strains of bacteria, such as *S. aureus*, *S. saprophyticus*, *S. epidermidis*, and *S. agalactiae*, on clinical dermatophytes isolated from patients suffering dermatophytosis in Shiraz, Iran.

## Materials and Methods

### *Study population and sample collection*

A subset of 84 clinical dermatophyte species

was isolated from more than 200 patients suspected of dermatophytosis referring to Laboratory of Medical Mycology of Faghihi Hospital and School of Medicine of Shiraz, Iran. The research project was approved by the Ethics Committee of Departmental Review Board (Ethical code: IR.SUMS.REC.1395.S610) of Shiraz University of Medical Sciences, Shiraz, Iran. A written informed consent was obtained from all the participants and patient details, including age, gender, place of residence, as well as history of exposure to animals or any suspected sources, were recorded.

Specimens from skin scrapping, hair, and nail clipping were taken by using a sterile scalpel blade, forceps, and tweezers. Direct microscopic examination with 10-20% potassium hydroxide (KOH) and lactophenol solution (for hair samples) was performed to determine the presence of hyphae and arthrospores.

### *Identification of the dermatophytes*

The biological materials were cultured on Sabouraud dextrose agar (Merck, Germany) supplemented with chloramphenicol and cycloheximide and incubated at 25°C for three weeks.

All the isolated dermatophytes were identified by the conventional methods based on gross colony morphology (texture, color, surface, reverse pigmentation, and topography). Microscopic characterization of microconidia and macroconidia and presence of accessory structures were determined using slide culture technique. A portion of each colony was transferred to 1.5 ml microtubes containing sterile distilled water and kept at room temperature for further study.

### *Hemolytic activity assay*

Hemolytic activity was evaluated with a Columbia agar medium (Oxoid CM 331, UK) supplemented with 5% sheep blood as described by Schaufuss et al. [7]. Before testing, all the strains were subcultured on Sabouraud glucose agar (Merck, Germany). The Columbia blood agar was poured into 100 mm plastic plates. A needle was used for punctiform inoculation of the dermatophyte colonies and incubated for seven days at 28°C and 1-5 days at 36°C. The test results were monitored each day and the presence of a distinctive translucent halo around the inoculum site was measured macroscopically. The results were expressed as complete (beta), incomplete alpha, and no zone hemolysis

around the colonies.

### CAMP test

Standard strains of bacteria including *S. aureus* (ATCC 25923), *S. saprophyticus* (PTCC1440), *S. pyogenes* (ATCC 19615), and *S. agalactiae* (ATCC 13813) were utilized for co-hemolytic effects. After incubation time for hemolytic activity, the edge of a loop was used to streak each bacterium in straight lines across the plate at a distance of 10 mm from the border of dermatophyte colony. The plates were then incubated at 27°C and inspected daily for 1-7 days. A distinct arrowhead of hemolysis at the intersection of the tester strain and the test dermatophyte colony streaks was considered as positive result for CAMP-like reactions.

### Assessment of proteolytic activity

Plate clearance assay was used for evaluation of proteolytic activity in dermatophyte isolates [11]. The 8% gelatin agar plates were prepared by adding 80 g gelatin and 25 g agar to 1000 ml distilled water. A pinpoint inoculum was spotted at the center of the plates and incubated at 28°C for up to ten days. For clear visualization, aqueous saturated solution of ammonium sulfate was added onto the surface of the agar for at least 30 min.

The clear zone around the colonies due to the complete degradation of gelatin indicated the presence of proteolytic activity. The index for proteolytic activity was measured based on colony diameter + clear zone divided by colony diameter and the results were expressed as 0-1 cm (+), 1-2 cm (++) , 2-3 cm (+++) , 3-4 cm (++++), and more than 4 cm (>++++).

### Statistical analysis

Chi-squared or Fisher's exact test was performed for data analysis. P-value less than 0.05 was considered statistically significant.

## Results

Of the 84 affected patients, 57 (69.7%) were male and 27 (32.1%) were female within the age range of 2-74 years. Skin was the predominant clinical manifestation in 44.04% of the cases (Table 1). The distribution of 84 identified dermatophyte species were as follows: *T. mentagrophytes* 27 (32.1%), *T. verrucosum* 20 (23.8%), *T. tonsurans* 10 (11.9%), *Microsporum canis* 7 (8.3%), *T. rubrum* 6 (7.1%), *Epidermophyton floccosum* 6 (7.1%), *M. gypseum* 5 (6%), and *T. violaceum* 3 (3.6%; Table 1).

### Hemolysin activity and CAMP test

All the isolates expressed the zone of incomplete alpha hemolysis. All the dermatophyte isolates had CAMP-positive reaction with *S. aureus*. Our results showed no CAMP reaction when using *S. saprophyticus*, *S. pyogenes*, or *S. agalactiae* (Figure 1).

### Proteolytic activity

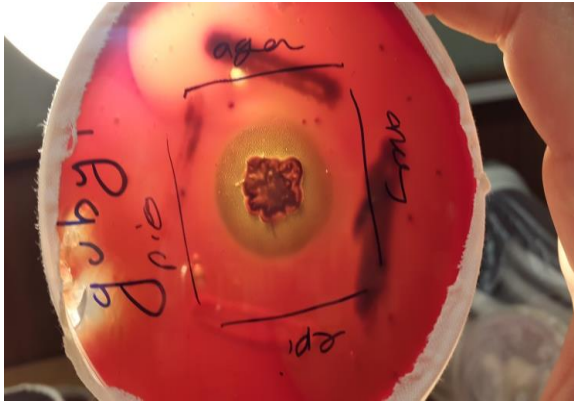
Proteolytic activity was detected in all the dermatophyte species. All the three genera of dermatophytes expressing proteolytic activity were given a score of 2 (Table 2). Scores 4 and higher were indicated only in genus *Trichophyton*. There were no significant differences between various genera of dermatophytes and intra-dermatophyte species in severity of proteolytic activity.

## Discussion

The distribution of dermatophyte species and the incidence rate of dermatophytosis vary according to region, and diverse parameters such as site of infection, type of society, lifestyle, weather conditions, and migration may affect the epidemiology of dermatophytic infection [12]. In our study, *T. mentagrophytes* and skin were the most prevalent causative agents and site of infection, respectively.

**Table 1.** Distribution and frequency of dermatophyte species according to clinical manifestations

Area	Genus								
	<i>Trichophyton</i>					<i>Microsporum</i>		<i>Epidermophyton</i>	
	<i>tonsurans</i>	<i>mentagrophytes</i>	<i>verrucosum</i>	<i>rubrum</i>	<i>violaceum</i>	<i>canis</i>	<i>gypseum</i>	<i>floccosum</i>	
Nail	0	0	1(50%)	0	0	0	1(50%)	0	2
Skin	2(5.4%)	12(32.4%)	11(29.7%)	3(8.11%)	1(2.7%)	4(10.8%)	2(5.4%)	2(5.4%)	37
Hair	8(57.1%)	1(7.1%)	1(7.1%)	0	1(7.1%)	3(21.4%)	0	0	14
Groin	0	2(25%)	2(25%)	0	0	0	0	4(50%)	8
Sole	0	8(72.8%)	1(9%)	1(9%)	0	0	1(9%)	0	11
Intertriginous	0	0	2(40%)	2(40%)	0	0	1(20%)	0	5
Dorsum	0	3(100%)	0	0	0	0	0	0	3
Face	0	1(25%)	2(50%)	0	1(25%)	0	0	0	4
Total	10	27	20	6	3	7	5	6	84



**Figure 1.** Haemolytic activity and CAMP-like effect: complete zone of hemolysin and CAMP-positive reaction with standard *Trichophyton* species (left) and CAMP-negative (right)



**Figure 2.** Proteolytic activity on agar plates containing gelatin as substrate (left), after ammonium sulfate decoloration (right)

There is a wide range of studies on epidemiology of dermatophytosis and its causative agents across Iran and other countries indicating different agents and clinical manifestations. *T. mentagrophytes* in Yazd [13], *T. rubrum* in Turkey [14], *T. sudanensis* in Nigeria [15], *E. floccosum* in Karaj [16], *T. verrucosum* in Isfahan [17], and *T. interdigital* in Tehran [18] were the most common etiological agents of dermatophytosis in those studies. Human skin is a large, heterogeneous organ that protects the body against pathogens while sustaining microorganisms that influence human health and disease [19]. Dermatophytic fungi were shown to have keratinolytic and other proteolytic and lipolytic activities [2]. It is evident that secreted proteolytic

activity is vital for their virulence [3]. Proteases play a crucial role in numerous pathologic processes [20].

Dermatophytes produce and secrete proteases in response to epidermal extracellular matrix components such as keratin during their invasion into the epidermal layer. These induced proteases may contribute to the potential of the dermatophytes to degrade components of deeper layers of dermis in dermatophytosis patients [21].

The role of exoenzymes as a virulence factor of fungi has been intensely studied. In case of dermatophytosis, their ability to secrete keratinolytic activity in vitro has attracted the attention of many researchers

**Table 2.** Distribution of proteolytic activity in dermatophyte species

Score	Agent								
	<i>Trichophyton</i>				<i>Microsporum</i>		<i>Epidermophyton</i>		Total
	<i>tonsurans</i>	<i>mentagrophytes</i>	<i>verrucosum</i>	<i>rubrum</i>	<i>violaceum</i>	<i>canis</i>	<i>gypseum</i>	<i>floccosum</i>	
+	3	10	9	1	0	3	2	1	29
++	7	14	7	4	3	4	2	3	44
+++	0	2	2	1	0	0	1	2	8
++++	0	0	2	0	0	0	0	0	2
>++++	0	1	0	0	0	0	0	0	1
Total	10	27	20	6	3	7	5	6	84

mainly to secreted fungal proteases [1]. Proteases play a crucial role in a wide range of pathologic processes and the identification of microbial proteases is prerequisite for understanding their role in the pathogenesis of infectious diseases [22]. Hemolysins were reported to have cytotoxic effects on membranes of erythrocytes and phagocytic cells and cause pore-formation and lysis effects on other eukaryotic cells and cellular structures [23].

Fungal hemolysins are potential virulence factors. Some fungal hemolysins belong to the family of aegerolysin proteins, which include cytolytins capable of lysing erythrocytes and other cells [14]. In our study, all dermatophyte species expressed hemolysin and proteolytic activity at different severity levels. In a study by Aktas et al. [14], more than 36% of isolates exhibited incomplete alpha hemolysis and not all dermatophytes could express hemolysin activity in the study by Schaufuss et al. [9], these findings were not congruent with our results. In addition, all dermatophyte species in our study revealed proteolytic activity, which could indicate that these enzymes probably play a role in pathogenesis of the isolates. The epidermis harbors a complex microbial environment. An initial step in the pathogenesis of cutaneous infections is the interaction of the microbial agents with the normal microbial flora and subsequent colonization of the stratum corneum [5, 22]. Schaufuss used clinical isolates of dermatophytes from humans and animals with ovine erythrocytes and found no hemolytic and CAMP reaction when using *S. epidermitis* and *S. hyicus* as normal bacterial flora of the skin [9]. Cooperative (membrane-active) processes such as CAMP reaction that lead to altered membranes are useful models to study the interaction of fungal products with host bacterial flora on host membranes [9].

In our study, for the assessment of the co-hemolytic effect of dermatophytes with different bacteria, we selected four species of beta-hemolytic bacteria including *S. aureus* and *S. pyogenes* as pathogenic bacteria that could be associated with infected fungal lesions such as tinea capitis and *S. saprophyticus* and *S. agalactiae* as normal flora of the skin. The co-hemolytic effect only occurs with *S. aureus* and no CAMP reaction could be detected with the other bacteria. Therefore, these bacteria that live as normal flora on the surface of the skin have no potential to aggravate secreted hemolysin in combination with different species of dermatophytes.

The Schaufuss study revealed that dermatophytes and bacteria can cooperate to destroy

skin protective barriers and an antifungal combined with antibacterial therapy must be prescribed to inhibit membrane damage [9]. For this reason, interaction of clinical dermatophytes with different bacteria seems to play a pivotal role that requires more research to achieve better treatments.

## Conclusion

All genera and species of clinical dermatophyte isolates secrete hemolysin and proteolytic enzymes that are potentially at play in their pathogenesis. In addition, *S. aureus* might be considered as the main bacterium creating a co-hemolytic effect associated with clinical dermatophytes.

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## Author's contribution

K. P contributed to study concept and design, drafted and revised the manuscript, and analyzed and interpreted the data. T. M aided in sample collection and laboratory examination. H. K contributed to study concept and design. M. M helped with bacterial preparation, analyzed and interpreted the data. K. Z contributed to study concept and design and interpreted the data. S. A cooperated with preparation of sample collection. M. M cooperated with data analysis and interpreted the data.

## Conflicts of interest

The authors declare that they have no competing interests.

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