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Molecular identification of *Trichophyton rubrum* Fungalysin gene of isolated from clinical sources by Multiplex PCR method

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ABSTRACT

Trichophyton rubrum is one of the most important causes of Dermatophytosis. The *Fungalysin* gene is an important factor in the incidence of dermatophytosis disease. The aim of this study was to investigate the presence of mep1 and mep2-generating *Fungalysin* genes using the multiplex PCR method. 150 scabs and nails were sampled from patients with symptoms of dermatophytosis. Samples containing Trichophyton rubrum were identified and isolated using a red pigment production test in DTM medium, without urea production, without hair piercing and sorbitol adsorption. Culture slide was performed to identify the microscopic structure. Then DNA extraction was performed and several PCR experiments were performed in these strains using specific primers of Fungalysin gene. Out of 150 samples isolated from patients, 30 Trichophyton strains were isolated based on morphological, biochemical and microbial tests. Among these, 20 strains had mep2 gene and 1 strain had mep1 gene. Results shows that the frequency of *mep2* genes is higher than mep1 and this abundance is involved in causing more pathogenicity of Trichophyton rubrum and is one of the major causes of treatment-resistant infections in medical centers. Now, with the help of the Multiplex PCR method, it is possible to detect the presence of pathogenic genes in the shortest time with high characteristics and sensitivity, and with appropriate and timely treatment, prevented the function of treatment resistance strains, recurrence of the disease and prolongation of the treatment period.

1. Introduction

The presence of fungi is one of the most important public health issues in developing countries and is recognized as a contagious fungal disease worldwide (Tarabees et al., 2015; Takiuchi et al., 1982). Dermatophytes are the most important keratinolytic filamentous fungi, which include the three genera *Trichophyton*, *Microsporum*, and *Epidermophyton*. Dermatophytes are filamentous fungi that can cause different types of dermatophytosis in animals and humans. An environment exposed to infection by a dermatophyte requires the secretion of creatinine enzymes that allow it to penetrate the host tissue (Larypoor et al., 2009; Sharma et al., 2014).

Some species of Trichophyton are antropophilic with global spread and is one of the most important causes of human infections.

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It is rarely isolated from animal infections, but there have been no reports of its separation from the soil. The *Fungalysin* motif is found in the bacterial peptide peptidase M4 and the fungal M36 propeptide is found in the opposite (Ebihara et al., 2009; Sanglard, 1997; Ueda et al., 2013).

Anthropogenic species live only on the surface of the human body and can be detected at the site of the lesion with mild chronic and inflammatory symptoms. Zoophilic species were primarily isolated and identified from animals and are associated with severe inflammatory reactions in humans in contact with cats, dogs, cows, horses, birds, or other infected animals. Soil-friendly soils are usually identified and isolated from the soil, but sometimes infect humans and animals. They cause a specific, limited inflammatory response to the site of infection that may improve spontaneously (Apodaca et al., 1989; Mignon, et al., 1998).

Based on studies on dermatophytes by the Genome Strategic Committee, based on criteria for identifying and investigating species infected with human infections, growth in specific environments, morphological genomic sequence and spore production, protoplast structure, drug sensitivity, five dermatophyte species Important for genomic analysis were identified (Tobin, et al., 1997; Komoto, et al., 2015).

Dermatophytes are primarily responsible for skin infections in various parts of the body, including the legs, trunk, scalp, and nails. Dermatophytes can cause fungal skin infections in the form of unpleasant clinical forms such as erythematous, scaling, Itchy lesion, and dermatitis on the surface of the skin (Tsuboi, et al., 1989). Inflammation, red and inflamed cracks with itching or burning, and in nail infections with increasing thickness, discoloration, and pain, as well as infection of the scalp with irreversible hair loss. It is very difficult to diagnose nail infections caused by *Trichophyton*, including *Trichophyton rubrum*, and can delay treatment for months or, in some cases, become chronic and resistant to treatment (O'sullivan, et al., 1971; Robati, 2019). *Fungolysins* have similar peptide roles to bacterial thermolysins, but have a similar sequence to this class of proteases. (Descamps, et al., 2002).

Fungolysins, such as thermolysins and bacterial proteins secreted, are expressed as inactive precursors containing N-terminal proteins. *Fungolysins* are intermolecular proteins that both inhibit activity and guide proteases in the right direction (Jousson, et al., 2004; Brouta, et al., 2002). The first described *Fungolysins* (Metalloprotease) was MEP42. Fungolysis exacerbate pathogenesis by destroying extracellular matrix proteins. Fungolysis is secreted by various fungi, including the *Trichophyton rubrum*, which causes skin diseases and is able to destroy the structural protein creative. (Miyajima, et al., 2013) *Fungalysin* studies of molecules also show that their expression plays a role in fungal diseases, but the host's goal is unknown (Brouta, et al., 2002).

Several proteases, including *Fungalysin* (*MEP1*-5), have been identified as a potential factor. In the present study, dermatophytes were isolated and identified from infected samples, and the samples were examined for the presence of these genes (*1mep*, *2mep*) using multiple polymerase chain reaction (PCR). This study was used to screen for the presence of these genes in dermatophytes isolated from local strains to investigate their exact role in dermatophyte infection (Tarabees, et al., 2013).

Finally, Trichophyton rubrum isolates were identified by specific in vitro and molecular PCR methods based on specific mep gene primers encoded in DNA. Trichophyton rubrum was confirmed by observing the 1100 genome fragment (Ueda, et al., 2013; Miyajima, et al., 2013; Tarabees, et al., 2013). This study aimed to identify the molecular identification of *Trichophyton* Fungalysin gene in front of the isolate isolated from clinical sources by the Multiplex PCR method.

2. Materials and Methods

2.1. Sampling

Out of 150 samples of suspected patients with dermatophytosis referred to Razi Skin Subspecialty Medical Center in Tehran, clinical sampling was performed with the permission of patients and after filling in the required questionnaire and information.

Sampling was performed using scalpel skin and nail chip removal methods and scalping of hair roots with pliers. After sampling and collection, they were transferred to the laboratory in a special envelope. Sampling

should be under a lamp or in natural light, and the patient should not bath for about a week before sampling or take oral or topical medications. A screening of isolated samples from dermatophyte-containing scales resulted in a total of 30 *Trichophyton rubrum* isolates in of Separation was achieved.

2.2. Perform biochemical tests to identify and isolate Trichophyton

Hair and skin samples were cultured on Sabouraud dextrose agar medium containing chloramphenicol and Cycloheximide (SCC) and kept in an incubator at 28°C for 4 weeks. Production of *Trichophyton rubrum* red pigment on SCC or Dermatophyte Test Medium (DTM) is one of the most important methods to identify this fungus (Sharma et al., 2014).

To study the colony shape and macroscopic morphological studies, including growth rate, colony surface color and colony background, (colony appearance, size and shape) in SDA and differential and nutritional medium, Sabouraud dextrose works containing rice extract and also, samples were cultured.

It was then incubated at 25 to 30°C temperatures, according to the type of dermatophyte studied over some time. Diagnosis of *Trichophyton rubrum* was made based on the appearance of the colony, preparation of a colony sample with Lactophenol cotton blue solution and slide culture technique (Apodaca and Mckerrow, 1989).

2.3. Hair piercing test

For this purpose, small pieces of sterilized human hair were incubated in a sterile container near a colony suspected of having dermatophyte at 25 ° C for 2 weeks. *Trichophyton rubrum* cannot pierce the hair.

2.4. Biochemical tests

The urea test was performed in Christian urea agar medium, in which *Trichophyton mentagraphytes* is able to digest urea and change the color of the environment. But this test is negative for *Trichophyton rubrum*.

Fermentation of sorbitol sugar is performed by *Trichophyton rubrum* and as a result the color of the environment changes to red.

2.5. DNA extraction with a column kit extracting a specific fungal genome

To extract the fungal DNA, its DNA extraction kit was used. With this kit, the extraction was done by the column method (mini column). In general, DNA extraction or purification is required to perform a good PCR reaction. PCR is a polymerase chain reaction that is used to propagate a specific genetic area in a specific biological agent that is detected by a specific primer. (Miyajima, Y., et al., 2013)

2.6. How to extract the DNA of a filamentous fungus

DNA extraction was used from 5-day cultured colonies in the culture medium (Potato dextrose agar (PDA). A small block measuring 0.5 by 0.5 mm was removed from the culture medium containing mycelium or fungal spores. One and a half milliliter tubes were dropped, 100 microliters of buffer lysis (containing 100 milliliters of triceps, 20 milliliters of EDTA 100 milliliters of NaCl, and 2 percent (SDS) were added.

2.7. Extracted DNA electrophoresis

After the extraction process, to ensure the presence of DNA and its purity, the extracted DNA was electrophoresed in 1% agarose gel for 45 minutes at 70 volts and then the resulting bands were observed under ultraviolet light after staining.

2.8. DNA quality control extracted

Quality control with Nano drapes: To control the quality of DNA extracted by the kinase kit, DNA adsorption nanoparticles were examined. This is a quantitative method and the DNA concentration can be calculated using light absorption at 260 nm. Absorption for an OD at 260 nm indicates the presence of 50 µg/ml of double-stranded DNA in solution. Using this method, the number of impurities caused by the presence of protein in the DNA solution can be detected. For this purpose, absorption should be

measured once at a wavelength of 260 nm (wavelength at which DNA has the maximum absorption) and once again at a wavelength of 280 nm (which has the maximum absorption protein) using the absorption ratio in 260. Nanometers to absorb at 280 nm were examined for impurities.

2.9. Preparing primers

After visiting the study site and searching in various articles, suitable primers for mep1 and mep2 genes were selected. The primers were compared and blasted on the site http://blast.ncbi.nlm.nih.gov/Blast.cgi) and were ordered from Macrogen. The primers were ionized with distilled water to a concentration of 100 picomoles and then to a suitable concentration of 10 picomoles. Table3 shows the primer sequence and the length of the propagation pieces (Komoto, et al., 2015).
Primers: According to studies, 2 pairs of suitable primers were selected and ordered to Bioneer for preparation (Table 1).
Multiplex PCR polymerase chain reaction: PCR temperature and time conditions this research is shown in table 2 after optimization.

Table 1. Sequence of primers used to propagate Fungalysin genes (Tsuboi et al., 1989)

Gene	*Trichophyton rubrum.* Primers (5´---> 3´)	Size (bp)
MEP1	F: GCCACTGAGCTGGTTAAG R: CTTTGGATCGAACTTAGC	1650
MEP2	F:AGAGTTCCTGACTCGGAC R:ACTCGTGGATGACAATACC	1400

Table 2.Temperature adjustment program and time to perform PCR steps

level	Temperature stage (degree of Celsius)	Time (seconds)	Return of cycle
Primary initial	95	180	-
initial	95	30	35
Connection	58.5	30	
Rearrangement (expansion)	72	60	
Final rearrangement	72	300	-

Agarose gel electrophoresis is a standard method for examining and isolating DNA fragments from Multiplex PCR. The location of the gel is detected by low-concentration staining of the Erythrogel color solution and its direct observation under the influence of ultraviolet light. The concentration of agarose gel is effective in better separation of different DNA sizes. Erythrogel is more sensitive and more stable than the toxic composition of ethidium bromide and is more environmentally safe. It should be noted that this substance does not have the cytotoxic and mutagenic effects of ethidium bromide.

3. Results

3.1. Sampling and isolation of fungal clinical specimens

A screening of isolated samples from dermatophyte-containing scales resulted in a total of 30 *Trichophyton rubrum* isolates.

3.2. Identification based on morphological and microscopic characteristics

From the colonies suspected of having Trichophyton, warm staining was performed and the presence of dermatophyte fungi was investigated. The results of the growth of *Trichophyton rubrum* fungus are seen of cotton and red pigment on the back of the colony. After two weeks and incubating the clutch slide at 30ºC, the mycelium grown in the culture medium was observed to separate the lamellae, then pour two drops of Lactophenol cotton blue on another clean slide and the lamellae with the fungal colony. *Trichophyton* was placed on the slide in front of me and the structure of the threads attached to it was examined. Isolation and identification results and dermatophyte culture slide technique are shown in figure 1.

3.3. Hair piercing test

The results of non-perforation of the hair by *Trichophyton rubrum* are shown in figure 2.

3.4. Biochemical tests

You can see the results of biochemical tests of urea enzyme production and sorbitol sugar absorption by Trichophyton rubrum in figure 3.

Figure 1. Results of identification of *Trichophyton rubrum* from clinical samples

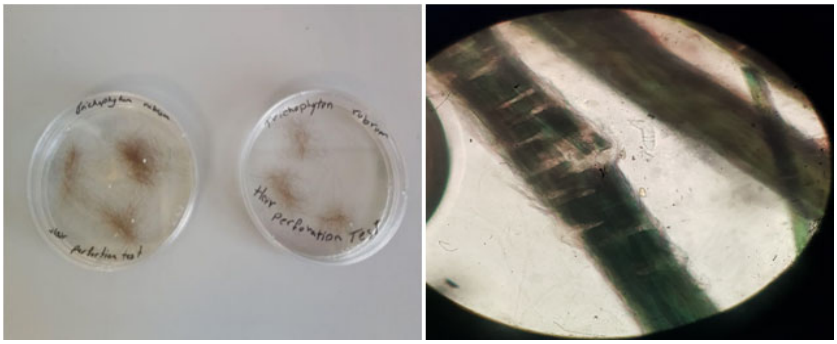

Figure 2. The figure on the left shows the hair piercing test. Figure on the right: No hair piercing by Trichophyton rubrum in front of the control sample

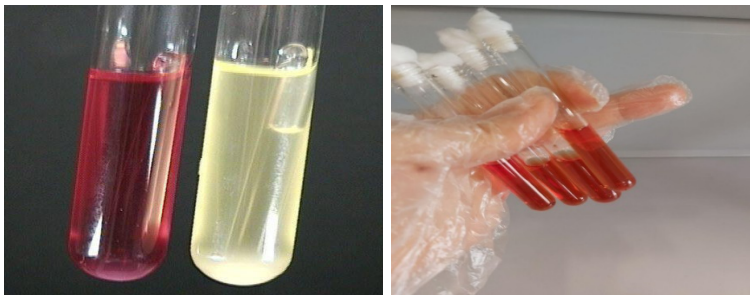

Figure 3. In the left figure, the red color indicates the lack of urea production by *Trichophyton rubrum*, the positive results of the sorbitol fermentation test by *Trichophyton rubrum* are shown in the figure on the right

3.5. Multiplex PCR test results for invasive gene

In figure 4, based on the results of Multiplex PCR technique in examining the presence of Mep1 and Mep2 genes related to Trichophyton rubrum strains, 20 samples were positive for Mep2 gene and Mep1 gene was observed only in sample 7.

3.6. Investigating the abundance of genes by Multiplex PCR

A total of 30 Isolated Trichophyton was isolated from the screening of clinical samples to the laboratory, which were identified based on morphological, microscopic, and biochemical tests. Multiplex PCR tests to determine the severity genes of the isolates showed that 20 strains carried the mep2 gene and 1 strain carried the mep1 gene.

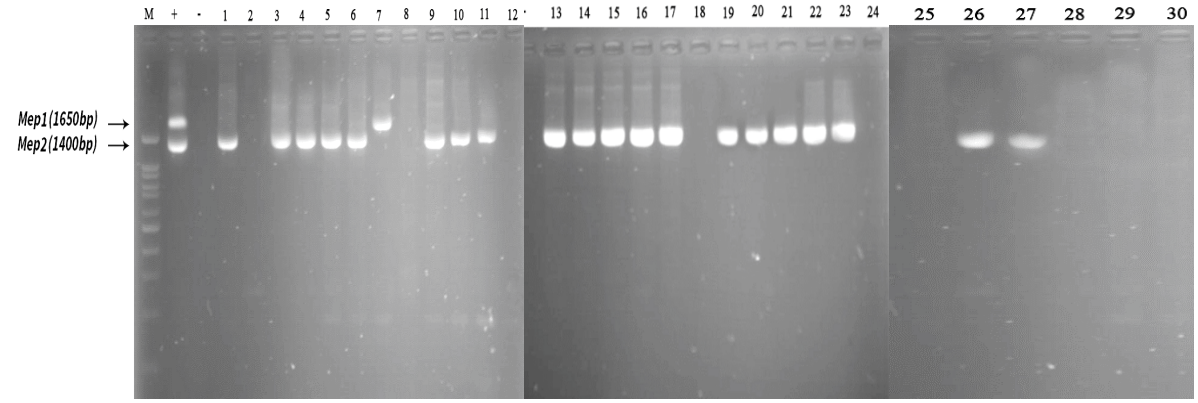

Figure 4. M-PCR test results for samples 1 to ٣٠, from left to right: Ladder 100bp - positive control - negative control of mep1 (1650 bp) and mep2 (1400 bp) genes. (Types 1, 3, 4, 5, 6, 9, 10, , 11,13 , 14 ,15, 16, 17, 19, 20, 21, 22, 23,27 and 28 had the Mep2 gene, and strains 7 had the Mep1 gene.)

Table 3. Results from Multiplex-PCR *Fungalysin* Gene *Trichophyton rubrum* Isolation

Genes studied	*mep1* 1650 bp	*mep2* *1400 bp*	Total number of isolates
Trichophyton rubrum	1 (3.3%)	20 (66%)	30

4. Discussion

Fungal infections are as an important health issue in the world. Fungal surface diseases in the world's population are currently on the rise, affecting more than 20-25% of the world's population. Social life, contact with animals, the use of antibiotics, corticosteroids, anticancer drugs, and some other factors help increase fungal infections, especially dermatophytosis (O'sullivan et al., 1981). The reason for the increase in fungal infections of the skin is also due to the economic conditions of the society and poor health (Takiuchi et al., 1982).

Dermatophytes are a group of keratinophilic fungi that cause fungal infections called dermatophytosis by invading the hair, nails, and stratum corneum. *Trichophyton* is an anthropophilic dermatophyte that is a common cause of groin, body, nail, and hand baldness (Tsuboi et al., 1989). Dermatophytosis is one of the most common fungal diseases caused by dermatophytes. The disease is transmitted directly and indirectly by contact with hair and skin infected with dermatophytes (Apodaca et al., 1989).

Fungulas are among the pathogens that produce the pathogenic gene *mep* in fungal fungi. Among the diagnostic methods, specific pathogenic PCR genes are often used. Multiplex PCR method for the simultaneous examination of multiple genes by saving the necessary materials and reducing the time of genetic studies and required patterns with several pairs of primers in general and exclusively with high reliability and speed for molecular identification of different genes the causative agent of rheumatoid arthritis is *Trichophyton* (Ueda et al., 2013).

Karami Robati 1397, this study was performed on 32 patients with fungal infections referred to Tehran University of Medical Sciences from July to September 2017. Based on microscopic and macroscopic observations, the *Trichophyton* species of *sub1*, *sub2*, *sub3*, *sub4*, *sub5*, *sub6*, and *sub7* molecular baroque of

multiplex polymerase chain reactions were replicated with specific primers. The relative percentage of each of the seven genomic sequences was also calculated. 7 species of *Trichophyton* were isolated from the clinical sample of skin (5 cases) and nails (2 cases). The subtype family (*sub1*-7) was reported to be 57% (Robati et al., 2019).

While the *sub*5 gene (100%) was found in all isolates, the SUB6 gene was reported with the lowest frequency (4 out of 7 cases) and the presence of seven subtilizing genes in species isolated from nail tissue. The presence or absence of subtilizing genes in the DNA of *Trichophyton* isolates is likely to indicate the importance of each gene at different stages of infection (binding, invasion, and inflammation) in dermatophytes and the type of nail and skin tissue (Robati et al., 2019). In contrast to the present study, the study looked at *Fungalysin* genes, which indicate the influence of other genes that are effective in infecting *the Trichophyton rubrum* in causing skin diseases.

Lemsaddek et al, 2012 Studied the presence of genes (*mep* 1-5), related to *Fungalysin*, and other subtilizing proteases (genes (*sub*1-7) using PCR. Reported the presence of at least one virulence gene in 212 of 233 (91%) dermatophyte isolates representing 14 species of mostly environmental origin (e.g., swimming pools from Portugal and Morocco), as well as reference isolate from Spain. However, the isolates analyzed in the current study mostly included human clinical and animal isolates. Reported that 44% and 27% of all isolates tested were positive for MEP and SUB genes, respectively. By comparison, we found these respective percentages were 32.9% and 5.1%. Further, in an earlier study. None of the tested genes could be amplified in 21 (9%) of the tested isolates, four of which belonged to the species *Trichophyton rubrum*. By comparison, none of the tested genes could be amplified in 11.3% of the tested *Trichophyton rubrum*.

Unlike the interspecies distribution of MEP and SUB genes in isolated depending on their origin, e.g., Turkey, Germany, and Japan, differences in the distribution of these genes were found in the current and previous studies at the isolate or species levels.

Olivier, Jason et al, 2004 in a study of common pathogenic fungal genes, including metallo-proteases, the MEP family studied *Fungalysin* in *Trichophyton rubrum* and *Trichophyton mentagrophytes*. Indeed, the four species analyzed here secreted substantial amounts of two orthologous *Meps* (*Mep3* and *Mep4*) in the soy protein medium. Phylogenetic analysis of the genome and protein sequence, the study clearly showed that in a culture medium containing soy protein as the only source of nitrogen, *MEP* gene proliferation occurs in dermatophytes and 19 to 36% of the total protein extract is secreted. Give; Determination of protein groups by proteolysis and mass spectrometry showed that three species of dermatophytes encode two genes, metalloprotease (*mep3* and *mep4*) (Jousson et al., 2004). In the present study, the presence of two other genes was investigated and more frequency was related to the *mep2* gene.

Brouta et al 2001, Analyzed one or more metalloprotease coding genes for *mep1* and other similar genes. At least one type of *mep* gene was used for identification, and the results showed that the high incidence of this gene, due to its role in infection, is in the early stages among a set of isolates (Brouta et al., 2002). This study is in line with the present study and has observed the presence of invasive genes.

Leng et al 2009, MEP4 and MEP5 reduced their activity in comparison with wild strains and different strains, while MEP3 is similar to wild strains and more active than observations with MEP1 and MEP2. These results showed that the MEP gene could affect keratine proteolytic activity. Differences in protein secretion levels, which play an important role in creatinine secretion, affect the digestive function of metallo-proteases over the years (Leng et al., 2009).

Tarabees et al. Studied dermatophytes in 2013 and showed that the Fungalysin gene is essential for disease development, which was assessed by PCR. Data collected from this study showed that 1-4 *MEPs* were detected in only 10% of screened samples, while MEP5 was 20%. This hypothesis seems to be wrong, because all specimens have been isolated from patients with dermatophytosis, and some strains do not carry metalloprotease genes, and more factors and genes must be involved. (Tarabees et al., 2013)

In the present study and previous studies, relying on clinical trials, only some of the causes

of skin diseases caused by *Trichophyton rubrum* were considered and more specialized research is needed to examine more closely the role of other genes and factors involved in dermatophytosis. The results of this study were not sufficient to investigate the genetic differences between the isolated species, but in a short period, it provided a platform for understanding the frequency of genomics to further investigate pathogenic genes and take necessary measures to combat the causes of disease they are a great help (Miyajima et al., 2013; Tarabees et al., 2013).

Conclusion

Trichophyton rubrum is an anthropophilic dermatophyte. This dermatophyte is one of the most important causes of human infections and is rarely isolated from animal infections. *Trichophyton rubrum* is the cause of body baldness and is often the cause of foot and nail baldness. Numerous studies have examined dermatophytes, genes, and the causes of dermatophytosis. In this study, the presence of *mep1* and *mep2* genes in *Trichophyton* of isolated isolates from patients were investigated, which resulted in more abundance of the *mep2* gene. In this study, with the Multiplex PCR method, in the shortest time with high characteristics and sensitivity, the presence of pathogenic genes was determined. The action will come.

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Refereces

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