Identification of trichophyton spp and detection of some virulence genes by using PCR

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Abstract

Background

The majority of dermatophytosis is caused by trichophyton. Infection establishment and tissue damage are caused by a number of pathogenicity, such as keratinase , phosphatase and transcription factor . The regulated expression of numerous genes governing host-fungus interactions controls the virulence .

Methods: Initial examination of 100 clinical specimens using the KOH microscopy procedure . To identify common pathogenic trichophyton spp, all samples were cultivated on Sabouraud Dextrose Agar at twenty eight °C for two weeks with chloramphenicol, gentamicin, and cycloheximide. These samples were then amplified using a multiplex PCR. Moreover, specialized primers can identify the virulence factor.

Results: The specific primer used to investigate the presence trichophyton spp ,that found in the clinical sample and the result show that all isolated were positive to phospholipase , Transcription factor PacC while only 26 isolated were positive to keratinase .

Conclusions: The present study was concluded the Detection of trichophyton spp and their virulence factor using specific primers are more accuracy and specific than phenotypic method .

Keyword : trichophyton spp , virulence factor , PCR

1- Introduction

The majority of dermatophytosis is caused by trichophyton , a frequent dermatophyte in medical settings. Tinea capitis, ringworm , athlete's foot, tinea cruris, and onychomycosis are only a few of the superficial illnesses caused by the organism's invasion of humans keratinous structures, which include the hairs, skins, and nail (Li *et al* .,2021).

Patients with these illnesses feel discomfort, including itching and pain, which can significantly lower their quality of life (Aylin *et al* ., 2008). Trichophyton is chronic, relapsing, and rarely life-threatening, but it can spread easily from one person to another person and having a substantial impact on the quality of life for people who have it. (Fekrazad *et al* ., 2017)

One of the main human pathogenic dermatophytes, Trichophyton rubrum, is responsible for roughly 69.5% of chronic dermatophytoses in people. Patients with diabetes mellitus, steroid therapy, bone marrowtransplants, or solid organ transplants take a lengthy time, and immunocompromised are all severely affected by this pathogen. Several virulence factors are used by Trichophytones to begin the infection. The most prevalent trichophyton of people nowadays is an anthropophilic fungus called Trichophyton rubrum (de Hoog *et al* 2016)

Sensitive methods , such as polymerase chain reaction used for detect Trichophyton spp pathogens . indeed Trichopytones DNA can be detected by polymerase chain reaction . the application of such sensitive assay are likely to contribute important new observation about the epidemiology and disease patterns of Trichophyton .(Elife Berk *et al* ., 2011)

The coordinated expression of several genes that mediate interaction between hosts and fungi determines fungal pathogenicity .Phospholipases is one of the pathogenic biomarker, help the trichophyton spp. colonize host cells by linking to the targeted site and degrading the cell membrane after hydrolyzing phospholipids, demonstrating the significance of this enzyme in the early stages of trichophyton spp. infection and fungal colonization. These enzymes might be crucial for maintaining a balance

between the host's immune system and the capacity of trichophyton species to suppress the immunological response.(Nilce *et al* ., 2021)

Trichophytone phospholipases damage host cell membranes , enhance disease .In addition to directly damage tissue ,these hydrolytic enzyme may also function in ways that increase pathogenicity .(Abdulkawi 2014)

Keratinase is the most importance virulence factor for trichophyton.It is produced only in the presence of keratin substrate. Trichophyton must develop in the host tissue, which depends on keratin. This group of critically significant fungus for medicine is characterised by the capacity to infiltrate keratinized tissues as a major virulence feature.

Trichophytones can cling to host tissue and use the available resources for their survival and growth thanks to a fungal metabolic that has been .modified as a result of the host - pathogen interactions

During growth, keratin is the only source of carbon for trichophyton , and as a result of its breakdown, the pH of the host's skin changes from acidic to alkaline. This adaptive reaction therefore creates a pH that is appropriate for the majority of the proteases released by Trichophyton to function at their best, which is necessary for their survival, pathogenicity, and dispersion in multiple host environments.(Abdulkawi 2014)

The transcription factor gene is crucial for the shipment and arrival of necessary components, for maintaining ion homeostasis, for participating in the direct regulation of salt tolerance via regulating efflux mechanisms, and for monitoring ion homeostasis

Material and Methods

A total of 100 samples were obtained from individuals who were thought to have a cutaneous fungal infection, including scrapings of the skin (n = 83) and nails (n = 17) . aged from 15 to 65 years old.

Patients were admitted to either Marjan medical city Dermatological advisory , Al‑Sadeq hospital Dermatological advisory and Al- zahawi Laboratory between April and October 2022

The diagnosis of trichophytones by noticed that 100 specimens were separated into three groups. One group under microscopic examination in 10% KOH, and the detection of pathogenic fungus isolated from clinical specimens has been attempted using molecular biological techniques . The second group was made on Sabouraud agar and cultured for two weeks at 28 °C .third group was detection by used multiplex polymerase chain reaction , which enables the simultaneous detection of several microbes, is frequently employed in clinical microbiology . Many fungi, including trichopytones spp , have been identified using this method (Ross IL *et al* .,2020)

This study's primary objective was to develop and assess an easy multiplex PCR test for the accurate detection and classification of sample isolates of trichophyton rubrum, trichophyton mentogrophytones , and trichophyton . violaceum and detection of some virulence factors for these trichopytones species

In this study, all individuals with uncomfortable and itching symptoms were included. Individuals having results of psoriasis, eczema, skin ulceration, or allergies to skin were excluded .

All ethical for taking a sample, all parties involved in this work are informed and verbal consent is acquired from everyone. With reference

number BMS/0231/016 and with the approval of the college of medicine at the University of Babylon in Iraq, the publishing ethics committee

accepted this work .

In this work. Primer designs were made using data from the NCBI's Blast database to help differentiate the three trichophyton strains from other dangerous fungi.

Molecular identification of Tichophyton spp was done by using gene-specific primers as show in table (1)

Table (1) specific primer for detection of trichophytone *spp*

Primer Sequence	Product	Trichoytones spp
F- RCGAGGAGAGGACCCRACHTCTGAC R- TTCCTTAGTACCRGCYTTG	925 bp	Trichoyton.rubrum
F- GATCCACAAGGTATGTATTAGTTA R- GGTGCCAGCCATGTCGTAGAC	421bp & 925bp	Trichoyton.violaceum
F- GCCTGTTGTTCCGCTCATTCTT R- CGGCTAGGAGGGCGTGGTAGAA	392 bp	Trichoyton . mentagrophytes

The PCR cycle settings were as chooses to follow: two minutes of pre - heating at 96C^{O}; thirty cycles at 96C^{O} for thirty second ; three seconds at 63C^{O}; and one hindered twenty second at 74C^{O}.

Primer Design of some virulence genes of trichophyton spp

The oligonucleotide primers for the studied genes were designed in this study according to an existing GenBank sequences for studied genes at (NCBI). These sequences used to design *gene's* forward and reverse primer by Primer3Plus software. Oligonucleotide primers were synthesized by Macrogen company (Korea).

Table (2) virulence genes of trichophyton spp

Gene name	Primer Sequence	Product	Study
keratinase	F-CACTGGTGTCGATGTCAACC R- AGTTAATACCGGCGACGATG	227 bp	New design
phospholipase	F-GGAGACATCGATCAGGTGGT R- GATGATGCGGTCAAGGAACT	162 bp	New design
PacC	F-ACCAATTCCTGGAGCAGATG R- GTGGTGTGACTGGTGGTGAG	152 bp	New design

PCR Amplification for virulence genes

New Uniplex-PCR conditions were used for each virulence gene by using gene-specific primer that listed in table (2). In this assay, Uniplex-PCR was done a total volume of 25 µl by using GoTaq® G2 Green Master Mix (Promega, USA), through Prime5 thermocycler (Techno, UK). All PCR mixtures and conditions for all genes were summarized in table

Table (3) Mixes for uniplex PCR as well as parameters for identifying specific gene.

PCR blend		PCR parameters		
Contents	**Volume**	**Type of cycle**	**Condition**	**No. of cycles**
Master Mix	12.5 micron	Initialization	94 °C for Five minute	1
Forward Primer	1.5 micron	Denaturation	94 °C for One minute	35
Reverse Primer	1.5 micron	Annealing	57 °C for One minute	
Template DNA	3 micron	Extension	72 °C for one minute	
Nuclase-Free Water	6.5 micron	Final Extension	72 °C for Ten minute	1

3 - RESULTS

A total of 100 specimens , including scrapings of skin and nails, were taken from patients who may have had cutaneous fungal infections. It should be noted that 100 samples were divided into three portions. One portion was used to determine or not fungal elements were present under a microscope in 10% KOH. Another fraction was grown on Sabouraud dextrose agar supplemented with gentamycin, chloramphenicol, and cyclohexemide and incubated at 28 °C for up to 4 weeks to identify the different forms. T. mentagrophytes produces colonies are the colour (white) and appearance of colonies (powdery or velvety) and distinguish T.rubrum during produce colonies that characteristic culture surface texture may vary from downy to suede-like; culture surface pigmentation

may vary from white to cream to deep red; culture reverse pigmentation may vary from colourless to yellowish to yellow-brown to wine red . Moreover, T. violaceum isolates were waxy, glabrous, wrinkled colonies and were slow-growing. The distinguishing color of colonies was a deep purple-red, and they occasionally had a white, waxy fringe. and the third part was used for PCR analysis and DNA extraction

The specific primer used to investigate the presence Trichopytones spp The clinical samples of patients suspected of having a cutaneous fungal infection contained a total of 60 DNAs were extracted . These DNAs were detected by the multiplex PCR designed in this work to simultaneously detect the infection and identify the causative organisms. T. rubrum, T. mentagrophytes, and T. violacem were all detected by multiplex PCR in Sixteen (55.5%) specimens , seven (25.9) specimens, and four (14.8%) specimens, respectively. For the 33 remaining samples, the result was show negative . Table 4, 5 Figure 1

The infectious trichophytone have been targeted for species identification using quick and precise molecular methods, primarily based on primary isolation by culture.

Table (4) Comparison of culture and multiplex PCR in terms of the identification of 60 clinical samples suspected of cutaneous fungal infection

			Detedted by multiplex PCR		
Clinical samples			T. rubrum	.T mentagrophytes	T. violacem
Detection by culture	Positive for trichophytone	60	16	7	4
	Positive for non trichohytone	25	0	0	0
	Negative	15	0	0	0
	Total	100			

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Table (5)

Recognitionof trichophytones species by PCR

Dermophyte type	Number N=27*	%	P. Value
T. rubrum	16	55.5	<0.0001*
T. violacem	4	14.8	
T. mentagrophytes	7	25.9	

* Number of samples is 27, some of samples with mix culture (have more than one species)

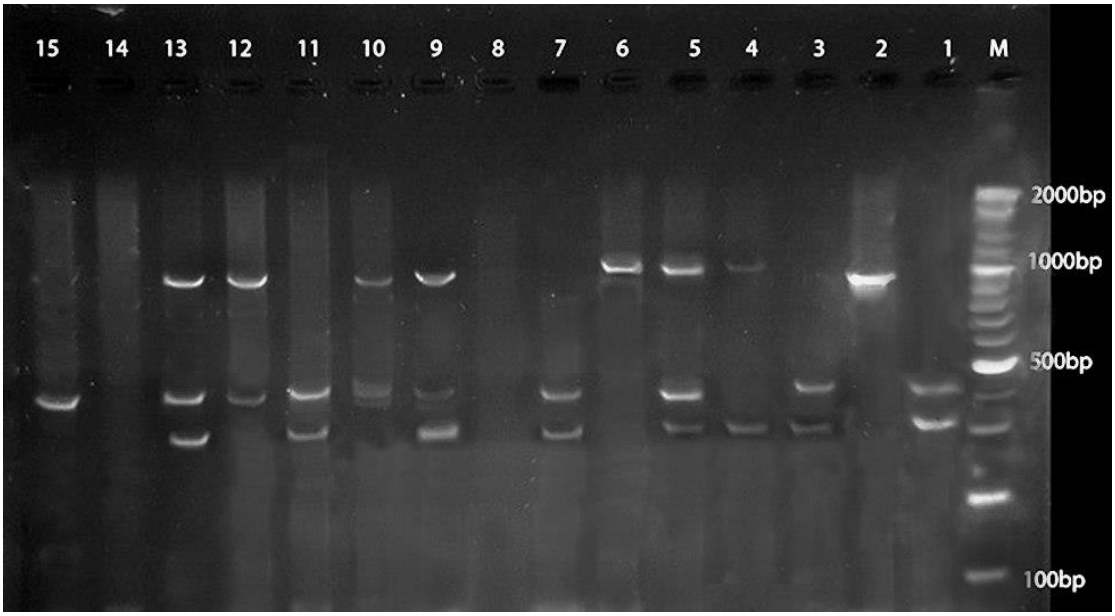

Figure 1: The electrophoresis of agarose gel of multiplex-PCR products obtained with trichophytones species -specific primers that generated: 925bp =T. rubrum, 925bp+421bp= T. violaceum, 392bp= T. mentagrophytes and 522bp . Lanes (1-15); represent the first isolated trichopytones, Lane M is a 100bp DNA ladder

On the other hand .some virulence factors were determined such as for phospholipase , transcription factor and keratinase genes of that result was shown that all samples were positive for phospholipase and transcription factor as shown in Table (6,7) and Figure (2,3)

Table 6 detection of phospholipase gene in trichophytones spp

Dermophyte type	Number N=27*	%
T. rubrum	16/16	100
T. violacem	4/4	100
T. mentagrophytes	7/7	100

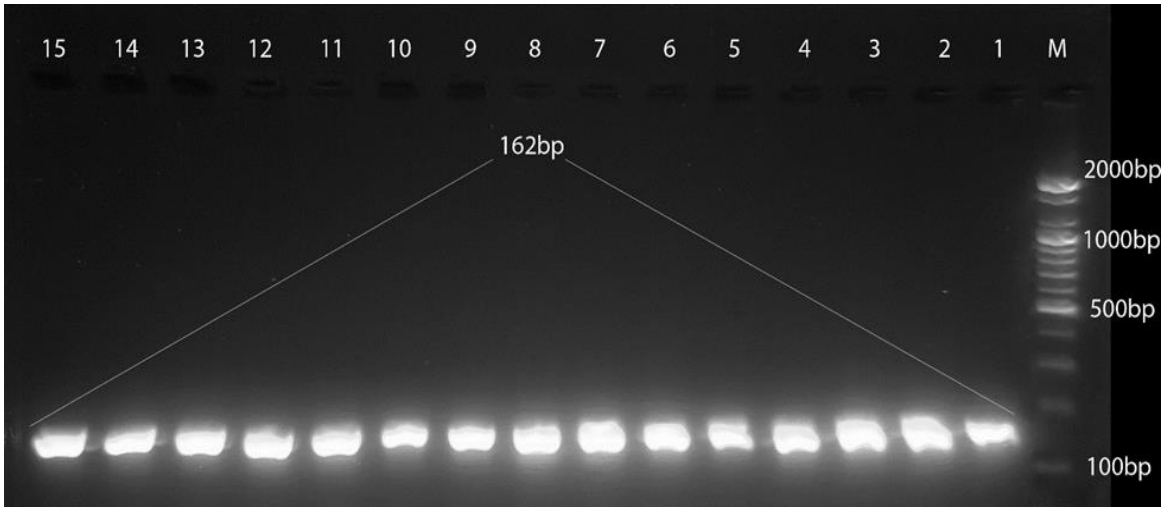

Figure 2 : Agarose gel electrophoresis of PCR product acquired with phospholipase-specific primers that generated 162bp. Lanes (1-9, and 11-15); represent the first positive phospholipase, Lane 10 is negative. Lane M is a 100bp DNA ladder.

Table 7 detection of PacC gene in trichohytones spp

Dermophyte type	Number N=27*	%
T. rubrum	16/16	100
T. violacem	4/4	100
T. mentagrophytes	7/7	100

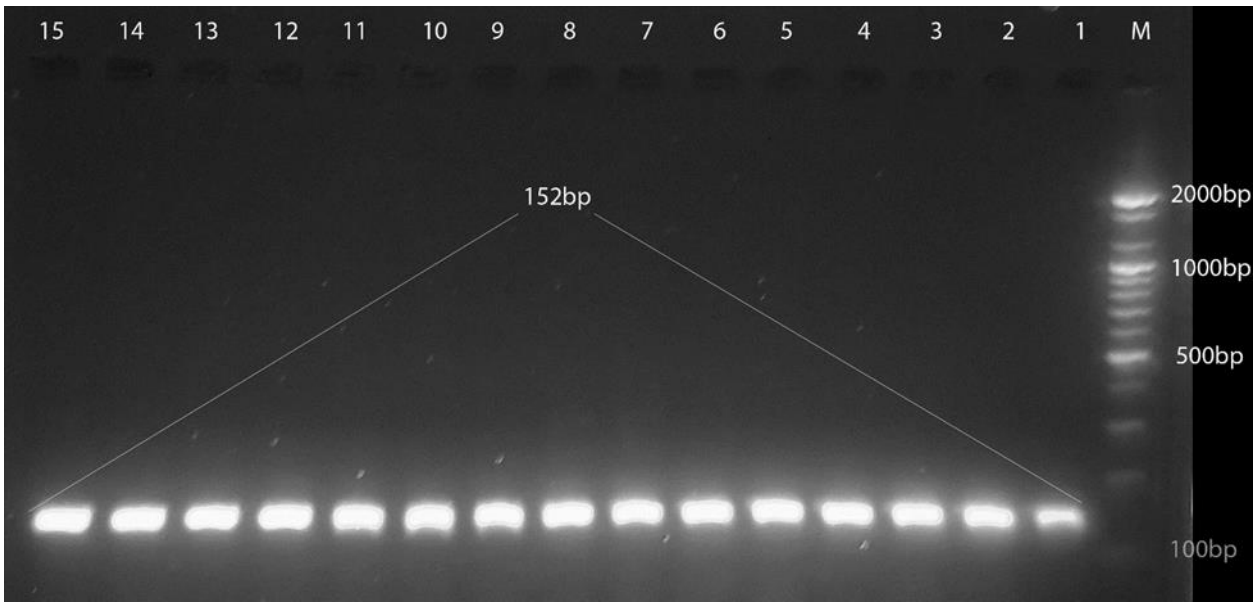

Fig. (3): Agarose gel electrophoresis of PCR product acquired with transcription factor PacC-specific primers that generated 152bp. Lanes (1-15); represent the first positive PacC. Lane M is a 100bp DNA ladder

On the other hand , keratinase gene was also determined , the result was show that only 26 isolates were positive for this gene as show in table 8 and figure 5

Figure (8) Table 5 detection of keratinase gene in trichophytones spp .

Dermophyte type	Number N=27*	%
T. rubrum	15/16	93.3
T. violacem	4/4	100
T. mentagrophytes	7/7	100

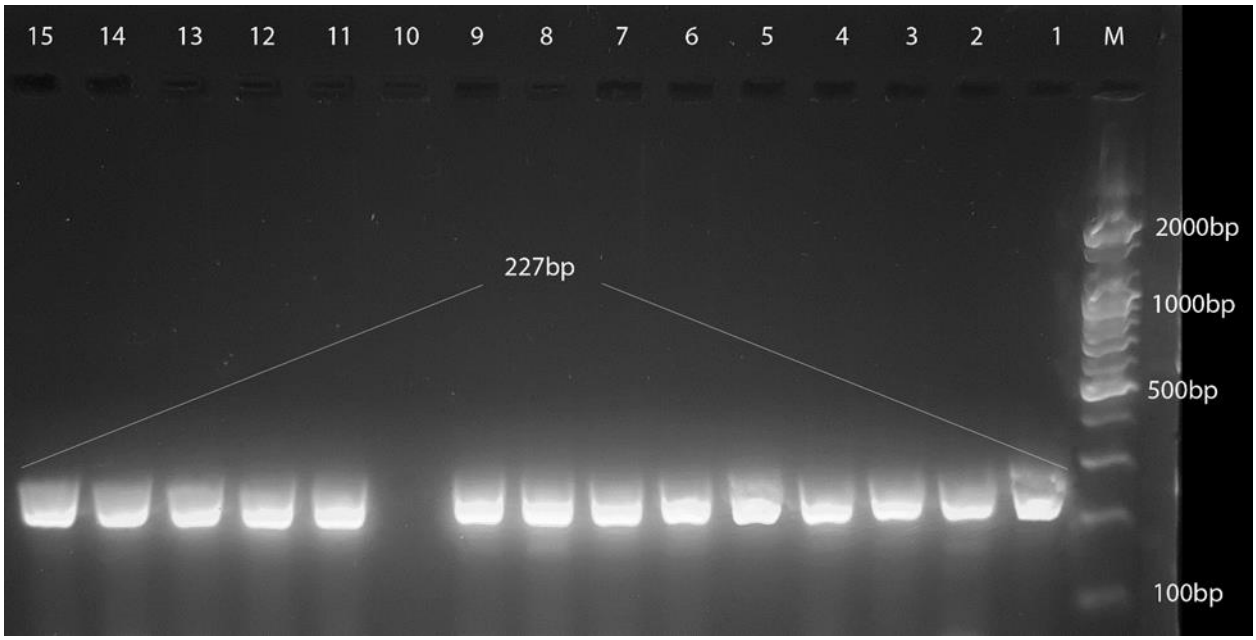

Fig. (4): The electrophoresis of agarose gel of PCR products acquired with keratinase-. specific primers that generated 227bp. Lanes (1-9, and 11-15); represent the first positive .keratinase, Lane 10 is negative. Lane M is a 100bp DNA ladder

Discussion

Trichophytones belong to the few fungi that cause infectious disease Previously, most trichophytones strains had a limited geography; however, Dermatophytosis has recently emerged to be one of the most prevalent infectious disorders affecting people worldwide. with a worldwide distribution. Trichophyton are not easy to identify based on clinical signs and symptoms.Trichophyton spp are also more complex to diagnose in immunocompromised people because their clinical presentations are frequently unusual.

Atopic dermatitis, erythrasma, psoriasis, Eczema and Seborrheic dermatitis are additional diseases with a challenging differential diagnosis from dermatophytoses.(Li *et al* ., 2021)

The objective of the current work was to create and evaluate a multiplex PCR approach that allowed the simultaneous identification of trichopyton spp .

The goal of the research was to create and evaluate a multiplex PCR approach that would enable simultaneous identification of three major pathogenic trichophyton species in a working day. For this procedure, the preparation of DNA from culture isolates would take two hours, PCR amplification would take two hours, and electrophoresis would take an hour. Because they are the species of human trichophyton, T. rubrum, T. mentagrophytes, and T. violacem are typically isolated .(Ebrahimi *et al* .,2019)

Culture failed to find any mixed trichophytone infections in the study's sample material. Sampling variance is a more likely explanation for this result because both trichophytones needed multiple samples in order to thrive in the culture. Another argument is that in cultured samples of various trichophyton species, the more common trichophyton species will probably perform better than the less frequent ones.

The clinical symptom, macroscopic colony examination from culture, microscopic examination of both macro- and micro-conidia, biochemical characteristics, and crossculture were once used to classify and identify trichohytones. However, because this method of classification and identification takes time, it could be difficult for non-experts to tell apart

the morphology of cultured colonies. Additionally, even the identical strains can result in morphologically different colonies, making it more difficult to identify the causative agent. (Ji Young Kim *et al* .,2011)

If culture-positive samples are taken into account as true positive , a multiplex PCR test is just as effective as conventional diagnostic techniques. Several samples that were positive for trichopyton spp in culture were found to be negative for multiplex PCR. These negative results are likely due to the fact that trichophytosis-causing agents in these samples came from species that weren't considered in this multiplex PCR. Another possibility might be that the positive compound was absent from the subsample kept for molecular analysis. It is important to highlight that such sample division issues have long been recognized as a potential problem in dermal mycology analysis (Aylin *et al* ., 2008)

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As a result, assessing the examination of the infection route, the differentiation between reinfection and relapse, the change of trichohytones, and the geographic distribution of strains is problematic. Several initiatives are currently being undertaken to generate a greater variety of primers and reaction conditions for the identification of trichophyton due to the simplicity and speed with which PCR (polymerase chain reaction) may be performed in common laboratories.

(Ji Young Kim *et al* .,2011)

So that . Fungal cells can be identified using as little as 1 pg of DNA because to the PCR method's high sensitivity and specificity. Multiplex PCR, which uses more than two primers for simultaneous amplification and is widely used in clinical microbiology, allows for the detection of several species from a single specimen. But when using the PCR method, it's crucial to select the primer and focus on the DNA that has to be amplified. When creating the primer, it is important to take into account the recurring base sequences that are shared by all dermatophytes, random base sequences with a high possibility of binding, and various specific base sequences found on a single chromosome. (Ebrahimi *et al* .,2019)

These characteristics cannot be utilized to distinguish one species from another due to the variability that the fungus can display in its morphologic characteristics with subcultures; therefore, more accurate methods, such as molecular tests, must be used.

In this investigation, we detected a genetic virulence factors for trichophyton species , This virulence factors was linked to an increased risk of skin and nail fungal infection .

In this study, found almost trichoytones species isolated have keratinase gene .Proteolytic enzymes necessary for the breakdown of keratinic waste products are produced by trichophyton spp . Keratin serves as the source of nitrogen and carbon for a large number of keratinophilic fungi, which live on keratinous substances. These fungi grow asexually and generate conidia. To penetrate the keratin substrates, boring hyphae are produced during the fungal colonization.

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So that , Trichophyton are known to infect keratinized tissues like skin, hair, and nails, hence their capacity to break down keratin is regarded as a key component of their virulence. An association between pathogenesis and keratinase activity .(Rebecca *et al* .,2012) .

it's probable that during infection, each species of trichophyton spp will have a different program for the development of virulence factors .

So that .In this Researchers have looked at the transcriptional response of trichophyton subjected to environmental conditions including pH variations in order to identify additional parameters that contribute to infection (Peres *et al* ., 2010).

Advanced, complex signal transduction networks in trichopytones enable them to adjust to pH changes and to stimulate the expression of a number of genes that let them survive in a variety of environmental situations.(Martins *et al* ., 2019)

The maintenance of infections of the skin growth depends greatly on the pH shift and the food source. (Martinez *et al* ., 2017)

Skin protein hydrolysis results in the release of amino acids like glycine, which helps shift the extra-cellular pH from acidic to alkaline circumstances. This promotes the colonization of dermatophytes and damages host tissue.(Silveira *et al* ., 2010)

The pathways for environmental adaptability as well as the transcriptional responses to food sources and pH differ. Moreover, strains that were obtained from patients could display various reactions with regards to the enzymatic activity profile . These variations might serve as factors that affect how frequently each species becomes infected (Gnat *et al* ., 2018)

Among the most commonly haemolysis-related enzymes is phospholipase, which hydrolyzes compounds found in common with cell membranes . Several Trichophyton species exhibit phospholipase activity (Elavarashi *et al* ., 2017)

In this study found all trichopyton isolated have phospholipase gene. The membrane disruption activities that take place during host cell invasion are believed to involve phospholipase. Phospholipases cause cell lysis by cleaving phospholipids, which destabilizes the membrane.

This enzyme's significance in the initial stages of trichophyton illness and fungus establishment has been confirmed. These enzymes might be crucial for maintaining a balance between the immune system of the host and trichophytones' immunosuppressive properties. (Rodwell *et al*.,2008)

Conclusion

The findings of the present study revealed that trichophytones are difficult to diagnose using clinical symptoms and common diagnostic techniques like culture

For the determination of trichopytones and pathogenicity, it is vital to rely on cutting-edge techniques like polymerase chain reaction. However, trichopytones spp isolates have phospholipase ,keratinase and transcription factor that increase risk of fungal infection .

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Not found

Conflicts of interest

There are no conflicts of interest.

Reference

-Abdulkawi Ali Al-Fakih . the Fungal Metabolites Involved in Mycopathy. Journal of Medical Microbiology.2014. Vol.4 No.1

-Aylin Ates, Kadri Ozcan .(2008) .Diagnostic value of morphological, physiological and biochemical tests in distinguishing Trichophyton rubrum from Trichophyton mentagrophytes complex, Macit Ilkit

Medical Mycology, Volume 46, Issue 8, , Pages 811–822.

-de Hoog et al. (2016). (mycology/fungal-descriptions-and-antifungal-susceptibility/dermatophytes/trichophyton#trichophyton-rubrum.

-Ebrahimi M, Zarrinfar H, Naseri A, Najafzadeh MJ, Fata A Parian M, et al. Epidemiology of dermatophytosis in northeastern Iran; A subtropical region. Curr Med Mycol.2019.

-Elavarashi, E., Kindo, A.J. and Rangarajan, S. (2017) Enzymatic and non-enzymatic virulence activities of dermatophytes on solid media. J Clin Diagn Res 11, 23–25 .

-Elife Berk , Semra Kuştimur, Ayşe Kalkancı, O Murat Oztaş . DNA extraction and identification of Trichophyton rubrum by real-time polymerase chain reaction from direct nail scraping specimens of patients with onycomycosis.2011 . Mikrobiyol Bul Jan;45(1):150-8

-Fekrazad, R., Poorsattar Bejeh Mir, A., Kahyaie Aghdam M., and Ghasemi Barghi, V. (2017) Comparison of photoinactivation

of T. rubrum by new methylene blue (NMB and indocyanine green (EmunDo®). Photodiagnosis Photodyn Ther 18: 208–212

-Gnat, S., Lagowski, D., Nowakiewicz, A., and Zieba, P. (2018). Phenotypic characterization of enzymatic activity of clinical dermatophyte isolates from animals with and without infection skin lesions and humans. J. Appl. Microbiol. 125, 700–709. doi: 10.1111/jam.13921

-Ji Young Kim, M.D., Yong Beom Choe, M.D., Kyu Joong Ahn, M.D., Yang Won Lee, M.D. Identification of Dermatophytes Using Multiplex Polymerase Chain Reaction . Vol. 23, No. 3, 2011 .

-Li, C., Jia, X., Bian, Y., Qi, D., and Wu, J. (2021) Different 1

susceptibility of spores and hyphae of Trichophyto

rubrum to methylene blue mediated photodynamic treatmen

in vitro. Mycoses 64: 48–54

-Martinez -Rossi, N. M., Peres, N. T. A., and Rossi, A. (2017). Pathogenesis of dermatophytosis: sensing the host tissue. Mycopathologia 182, 215–227. doi: 10.1007/s11046-016-0057-9

-Martins, M. P., Martinez-Rossi, N. M., Sanches, P. R., Gomes, E. V., Bertolini, M. C., Pedersoli, W. R., et al. (2019a). The pH signaling transcription factor PAC-3 regulates metabolic and developmental processes in pathogenic fungi. Front. Microbiol. 10, 2076. doi: 10.3389/fmicb.2019.02076

- Nilce M. Martinez-Rossi 1. J. Fungi 2021, 7(8), 629.

-Rebecca Rashid Achterman1and Theodore C. White . Dermatophyte Virulence Factors: Identifying and Analyzing Genes That May Contribute to Chronic or Acute Skin Infections . International Journal of Microbiology 2012

-Peres N.T, P. R. Sanches, J. P. Falco et al., "Transcriptional profiling reveals the expression of novel genes in response to various stimuli in the human dermatophyte Trichophyton rubrum," BMC Microbiology, vol. 10, article no. 39, 2010

- Rodwell GE, Bayles CL, Towersey L, Aly R. The prevalence of dermatophyte infection in patients infected with human immunodeficiency virus. Int J Dermatol. 2008;47(4):339–43.

- Ross IL, Weldhagen GF, Kidd SE. Detection and identification of dermatophyte fungi in clinical samples using a commercial multiplex tandem PCR assay. Pathology. 2020; 52(4):473-7.]. A unique profile has not been found for differentiating the three prevalent species of T. rubrum, T. interdigitale

-Silveira, H. C. S., Gras, D. E., Cazzaniga, R. A., Sanches, P. R., Rossi, A., and Martinez-Rossi, N. M. (2010). Transcriptional profiling reveals genes in the human pathogen Trichophyton rubrum that are expressed in response to pH signaling. Microb. Pathog. 48, 91–96. doi: 10.1016/j.micpath.2009.10.006