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ORIGNAL ARTICLE

Emergence of Antifungal Azole Resistance in the Fungal Strains of Tinea corporis, Tinea capitis, Tinea cruris and Tinea pedis from the Locality of Southern Punjab, Pakistan

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ABSTRACT

Background: Dermatophytes are the most common group of fungi causing fungal infections all over the world. They are classified into three main groups Trichophyton, Microsporum and Epidermophyton. Among these, Trichophyton has the highest prevalence rate (70-90%) as compared to the others. The global emergence of fungal infections is varied due to the socio-economic conditions throughout the world. Developing countries, like Pakistan, are facing an increase in the number of dermatophytoses, including frequent relapses and treatment failures.

Objectives: The study have been conducted to identify the emerging fungal species, the role of commonly available antifungals such as azoles including voriconazole, ketoconazole, fluconazole and amphotericin B were used to determine the drug resistance among these species.

Methodology: Nine groups of dermatophytes and non-dermatophyte fungi isolated from the patients of tinea corporis, tinea cruris, tinea capitis and tinea pedis infections were analyzed for phenotypic diversity, antifungal susceptibility and strains identification, was performed by cultural characteristics and microscopy.

Results: Nine groups of isolated fungal strains were identified as *Trichophyton interdigitale, Trichophyton mentagrophyte, Trichophyton rubrum* amongst dermatophytes class and *Aspergillus terreus, Aspergillus verisocolor, Aspergillus niger, Acretonium sordidulum* and *Acremonium sclerotigenum* of non-dermatophytes class.

Conclusion: The study revealed *Trichophytone interdigitale* group as more frequent dermatophytes. Whereas, among the antifungal drugs, fluconazole that targets the Erg 1 gene of ergosterol biosynthesis in fungi is less effective most common antifungal drugs available locally.

Keywords
Antifungal Drug Resistance, Azoles, Dermatomycosis, Filamentous fungi, Sensitive Phenotype, Sterol biosynthesis.

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INTRODUCTION

Fungal infections have a wide impact on global health. Fungi threats nearly a billion people suffer from superficial infections of skin hair and nail, 100 million people suffer from mucosal candidiasis,10 million people developed severe allergic reactions result in million deaths each year reported are all linked with fungal infections[1,2]. The worldwide death rate due to fungal infections is higher than malaria and breast cancer and is comparable to

Tuberculosis (TB) and HIV[3]. The infections caused by fungi are divided into three main groups: cutaneous, superficial and systematic mycosis[4]. Dermatomycosis is the most common in superficial mycosis reported worldwide. It is a well defined infection of the skin, hair and nails[5]. Based on location and mode of transmission, dermatophytes are further classified into three groups i.e. zoophilic, geophilic and anthropophilic associated with the animal, soil and human beings, respectively. The geophilic group including *Nannizzia gypsea*, *Epidermatophyton floccosum* and *Alternaria* is worldwide recognized as a common plant pathogen and airborne allergen. It is the typical aeroallergen species of the genus and in a majority of cases, the most frequent species associated with human and animal health problems. Similarly, the zoophilic group include *Microsporum canis, Trichophyton equinum, Trichophyton verrucosum, Trichophyton erinacei* and *Microsporum manum whereas, anthropophilic group* include *Trichophyton interdigitale, Trichophyton mentagrophyte, Trichophyton tonsurans, Trichophyton soudanense, Trichophyton megninii* and *Trichophyton violaceum*[6]. These species invade the skin due to their ability to digest keratin used as a substrate[7].

Dermatophytosis is a disease of overall significance and a general medical issue in numerous parts of the world, especially in developing countries[8, 9]. It is an increasing threat in immune-compromised individuals[10]. Increasing number of population, low financial status, and insufficient health condition and trading of inappropriately cleaned or used foot-wears, garments, and barbershop supplies etc. among individuals have been perceived as potential hazardous factors for the multiplication of the disease[11]. The types of fungi *Epidermophyto*n, *Microsporum*, *Trichophyton*, non-dermatophyte molds and yeast have been considered as significant cause of the mycosis[12]. The worldwide weight of cutaneous contamination was evaluated to be ~1,001,000,000[13]. These contaminations are more typical among rural than urban population, and the disease, *tinea capitis* is reported to be more prevalent in males[14]. Literature has indicated that the worldwide burden of dermatophyte disease explicitly was evaluated to be 20-25%[14].

There are fewer studies conducted on antifungal drug resistance and on fungal stress response. Therefore, to this end, investigating human dermatophytosis causing superficial mycosis appears to be one of the priorities in health-related studies. The aim of the study is to investigate the more invasive and frequent types of fungal infections and the fungal species involved, and to find their possible resistance against current azoles and amphotericin B antifungals.

MATERIAL & METHODS

Sample Collection

Initially, 74 samples were collected from suspected patients of dermatophytosis at the Civil hospital Bahawalpur. Before the collection of skin scrapings and scalp samples, infection site was cleaned with 70% ethanol. The hair were removed from the scalp in case of infection of *tinea capitis* and nails were scraped and clipped from *tinea unguium* patients as previously described[15]. Lactophenol cotton blue staining was used for the microscopic observation of dermatophyte.

Culture Media

The samples were cultured on sterile Sabouraud dextrose Agar (SDA), fungal specific medium containing chloramphenicol (0.5g/L) which inhibits most of the bacterial growth, and cycloheximide (0.4g/L) that inhibits saprophytic fungi, and plates were incubated at 30°C for 7 days. Spores were collected from the SDA slant and spore suspension was prepared in 2ml sterilized water. Spores collection was done after oscillating at vortex and transferred into the 2ml sterilized tubes. They were then centrifuged at 4000rpm for 2min. Supernatant was discarded and 200ml sterilized water was added into conidial suspension. Conidial suspension (2µl) was transferred into 2ml tube adding sterilized H_2O to reach up to 2×10^6 conidial suspension[16, 17].

Identification of agents causing Dermatophytosis using Microscopy

All the isolates were undertaken for the phenotypic examinations. The conidial suspension was prepared as previously describes by Samuel *et al*[18]. Conidia were examined under the light microscope at 100X magnification.

Identification of agents causing Dermatophytosis by ITS Region

The DNA of these isolates was extracted using the method described[19]. Species identification was done using fungal universal primers for intra transcribed spacer regions (ITS1), The forward and reverse primers used (Forward primer: ITS1 (5'TCCGTAGGTGAACCTGCGG3') and the (Reverse primer: ITS4 (5'TCCTCCGCTTATTGATATGC-3') was used for PCR (Mygene L Series Peltier Thermal Cycler by UniEquip. The PCR cycling conditions were 35 cycles of 95ºC for 1 min, 55ºC for 1 min, and 72ºC for 2 min, followed by an extension step of 72ºC for 10 min[20]. Intra transcribed spacer regions are called the fungal bar code they are non-coding regions which are considered the most standardized nucleotide regions to identify the fungal taxonomy at species levels. Amplified product was confirmed by running on 2% agarose gel electrophoresis. Amplified PCR products were sent for Sanger sequencing to Beijing Institute of Genomics (BIG). The sequences obtained were analyzed by nucleotide NCBI blast and aligned by multiple sequence alignment tool (Clustal W and MEGA6).

Antifungal Drug Susceptibility

Sabouraud dextrose agar (SDA) plates were prepared using antifungal drugs stock solutions: 2mg/ml ketoconazole, 10mg/ml fluconazole 10mg/ml amphotericin B, and 5mg/ml voriconazole. Drugs were dissolved in Dimethyl sulfoxide (DMSO) and added to autoclaved media before pouring onto the petri plates. The concentrations of drugs such as ketoconazole, fluconazole, amphotericin B and voriconazole selected according to their Minimum inhibitory concentration (MIC) points. The drug sensitivity was conducted on petri plates and inoculated with 2µl spores (2 x 10^6/ml) conidial dilution and incubated at 30ºC for 7 days. The control was inoculated with no drug. Each sensitivity test has been done three times, independently.

Oxidative Stress

The investigation was conducted to identify the effect of stress generated by different chemical compounds individually. Firstly, the effect of Hydrogen peroxide (H_2O_2) was tested by preparing 2, 4 and 6mM concentrations of hydrogen peroxide and added in SDA media for identification of the effect of H_2O_2 on the growth of the dermatophytes. The media (20ml) was poured in each petri plate and 2µl spore suspension was inoculated and incubated at 30ºC for 7 days. The growth after 4 days on different concentration hydrogen peroxide in media and control was compared.

Similarly, different concentrations of benzoic acid (2, 4 and 6mM) were used in SDA media for identification of the phenotypic effect on the growth of the dermatophytes. The stress responses with growth differences was considered at 6mM benzoic acid (dissolved in DMSO). Twenty milliliter (20ml) media was poured in each petri plate, and 2µl conidial suspension was added and incubated at 30ºC for 7 days. Fungal phenotype on different concentration of benzoic acid media and control was compared. Each test has been done three times independently.

Lastly, stress effect of sodium chloride (NaCl) was examined using 4% NaCl which was adjusted in the media. Conidial suspension (2µl) was inoculated on the media plates and the growth of dermatophytes and non-dermatophytes on SDA medium was observed. The plates were incubated at 30ºC for 7 days and the effect of sodium chloride on fungal species was observed by comparing with the controls. Each test has been done three times independently.

Enzymatic Activity

The Protease activity was performed as described previously[21]. For this purpose, a medium was prepared that contain contained dextrose 2%, potassium dihydrogen phosphate 0.1%, magnesium phosphate 0.05% and agar 2%. The media was sterilized and cooled up to 50ºC and 1% Bovine serum albumin (BSA) was added. The enriched medium was mixed thoroughly and poured in a sterile petri plates. The spore suspension (2µl) was inoculated and incubated at 30ºC for 7 days. The control was inoculated with no BSA in the media. Each test has been done three times independently. *Candida albicans* was used as a positive control. A clear zone around the colony indicated protease production.

RESULTS

A total of 74 clinically diagnosed cases of dermatophytosis were studied from Bahawalpur. The age group in this study ranged from 1-50 years. The most common age group affected was 21-30 years (Table **1**). The highest

percentage of patients were found to have the infection of Tinea corporis 39% followed by tinea capitis 20%, tinea cruris 18%, tinea ungium 11%, tinea faciei 5%, tinea pedis 3%, tinea manum 3%, tinea versicolor 1% as mentioned in (Fig. **1**). The tinea corporis (39%) was the most common of all tinea patients included in this study. All 74 samples of skin, scalp scrapings and hairs were grown on SDA media containing cycloheximide and chloramphenicol.

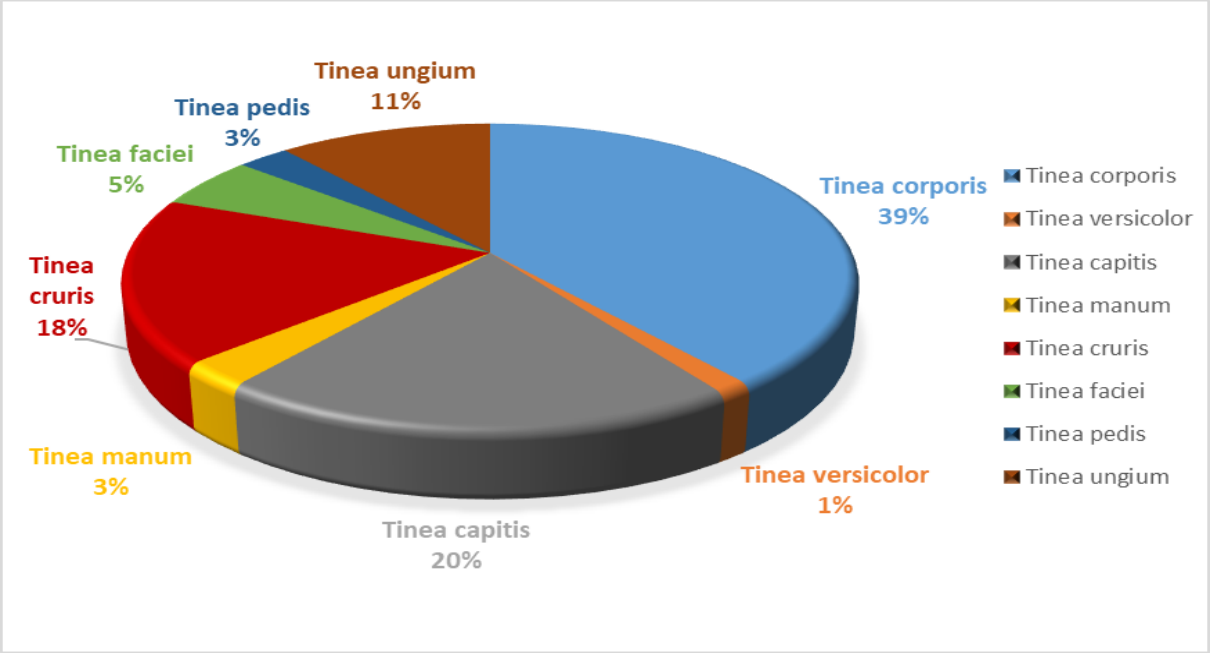

Figure 1. Pie-chart of diseases caused by dermatophyte. Infection due to *tinea corporis* has the highest percentage of 39% followed by tinea capitis 20%, tinea cruris 18%, tinea ungium 11%, tinea faciei 5%, tinea pedis 3%, tinea manum 3%, tinea versicolor 1%.

Table 1. Age and Sex-wise Distribution of Dermatophytosis in Clinical Samples.

S. No.	Clinical Types	Age Group (In Years)					Sex		Total	%age
		0-10	11-20	21-30	31-40	41-50	Male	Female		
1.	*Tinea corporis*	3	3	11	9	3	12	17	29	39%
2.	*Tinea unguium*	-	2	5	-	1	2	6	8	11%
3.	*Tinea capitis*	14	-	-	1	-	5	10	15	20.2%
4.	*Tinea cruris*	-	1	5	6	1	3	10	13	17.5%
5.	*Tinea faciei*	2	1	1	-	-	1	3	4	5.4%
6.	*Tinea pedis*	-	-	1	1	-	1	1	2	2.7%
7.	*Tinea versicolor*	-	-	1	-	-	-	1	1	1.3%
8.	*Tinea. Manum*	1	-	1	-	-	-	2	2	2.7%
	Total	22	7	26	18	7	24	50	74	100%

The results showed the highest percentage of tinea corporis infection (39.3%) as compared to others. Females are affected more than males and 21-30 years is a more common age group found.

Macroscopic and Microscopic Identification of Dermatophytes

Fungal isolates were identified on the basis of macro-morphology (forward and reverse color) and micro-morphology (microconidia and macroconidia). Dermatophytes and non-dermatophyte isolates were separated on the basis of color, shape and number of micro and macroconidia such as *Trichophyton mentagrophytes* (yellow brown to reddish brown colony with numerous microconidia and hyaline macroconidia), *Trichophyton interdigitale* (White to brown colour become reddish brown with age from reverse pigment with pyriform microconidia); non-dermatophyte causing dermatophytosis like *Acremonium sclerotigenum* (white center with large hypha) and *Aspergillus versicolor* showed various color from orange yellow to tan green and penicilli-like conidia. Among all the collected fungal samples, nine different species *(Trichophyton mentagrophyte*, *Trichophyton interdigitale, Trichophyton rubrum, Aspergillus terreus, Alternaria alternata, Aerentonium sordidulum, Alternaria mean versicolor* and *Acremonium sclerotigenum* were identified **(**Table **2)**.

Identification from PCR and DNA Sequencing

Dermatophytes and non-dermatophytes isolates were identified by using PCR for amplification of ITS1 and ITS4 region on the DNA gel electrophoresis (Fig**. 2**). Seventeen isolates including one from each fungal group and eight unidentified fungal species were selected for further verification. Identified fungal sub groups were selected and sequenced on Sanger sequencing. DNA sequencing was run on BLAST. All the strains are mentioned with percentage (%) identification by BLAST and with accession number provided in (Table **2)**. The sequence of nine species identified from BLAST were aligned by Clustal W. Phylogenetic tree was prepared based on nine different species of fungal strains (Fig**. 3**).

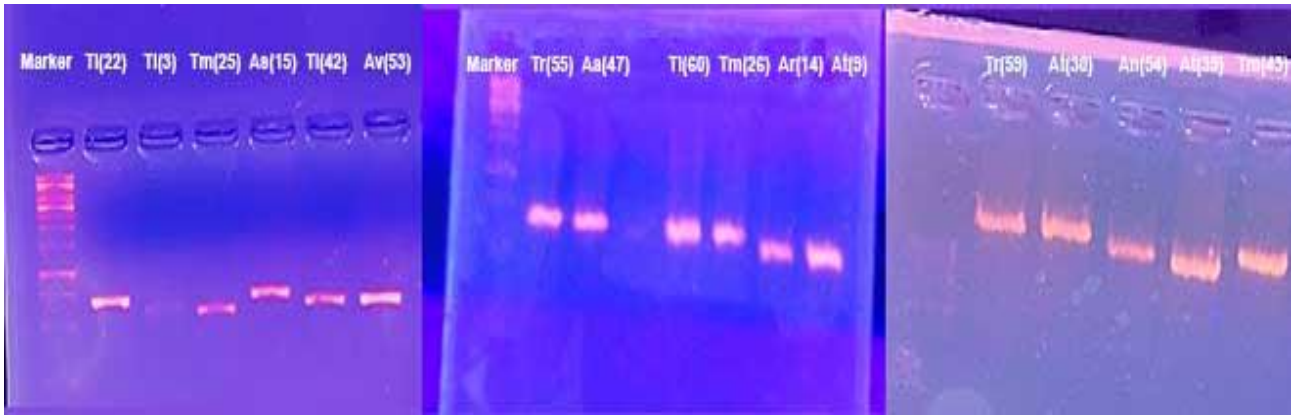

Figure 2. ITS gene PCR amplifications on gel electrophoresis.

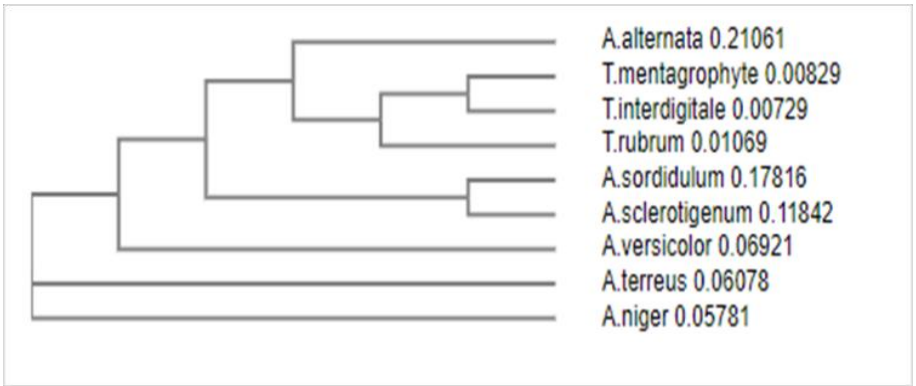

Figure 3. Phylogenetic tree of 9 representative dermatophytes and non-dermatophyte species based on analysis of ITS1 region sequences. The evolutionary history was inferred using the Neighbor–Joining (NJ) method based on the Tamura–Nei model.

Table 2. DNA Sequence of Eight Dermatophytes and Non-Dermatophytes Aligned on NCBI Gene bank.

S. No.	Strain No.	Clinical Types	Identification According to ITS Sequence	Percentage % Identification by BLAST	Accession
1	Tm(25)	*Tinea corporis*	*Trichophyton mentagrophyte*	98.21%	MN661259.1
2	At(30)		*Aspergillus terreus*	99.48%	KY053121.1
3	Tr(55)		*Trichophyton rubrum*	99.69%	MN691068.1
4	Tm(43)		*Trichophyton mentagrophyte*	99.53%	MN661259.1
5	Ti(22)		*Trichophyton interdigitale*	99.70%	MH517559.1
6	Ti(3)		*Trichophyton interdigitale*	99.56%	MH517559.1
7	Tm(26)	*Tinea cruris*	*Trichophyton mentagrophyte*	98.22%	MN661259.1
8	An(54)		*Aspergillus niger*	99.65%	MG654699.1
9	Av(53)		*Alternaria versicolor*	100%	MH712290.1
10	Ti(42)		*Trichophyton interdigitale*	99.44%	MT497400.1
11	Ti(60)		*Trichophyton interdigitale*	100.00%	MN178659.1
12	At(39)	*Tinea capitis*	*Aspergillus terreus*	99.34%	MN099077.1
13	Aa(47)		*Alternaria alternata*	88.89%	GU004283.1
14	At(9)		*Aspergillus terreus*	100.00%	KF971363.1
15	Tr(59)		*Trichophyton rubrum*	100.00%	MN176601.1
16	Ar(14)		*Aerentonium sordulum*	87.67%	MK513818.1
17	As(15)	*Tinea pedis*	*Acremonium sclerotigenum*	99.65%	MK732096.1

Result showed nine different species of fungi causing dermatophytosis. Four group of tinea infection has been focused. ITS sequence analysis has been used for the identification of four genera of fungi belong to species of Aspergillus, Trichophytone group, *Alternaria alternate*, *Acrentonium sordulum* and *Acremonium sclerotigenum*.

Antifungal Drug Susceptibility

In this study, all the isolates form tinea corporis, tinea cruris, tinea capitis and tinea pedis were tested for drug sensitivity of fluconazole (10µg/ml), ketoconazole (2µg/ml), voriconazole (3µg/ml) and amphotericin B (2µg/ml). Only one isolate (Ti3) tinea corporis showed resistance against fluconazole at 10µg/ml while others were sensitive against ketoconazole, voriconazole and amphotericin B. One isolate (Av53) from tinea cruris showed resistance against fluconazole and amphotericin B at 10µg/ml and 2µg/ml, respectively. Tinea capitis (Tr59) showed resistance against fluconazole and amphotericin B. The isolate of tinea pedis showed inhibition against all the drugs used in this study **(**Table **3.1, 3.2** and **3.3)**. The minimum inhibitory concentrations of nine different species of dermatophyte and non-dermatophyte isolates of tinea corporis, tinea cruris, tinea capitis and tinea pedis were carried out. Results showed that fluconazole is the least effective antifungal drug in contrast to ketoconazole which is highly effective drug against dermatophyte and non-dermatophyte **(**Table **4)**.

Fungal Adaptations to Oxidative Stresses

The effect of hydrogen peroxide on the growth of dermatophyte and non-dermatophyte species isolated from tinea corporis, tinea cruris, tinea capitis and tinea pedis infections have been studied. The growth of all isolates were inhibited at 6mM H_2O_2 except *Aspergillus terreus* (At30) and *Trichophyton interdigitale* (Ti60) belongs to tinea corporis and tinea cruris, which showed no inhibition in growth and adapted hydrogen peroxide stress. In order to conduct the fungal oxidative stress response, benzoic acid sensitivity test was also conducted. Benzoic acid stress inhibited the growth of all isolates except *Acremonium sclerotigenum* (As15), *Aspergillus terreus*

(At30) and *Acrentonium sordidulum* (Ar14) from tinea pedis, tinea corporis and tinea capitis, respectively at 6mM. However, the effect of salt stress on 4% sodium chloride inhibited the growth.

However, the effect of salt stress on 4% sodium chloride inhibited the growth of all isolates except *Aspergillus terreus* (At30) isolated form tinea corporis and *Trichophyton interdigitale* (Ti60) isolated from tinea cruris. Oxidative stress affect the cellular pH that lead to the alterations in metabolic regulations, which arrest the cell growth in the fungal isolates. However, the isolates of *Aspergillus terreus* (At30) form tinea corporis and *Trichophyton interdigitale* (Ti60) from tinea cruris tolerated the salt stress due the possible adaptations as **(**Table **5).**

Table 3.1. Antifungal Drug Susceptibility against Different Dermatophytes and Non Dermatophytes

S. No.	Strain No.	Clinical Types	Species	VOR	KTC	FLU	AMP
1	Tm26		*Trichophyton mentagrophytes*	Sensitive	Sensitive	Similar growth	Resistance
2	An54		*Aspergillus niger*	Sensitive	Sensitive	Sensitive	Similar growth
3	Ti60		*Trichophyton interdigitale*	Sensitive	Sensitive	Similar growth	Similar growth
4	Ti35		*Trichophyton interdigtale*	Sensitive	Sensitive	Similar growth	Similar growth
5	Tm42		*Trichophyton mentagrophyte*	Sensitive	Sensitive	Similar growth	Similar growth
6	Av53	*Tinea cruris*	*Aspergillus versicolor*	Sensitive	Sensitive	Similar growth	Resistance
7	Ti33		*Trichophyton interdigitale*	Sensitive	Sensitive	Sensitive	Sensitive
8	28		*No growth*	-	-	-	-
9	49		*No growth*	-	-	-	-
10	52		*No growth*	-	-	-	-
11	65			Sensitive	Similar growth	Sensitive	Sensitive
12	56		*No growth*	-	-	-	-
13	57		*No growth*	-	-	-	-
1	69		*Trichophyton rubrum*	Sensitive	Sensitive	Sensitive	Sensitive
2	Ti73		*Trichophyton interdigitale*	Sensitive	Sensitive	Resistant	Sensitive
3	Ti74	*Tinea faciei*	*Trichophyton interdigitale*	Sensitive	Sensitive	Sensitive	Sensitive
4	76		*Microsporum canis*	Resistant	Sensitive	Sensitive	Resistant
1	Tm11		*Trichophyton mentagrophytes*	Similar growth	Sensitive	Similar growth	Sensitive
2	Ca20		*Candida albicans*	Sensitive	Sensitive	Similar growth	Sensitive
3	Ca23	*Tinea unguium*	*Candida albicans*	Similar growth	Sensitive	Sensitive	Sensitive

Contd…

4	13	*No growth*	-	-	-	-
5	37	*No growth*	-	-	-	-
6	38	*No growth*	-	-	-	-
7	45	*No growth*	-	-	-	-
8	51	*No growth*	-	-	-	-

Drug susceptibility against different species of dermatophyte has been presented on basis of phenotypic growth as comparison with control.

Table 3.2. Antifungal Drug Susceptibility against Trichophyton, Alternaria and Aspergillus species isolated from tinea corporis infection.

S. No.	Strain No.	Clinical Types	Species	VOR	KTC	FLU	AMP
1	Ti3		*Trichophyton interdigitale*	Sensitive	Sensitive	Sensitive	Sensitive
2	Tm19		*Trichophyton mentagrophytes*	Similar growth	Sensitive	Similar growth	Sensitive
3	Ti30		*Trichophyton interdigitale*	Sensitive	Sensitive	Sensitive	Sensitive
4	Ti21		*Trichophyton interdigitale*	Sensitive	Sensitive	Similar growth	Sensitive
5	Ti22	*Tinea corporis*	*Trichophyton interdigitale*	Sensitive	Sensitive	Sensitive	Sensitive
6	Ti46		*Trichophyton interdigitale*	Similar growth	Sensitive	Sensitive	Sensitive
7	Tm25		*Trichophyton mentagrophytes*	Sensitive	Sensitive	Sensitive	Sensitive
8	Aa41		*Alternaria alternata*	Sensitive	Sensitive	Sensitive	Sensitive
9	70		*Trichopyton rubrum*	Sensitive	Sensitive	Similar growth	Sensitive
10	Av43		*Aspergillus versicolor*	Sensitive	Sensitive	Sensitive	Sensitive
11	Ti58		*Trichophyton interdigitale*	Similar growth	Sensitive	Similar growth	Sensitive
12	Ti34		*Trichophyton interdigitale*	Sensitive	Sensitive	Sensitive	Sensitive
13	Ti59		*Trichophyton interdigitale*	Similar growth	Sensitive	Sensitive	Sensitive
14	8		*No growth*	-	-	-	-
15	10		*No growth*	-	-	-	-
16	18		*No growth*	-	-	-	-

Contd…

17	27		*No growth*	-	-	-	-
18	77		*Trichophyton. rubrum*	Sensitive	Sensitive	Similar growth	Resistant
19	29		*No growth*	-	-	-	-
20	32		*No growth*	-	-	-	-
21	33		*No growth*	-	-	-	-
22	36		*No growth*	-	-	-	-
23	40		*No growth*	-	-	-	-
24	44		*No growth*	-	-	-	-
25	50		*No growth*	-	-	-	-
26	55		*No growth*	-	-	-	-
27	61		*No growth*	-	-	-	-
28	64		*Trichophyton interdigitale*	Similar growth	Similar growth	Sensitive	Sensitive
29	67		*Trichophyton interdigitale*	Similar growth	Similar growth	Resistant	Sensitive

Results has been presented on bases of phenotypic growth in comparison with control.

Table 3.3. Antifungal Drug Susceptibility against Different Species of Dermatophytes and Non Dermatophytes.

S. No.	Strain No.	Clinical Types	Species	VOR	KTC	FLU	AMP
1	At39		*Aspergillus terreus*	Sensitive	Sensitive	Sensitive	Sensitive
2	An48		*Aspergillus niger*	Sensitive	Sensitive	Similar growth	Resistant
3	Aa47	*Tinea capitis*	*Aspergillus alternata*	Sensitive	Sensitive	Sensitive	Similar growth
4	At9		*Aspergillus terreus*	Sensitive	Sensitive	Sensitive	Sensitive
5	Aa1		*Aspergillus alternata*	Sensitive	Sensitive	Sensitive	Sensitive
6	Aa2		*Aspergillus alternata*	Sensitive	Sensitive	Sensitive	Sensitive
7	Tr55		*Trichophyton rubrum*	Sensitive	Sensitive	Similar growth	Resistant
8	As14		*Aspergillus sordidulum*	Sensitive	Sensitive	Sensitive	Sensitive
9	17		*No growth*	-	-	-	-
10	24		*No growth*	-	-	-	-
11	62		*Trichophyton mentagrophyte*	Sensitive	Sensitive	Sensitive	Sensitive
12	63		*Aspergillus terreus*	Resistant	-	Resistant	Sensitive
13	71		*Trichophyton interdigitale*	Sensitive	Sensitive	Sensitive	Sensitive
14	72		*Trichophyton mentagrophyte*	Sensitive	Similar growth	Sensitive	Sensitive
15	78		*Candida albicans*	Sensitive	-	Similar growth	Sensitive
1	As15	*Tinea pedis*	*Aspergillus sclerotigenum*	Sensitive	Sensitive	Sensitive	Sensitive

Contd…

2	68		*Trichophyton rubrum*	Sensitive	Similar growth	Similar growth	Resistant
1	An4	*Tinea versicolor*	*Aspergillus niger*	Sensitive	Sensitive	Similar growth	Similar growth
2	66	*Tinea manum*	*Trichophyton mentagrophyte*	Sensitive	Similar growth	Similar growth	Sensitive
3	75		*Trichophyton rubrum*	Similar growth	-	Resistant	Sensitive

Drug susceptibility against **nine different species of dermatophyte and non-dermatophyte** has been presented on the basis of phenotypic growth as comparison with control.

Table 4. The MIC Pattern of Nine Different Species of Dermatophytes and Non-Dermatophytes.

S. No.	Strains	No.	MIC (Minimum Inhibitory Concentration)			
			FLU	KTC	AMB	VOR
1	*Trichophyton interdigitale*	4	>10	1.4	>2	1.8
2	*Trichophyton mentagrophyte*	3	>10	2	>2	1.4
3	*Trichophyton rubrum*	2	>10	1.8	>2	1.4
4	*Aspergillus terreus*	3	>10	1	>2	2
5	*Acretonium sclerotigenum*	1	>10	1.4	>2	1
6	*Aspergillus versicolor*	1	8	1	2	2
7	*Acrentonium sordidulum*	1	>10	2	2	3
8	*Alterneria alternata*	1	>10	>2	>2	3
9	*Aspergillus niger*	1	>10	2	>2	2

The MIC (Minimum inhibitory concentration) of nine different species of dermatophytes and non-dermatophytes obtained using FLU (Fluconazole), KTC (Ketoconazole), AMB (Amphotericin B), VOR (Voriconazole). MIC has been conducted using CLSI, 2020 guideline.

Table 5. Comparison of Effect of Oxidative Stress (H_2O_2, Benzoic acid and NaCl Stress) in Dermatophytes with Controls.

S. No.	Strains	Clinical Types	Identified Species	Effect of Stress on Growth Compared to Control		
				Benzoic Acid 6mM	H_2O_2 6mM	NaCl 4%
1	Ti(3)	*Tinea corporis*	*Trichophyton interdigitale*	Sensitive	Sensitive	Sensitive
2	Tm(25)		*Trichophyton mentagrophyte*	Sensitive	Sensitive	No effect
3	At(30)		*Aspergillus terreus*	No effect	No effect	No effect
4	Tr(55)		*Trichophyton rubrum*	Sensitive	Sensitive	Sensitive
5	Tm(43)		*Trichophyton mentagrophyte*	Sensitive	Sensitive	Sensitive
6	Ti(22)		*Trichophyton interdigitale*	Sensitive	Sensitive	Sensitive

Contd…

7	Tm(26)		*Trichophyton mentagrophyte*	Sensitive	Sensitive	Sensitive
8	An(54)		*Aspergillus niger*	Sensitive	Sensitive	Sensitive
9	Ti(60)	*Tinea cruris*	*Trichophyton interdigitale*	No effect	No effect	No effect
10	Ti(42)		*Trichophyton interdigitale*	Sensitive	Sensitive	Sensitive
11	Av(53)		*Aspergillus versicolor*	Sensitive	Sensitive	Sensitive
12	At(39)		*Aspergillus terreus*	Sensitive	Sensitive	Sensitive
13	Aa(47)		*Alternaria alternate*	Sensitive	Sensitive	Sensitive
14	At(9)	*Tinea capitis*	*Aspergillus terreus*	Sensitive	Sensitive	Sensitive
15	Tr(59)		*Trichophyton rubrum*	Sensitive	Sensitive	Sensitive
16	Ar(14)		*Acretonium sordidulum*	No effect	Sensitive	Sensitive
17	As(15)	*Tinea pedis*	*Acremonium sclerotigenum*	No effect	Sensitive	Sensitive

Oxidative stress of H_2O_2, benzoic acid and NaCl on fungal isolates isolated from *Tinea corporis*, *Tinea cruris*, *Tinea capitis* and *Tinea pedis* has been conducted in comparison to controls.

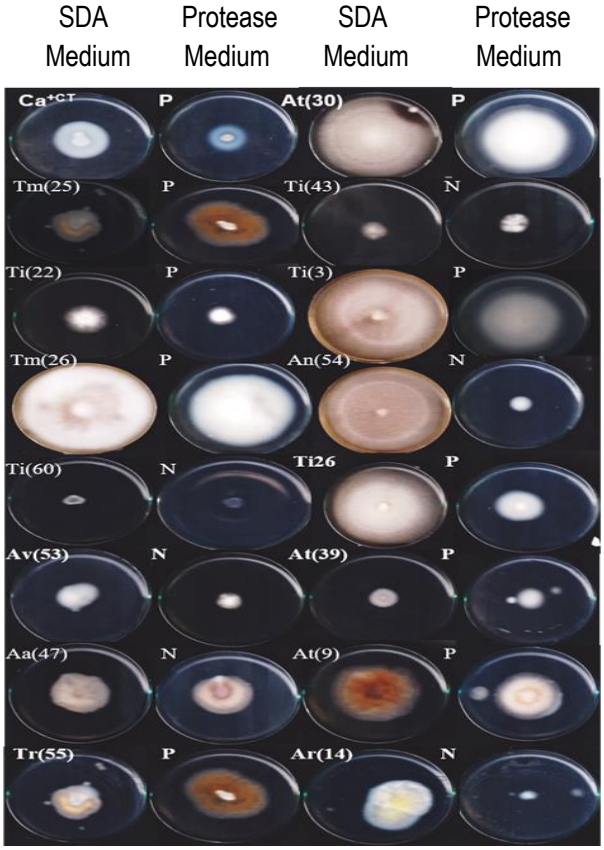

Figure 4. Protease activity on Bovine serum albumin (BSA) medium gave a clear halo zone around the colony and indicated protease production {P (Protease production), N (No protease production)} *Ca*$^{+CT}$ (*Candida albicans)* as + ve control.

Enzymatic Activity

In order to know whether fungal pathogenicity depend on enzymatic secretions (particularly protease enzyme during dermatophytosis), the enzyme activity such as protease was analyzed for all isolates on protease media, the result showed that dermatophyte and non-dermatophyte species such as *Trichophyton mentagrophyte* (25), *Aspergillus terreus* (30), *Trichophyton interdigitale* (22), *Trichophyton interdigitale* (3) The non-dermatophytes like *Alternaria alternate* (47) and *Aspergillus terreus* (9) isolated from tinea capitis produced protease enzyme **(**Fig**. 4**).

DISCUSSION

Dermatophytosis annually affects millions of people all over the world. The prevalence of dermatophytosis has been enhanced in immuno-compromised patients suffering from diseases like diabetes mellitus, AIDS, morbid obesity diseases, and transplant patients also reported by Pierard *et.al*[22]. Antifungal drug-resistance has become a major hurdle in the treatment of fungal infection. This study was conducted in Bahawalpur, Pakistan. Clinically diagnosed fungal isolates were collected. The fungal infections found to be common in the age group of 21-30 years and more frequent in female as compared to male. *Tinea corporis* was found as the most common type of infection (35%), followed by tinea cruris (29%), tinea capitis (30%), and tinea pedis (6%). Initially, phenotypic identification was done on the basis of color of colony and shape of microconidia and macroconidia such as *Trichophyton mentagrophytes* (yellow brown to reddish brown colony with numerous microconidia and hyline macroconidia), *Trichophyton interdigitale*, (white to brown colour that become reddish brown with age) with pyriform microconidia) and non-dermatophytes causing dermatophytosis like *Acremonium sclerotigenum* (white center and thin hyphae) and *Aspergillus Versicolor* (color varies from orange yellow to tan green)[23]. In addition, nine different species including *Trichophyton interdigitale*, *Trichophyton mentagrophyte*, *Aspergillus versicolor*, *Trichophyton rubrum, Aspergillus terreus, Aspergillus niger, Alternaria alternata, Acrenotinum sordidulum* and *Acremonium sclerotigenum* were identified by sequencing ITS region[24]. The results shown similarity with the previous studies in which dermatophyte species (*Trichophyton interdigitale, Trichophyton mentagrophyte* and *Trichophyton rubrum*) non-dermatophyte species (*Aspergillus versicolor, Aspergillus terreus, Aspergillus niger, Alternaria alternata, Acrenotinum sordidulum* and *Acremonium sclerotigenum*) caused dermatophytosis in human beings[25]. The sequence of nine different species among 17 of dermatophyte and non-dermatophyte causing dermatophytosis were aligned by Clustal W. For the phylogenetic tree the isolates of common fungal group were omitted. Phylogenetic tree relationship revealed the homology between identified strains of dermatophytes and non-dermatophytes[26].

Antifungal drug susceptibility tests showed only two fungal groups (*Aspergillus versicolor* (Av53) and *Trichophyton rubrum* (Tr59) non-responsive against Amphotericin B, three isolates (*Trichophyton interdigitale*) Ti3, (*Trichophyton rubrum*) Tr59 and *Aspergillus versicolor*) Av53 were resistant against fluconazole probably due to the possible mutation in the fluconazole drug target gene squalene epoxidase. All other isolates were sensitive against voriconazole and ketoconazole due to their potential broad spectrum of the molecular target. Such as the azoles target cytochrome P-450 dependent enzymes in fungi. Inhibition of the membrane bound enzymes of P450 family accumulate the toxic intermediate such as lanosterol, eburicol and the toxic 14α-methyl-3,6-diol, which reduces the permeability of cell membrane and inhibit the growth of fungi[27, 28].

In order to examine the adaptability of the oxidative stress (Hydrogen peroxide, Benzoic acid and NaCl stress), 17 various fungal groups were analyzed at a concentration of 6mM. The results showed that all the isolates except *Aspergillus terreus* (At30) and *Trichophyton interdigitale* (Ti60) were inhibited against hydrogen peroxide under oxidative stress. The mechanism of hydrogen peroxide involved in the cytotoxicity in oxygen reduction which generates more reactive and cytotoxic oxygen species such as the hydroxyl radical (•OH) which is a powerful oxidant and can initiate oxidation and cause damage to nucleic acids, proteins, and lipids. These results were similar to the previous study[28] in which most of the isolates of dermatophytes shown inhibition against hydrogen peroxide. However the oxidative stress of benzoic acid inhibited at 6mM benzoic acid except *Acremonium sclerotigenum* (As15), *Trichophyton interdigitale* (Ti60) and *Aspergillus terreus* (At30), which does not show any effect

on growth. The mechanism of benzoic acid stress induce the accumulation of benzoic acid in cells, which decrease the cytoplasmic pH of the cell and lead to cell death[29]. The reason for their resistance against benzoic acid should be their adoption to low pH[30]. A high concentration of sodium chloride in the environment may cause an intracellular imbalance in the Na^+/K^+ ratio resulting in a loss of potassium and a metabolic disturbance, which reduces the growth of dermatophytes[31]. This study showed the salt stress at high concentration diminished the fungal growth. Study identified that pH 5.6 is a suitable pH for the growth of dermatophyte (data not shown). The mechanism of pH alterations in bioprocess of cytoplasmic enzymes in microorganisms' effect on the ion uptake from the nutrient medium[32]. Protease enzyme essay was also examined and it was identified that dermatophyte and non-dermatophyte species such as *Trichophyton mentagrophyte* (25), *Aspergillus terreus* (30), *Trichophyton interdigitale* (22), *Trichophyton interdigitale* (3), *Alternaria alternate* (47) and *Aspergillus terreus* (9) produce protease enzyme, which helps in pathogenesis by breaking down the protein on a surface layer of the host and form the colony on the Stratum corneum of the skin. These results were identical to the study of Elavarashi *et al*[33].

CONCLUSION

Study identified tinea corporis as major cause of fungal infection, more common in the age group of 21-30 years, and the high prevalence rates were found in female as compared to male in the region of southern Punjab Pakistan. Selected isolates from nine fungal groups were further identified by the identification of ITS gene sequencing. The resistance to azole and polyene group was determined. Study revealed amphotericin B and fluconazole found to be the least effective against *Aspergillus terreus, Trichophyton mentagrophyte, Aspergillus niger, Aspergillus versicolor, Trichophyton rubrum* and *Trichophyton interdigitale.* The role of various oxidative stresses on fungal morphology was determined and found out that hydrogen peroxide and benzoic acid produce less stress on the growth and morphology of *Aspergillus terreus* and *Trichophyton metagrophyte.* Whereas, the 4% NaCl stress had not been tolerated by fungal isolates except *Aspergillus terrus* and *Trichophyton interdigitale* which reduced the cellular nutritional uptake and did not ponder the growth retardation. Thus, among the four groups of tinea infections, we concluded that in tinea corporis, *Aspergillus terreus* is more resistant to most of the stress responses and do not induce growth arrest in fluconazole and amphotericin B exposure and adapt to the oxidative stresses. The *Trichophyton interdigitale* isolated from tinea cruris infection have stress adaptations and do not induce growth retardation in physiological stress responses. In tinea capitis and tinea pedis *Acremonium sordidulum* and *Acremonium sclerotigenum* were non-responsive to benzoic acid stress, respectively. Fungal protease production concluded that *Aspergillus terrues, Trichophyton mentagrophyte, Trichophyton interdigitale* and *Alternaria alternate* excrete protease production for the possible adaptations to the oxidative stresses in tinea infections invading. Hence, this can be concluded that in dermatophytosis, fungi adapt to the physiological stress and excrete protease enzyme to invade on the human epidermal layer. Whereas, the adaptations of the antifungal azole in dermatophytes and non-dermatophytes may be due to the abnormal production of the sterol derivatives in fungal cell. Thus, it is suggested to analyze the possible abnormal production of fungal sterols particularly in *Aspergillus terreus* and *Trichophyton interdigitale*.

ETHICAL APPROVAL

The ethical approval was taken from Institutional Bioethical Research Committee (IBC), The Islamia University of Bhalwalpur, Pakistan.

CONFLICTS OF INTEREST

None.

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LIST OF ABBREVIATIONS

AMB	Amphotericin B
BSA	Bovine Serum Albumin
CLSI	Clinical Laboratory Standard Institute

DMSO	Dimethyl sulfoxide
FLC	Fluconazole
HIV	Human Immunodeficiency Virus
ITC	Itraconazole
ITS	Internal Transcribed Spacer
KTC	Ketoconazole
MIC	Minimum Inhibitory Concentration
SDA	Sabouraud Dextrose Agar
TB	Tuberculosis
VOR	Voriconazole

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