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ORIGINAL ARTICLE

Clinico-mycological profile and Antifungal Susceptibility Testing of Dermatophytic Fungi isolated from Clinical Samples in Zagazig University Hospital, Egypt

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ABSTRACT

Background*. Dermatophytes encompass over 40 species in three genera: Trichophyton Microsporum, as well as Epidermaphyton. Infections due to dermatophytes are generally known as "tinea" or "ring-worm" infections because of characteristic ringed lesions. Many antifungal agents can be used to treat these infections. Unfortunately, drug resistance can lead to treatment failure.* ***Objectives*** *of this study is exploration of clinico-mycological pattern of dermatophytic infections in patients with clinically suspected dermatophytosis attending the Dermatology Outpatient Clinic and characterization of antifungal susceptibility pattern for various dermatophytes species isolated from these patients.* ***Methodology:*** *A total of 62 dermatophytosis specimens (skin scrapings, nail, hair) were collected from 85 patients clinically suspected to have dermatophytosis. All the specimens were subjected to direct examination (10% KOH mount) and culture on Mycobiotic agar media then identification by macroscopic and microscopic characters. In vitro antifungal sensitivity testing to (fluconazole, ketoconazole, itraconazole, terbinafine and voriconazole) was done on species isolated from a culture with E. test method.* ***Results:*** *Dermatophytosis had a higher incidence in men (n=42, 68%) and more common in age group from (6-20 years) (40%). Tinea corporis was the most common clinical type 23 (37%), followed by tinea capitis 20 (32%). Sixty two isolated dermatophytes species were distributed as follows: T. mentagrophytes 20 (32%), T. rubrum 16 (26%), T.violaceum 14 (23%), T.schoenlinii 7 (11%), M.canis 5 (8%). Voriconazole and terbinafine were the most active antifungal agents, while fluconazole showed the least antifungal activity.* ***Conclusion:*** *highest incidence of cases falling in the 6–20 years old age group with male predominance. Tinea corporis was the most common clinical type followed by tinea capitis, tinea pedis, tinea ungums and tinea cruris. T.mentagrophytes was the most prevalent species isolated followed by T.rubrum, T. violaceum,T. Schoenlinii, and M.canis. Voriconazole showed notably low MIC values and was the most active azole against all dermatophytes isolates, followed by terbinafine, itraconazole ketoconazole. While fluconazole showed the least antifungal activity with high MICs values*

INTRODUCTION

Dermatophytes are the most commonly confronted fungi in humans and other vertebrates that spread through direct contact with infected animals, humans, and soil. Having considered as keratinophilic hyaline molds, dermatophytes may lead to disease in keratinized tissues such as skin, hair, as well as nail. According to reservoir and transmission route, dermatophytes origin can be anthropophilic, zoophilic, or geophilic or also referred to as human, animals, and soil origin, respectively. Furthermore, dermatophytes contain over 40 species in three genera: *Trichophyton, Microsporum*, in addition to *Epidermaphyton* [1].

Dermatomycosis (tinea or ringworm) is a generic name for acute to mild and chronic lesions of the outer layers of the keratinized tissue caused by the skin fungus. The name of such organisms is based on the body site affected as follows: *T. corporis* (trunk), *T. capitis* (head) *T. cruris* (perianal area), *T. pedis* (foot as well as interdigital area), and *T. unguium* (nail). Such a disease has adverse impact on individuals of diverse ages; however, the most prevalent among children are *T. corporis and T. capitis*, while *T. pedis* is the most popular among adults [1].

Dermatophytosis are highly frequent worldwide, affecting 20–25% of the universal population. World Health Organization (WHO) in 2005; reported a prevalence of up to 19.7% for tinea capitis in the general population of developing countries. The disease

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is more common among individuals with immune system disorders and diabetes [2].

Trichophyton species are the principal causative agents responsible for dermatophytosis with a prevalence rate of 70-90% for onychomycosis and 53-86% for rest of the tinea infections [3]. Of these, *Trichophyton rubrum* is the main etiological agent followed by *T. mentagrophytes* complex, *Microsporum canis*, and *M. gypseum* [4].

Several antifungal drugs have been developed and used in the management of cutaneous fungi. Azole antifungal drugs, such as itraconazole, ketoconazole and fluconazole inhibit lanosterol 14 a-demethylase and the mass of the synthesis of the fungal membrane ergosterol in the cell [5].

There is not much data available regarding the antifungal susceptibility of dermatophytes in Middle Eastern countries, where the occurrence of dermatophytosis is endemic [6,7,8,9]. Due to recent increase in the reports of antifungal drug resistance in dermatophytes, so performing the antifungal drug susceptibility testing especially for the dermatophytes isolated from recalcitrant chronic/recurrent cases [10-11].

The E-test is a simple, less laborious, agar-based, quantitative minimal inhibitory concentration (MIC) method that is satisfactorily used to test fungi, mainly Candida spp. and Cryptococcus spp [12].

This study aimed to determine antifungal sensitivity pattern of isolated dermatophytic fungi isolated from patients with suspected dermatophytic fungi by E-test method and also, explore the clinico-mycological pattern of these patients.

METHODOLOGY

Design of the study and patients:

This current prospective study was performed in Department of Medical Microbiology & Immunology and Dermatology, Venereology & Andrology Department, Faculty of Medicine, Zagazig University, Egypt, from January 2018 to May 2019. A total 85 clinically suspected patients with dermatophytes attending the Dermatology Outpatient Clinic were enrolled in this research, regardless of gender or age. Exclusion criteria include patients receiving antifungal treatment (systemic or topical) in one-month period prior to sampling. Complete history was taken from patients. Once elaborate history was recorded, patients were subjected to a clinical examination in good light, and this includes lesion site, lesions number and types, in addition to inflammatory margin existence. Following patients' consent, collected samples were eligible to further examinations. The current research obtained an approval form ethical committee of Zagazig University, Faculty of Medicine.

Collection of the samples:

Hair specimens were obtained with epilating forceps, which were then plucked along the hair shaft base, while scraping scales from surface was performed with a sterile surgical blade, particularly the blunt edge. Suspected skin lesions were subjected to alcoholic cleaning by means of ethanol (70%) to eliminate any contaminating bacteria and dirt. In the case of skin scales as well as crusts, they were obtained from the actively growing, erythematous, peripheral margins of lesions through scraping across inflamed lesion margin into seemed healthy tissue on clean glass slides by means of a sterile surgical blade, utilizing the blunt edge in particular. To obtain nail specimens, brittle or dystrophic parts of the nail was cut superficially as far back as possible by means of surgical blade involving any crumbly items. Regarding superficial involvement (as in white superficial onychomycosis), nail scrapings were obtained [13]. After that, in Petri dishes (sterile, dry), the gathered specimens were sealed, followed by labelling step, including patient's name, sex, age, site of infection, collection date and then sent to laboratory towards mycological test. Upon splitting samples into two portions: the first one was ready for culture, while the other was employed for microscopic examination.

Microscopic examination, fungal culture and identification:

All samples were examined microscopically using 10% KOH for screening of fungal elements (hyphae and/or arthroconidia). On a grease-free, clean glass slide, the sample was added in KOH (2-3 drops), followed by coverage with a clean slide. The above slide was pressed for inhibition of air bubbles formation, keeping the sample for further observation after 5–8 minutes for skin scraping and 30 minutes for nail [13]. After a direct microscopic examination, samples were inoculated on culture media Mycobiotic agar media (CONDA, Spain).

Cultures were incubated at room-temperature (25°C) for one month, followed by examination for growth every three days. Positive cultures were subjected to macroscopic and microscopic examination. The first test includes surface color and reverse, topography, as well as texture, while the latter includes Lactophenol cotton blue stain, where two conidia kinds were originated from dermatophytes: small unicellular microconidia in addition to larger septate macroconidia. Such stain is devoted to identifying species by means of cellotape flag approach in addition to the slide culture procedure [14,15] and physiological tests (urease test and *in-vitro* hair perforation test). The culture was considered negative, when any growth is absent after one month.

Antifungal susceptibility testing (E-test):

The utilized test medium was RPMI 1640 with L-glutamine, in the absence of bicarbonate adjusted to pH 7, supplemented with 0.2% glucose, buffered with 0.165 M morpholine propanesufonic acid (MOPS)

(HIMEDIA, USA) and solidified with 1.8% agar. Petri plates with 15-cm diameter comprised RPMI 1640 at a 4.0 mm depth. The utilized E-test strips were supplied by (HIMEDIA, USA and stored at -20^{0}C until conducting the tests. The concentrations assayed are in the range between 0.002 and 32 µg/mL for terbinafine, itraconazole, voriconazole, ketoconazole and from 0.016 to 256 µg/mL in the case of fluconazole [16].

Based on the manufacturer's guidelines, the isolates were subjected to E-test against five antifungal agents. After inoculums suspensions preparation, they were adjusted to transmittance of 65-70% at a 530 nm wavelength, which corresponds to a 10^5-10^6 CFU/mL concentration, as confirmed using quantitative plate counts. To inoculate the surface of RPMI agar, a sterile swab was dipped into inoculums suspension, followed by consistent streaking in three directions. Following absorption of the excess moisture from agar and complete dryness of the agar surface, each plate was subjected to E-test strip. Finally, incubation of plates was done at 28^{0}C and the resultant findings were read at 72-96 hrs [16] .

Minimal inhibitory concentration (MIC) endpoints determination:

By definition, MIC represents the lowest concentration of drug at which elliptical inhibition zone border intercepted MIC scale on E-test strip. The MIC highest value was read upon observation of various intersections on either side of strip. Observation of a double halo of growth resulted in reading MIC upon complete inhibition of growth [16]. The MIC data were reported as MIC ranges, MIC at which 50% (MIC_{50}) and 90% (MIC_{90}) of the strains were inhibited. Quality control (QC) strains (*C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258; American Type Culture Collection, Manassas, VA, USA) were included as recommended manufacturer's instructions.

Statistical analysis:

Data were collected, tabulated, and analyzed using SPSS version 16.0.

RESULTS

In this study, 85 cases with clinically suspected dermatophytosis were involved. Dermatophytes were proven by culture and direct microscope in 62 cases (73%). Male accounts for (n=42, 68%) and female (n=20, 32%) with male to female ratio (2.1:1). The mean age of the study group (31.8 ±19.1) ranging from 6 to 62 years, and the highest incidence of cases is in the age group of 6–20 years (40%), followed by 31-40 years (24%), 21-30 years (19%) ,41-50 years old and 51-62 years old 13%, 3% respectively (Figure-1).

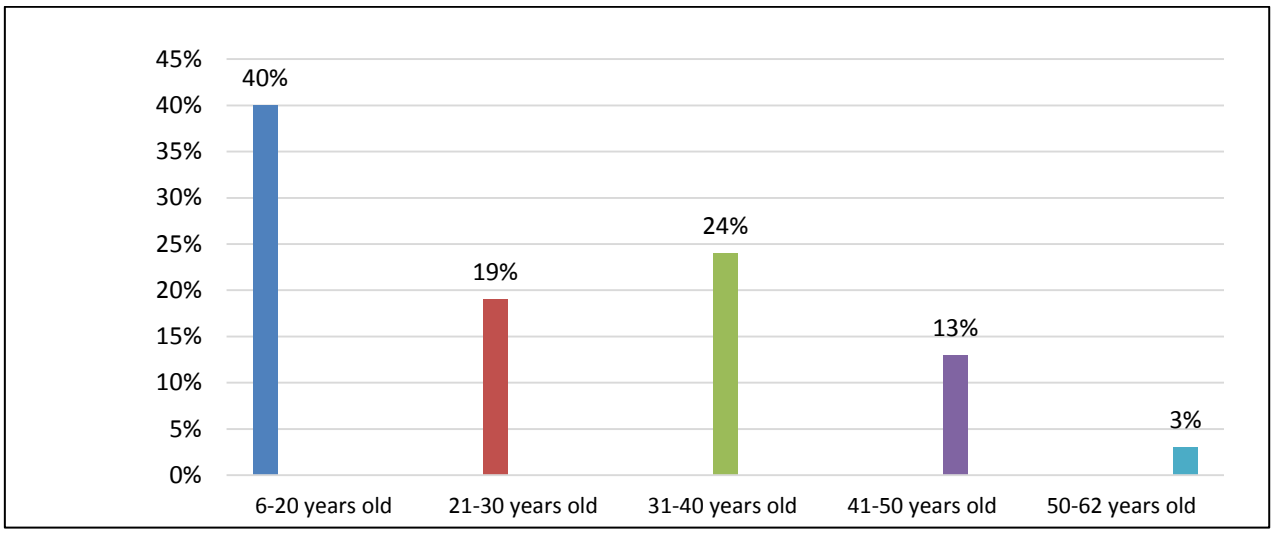

Fig. 1: Age distribution of the study group n=62

In our study 62 dermatophytes strains were obtained from different clinical types. Tinea corporis was the most prevalent clinical type 23 (37%), tinea capitis 20 (32%), tinea pedis 12 (19%), tinea ungium 4 (6%), and tinea cruris 3 (5%). Sixty two isolated dermatophytes species were distributed as follows: *T. mentagrophytes* 20 (32%), *T. rubrum* 16 (26%), *T. violaceum* 14 (23%), *T. schoenlinii* 7 (11%), *M. canis* 5 (8%) (Table 1, figure 2,3).

Table 1: Frequency distribution of dermatophytes strains in respect to clinical types

Dermatophyte strain	N	%	Tinea corporis	Tinea pedis	Tinea capitis	Tinea ungium	Tinea cruris
T.mentagrophytes	20	32%	8	7	2	1	2
T.rubrum	16	26%	9	3	0	3	1
T. violaceum	14	23%	2	2	10	0	0
T. Schoenlinii	7	11%	0	0	7	0	0
M.canis	5	8%	4	0	1	0	0
Total	62	100	23(37%)	12(19%)	20(32%)	4(6%)	3(5%)

Fig. 2: Microscopic appearance of *M.canis* showing Macrocondia and microcondia

Fig. 3: Microscopic appearance of *T.schonleinii* showing favic chandlers like hyphae

The antifungal susceptibility data of 62 strains belonging 5 species *T. rubrum*, *T. mentagophytes*, *T.* violacieum, *T. scholenii* and *M. canis* are summarized in (Table -2), involving ranges of MIC and MIC_{50}, MIC_{90}. Because of the insufficient number of *T. schonlenii* strains (n = 7) and *M.canis* (n=5), calculation of MIC_{90} and MIC_{50} values cannot be obtained for *T. schonlenii* and *M.canis.*

Table 2: Susceptibility data for dermatophytes species included in the study N(62) using the E-test method .

	Antifungal (ug/ml)	**[a]MIC range**	**[b]MIC $_{50}$**	**MIC $_{90}$**
T.mentagrophytes	Fluconazole	16—256	128	256
	Ketoconazole	0.04-8	0.5	8
	Itraconazole	0.03-16	2	8
	Terbinafine	0.04-16	0.5	2
	Voriconazole	0.003-0.06	0.06	0.06
T.rubrum	Fluconazole	8-128	64	128
	Ketoconazole	0.06-24	0.5	8
	Itraconazole	0.04-2	0.5	2
	Terbinafine	0.03-1	0.5	1
	Voriconazole	0.015-0.06	0.06	0.06
T. violaceum	Fluconazole	16-24	16	24
	Ketoconazole	0.09-4	0.5	2
	Itraconazole	0.04-2	0.5	1
	Terbinafine	0.03-1	0.5	0.5
	Voriconazole	0.004-0.125	0.06	0.06
T. Schoenlinii	Fluconazole	32-192	--	--
	Ketoconazole	0.2-2	--	--
	Itraconazole	0.06-2	--	--
	Terbinafine	0.03-2	--	--
	Voriconazole	0.004-0.06	--	--
M.canis	Fluconazole	2-8	--	--
	Ketoconazole	0.2-2	--	--
	Itraconazole	0.06-4	--	--
	Terbinafine	0.06-0.125	--	--
	Voriconazole	0.003-0.125	--	--
Total	Fluconazole	2-256	16-128	24-256
	Ketoconazole	0.04-24	0.5	2-8
	Itraconazole	0.03-16	0.5-2	1-8
	Terbinafine	0.03-16	0.5	0.5-2
	Voriconazole	0.003-0.125	0.06	0.06

[a]MIC_{50} and [b]MIC_{90} are the lowest concentrations of the antifungal drug that caused growth inhibition of 50% and 90% of tested isolates, respectively.

Fluconazole showed the least activity having MIC range of 2-256 µg/mL. MIC ≥64 µg/ml was considered resistant. Thus, in our study, 24 (39%) of strains were found to be resistant to fluconazole (Figure-4).

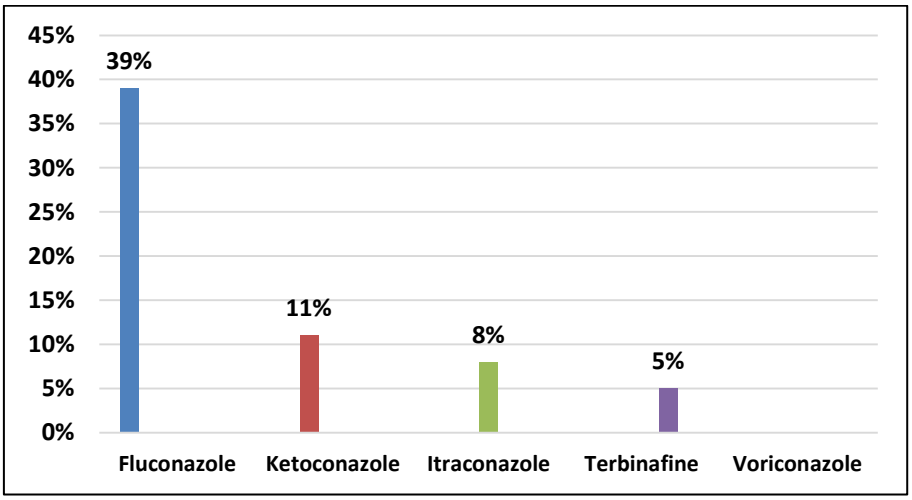

Fig. 4: Percentage of resistant strains

Minimum inhibitory concentration of ketoconazole ranged from 0.04 -24 µg/ml. In accordance to CLSI guideline for filamentous fungi is that MIC ≥8 µg/ml is resistant to ketoconazole. Following this guideline, we report seven strains (11%) to be resistant to ketoconazole.

Itraconazole MIC is in the range of 0.03 -16 µg/ml. According to the Clinical and Laboratory Standard Institute standards, sensitive strain has minimum inhibitory concentration between 0.01 and 8 µg/ml. Only five strains (8%) had MIC ≥8 µg/ml, which regarded as resistant to itraconazole.

Regarding terbinafine MIC, it was in the range of 0.03 -16 µg/ml. In the case of strains with MIC >1 µg/ml, it was found to be resistant, and we found 3 (5%) resistant to terbinafine.

Voriconazole manifested the highest activity towards all dermatophytes species, featuring MIC range of 0.003-0.125 µg/mL. There is no resistance for voriconazole for dermatophytes strains tested in this study.

DISCUSSION

Dermatophytosis are infectious and on their direction for being chronic. Presently infected cases comprise a substantial patients' group in dermatology clinics. Despite the promising results based on in vitro results preventing relapsing infections and improving the clinical care will need an additional determination of relapses causes in addition to treatment failures and influential surveillance with respect to resistance development [17].

The correct control over dermatophytic infections includes personal hygiene, infection awareness, adequate treatment and appropriate diagnosis. According to clinical response by patients, vulnerable nature of drugs is decided, instead of in vitro testing. Examining dermatophytes towards antifungal susceptibilities in addition to resistance emergence prevention is highly required by means of a standard reference approach [17].

In this study, majority of patients fall in a group (6-20) years. In other previous studies [18,19,20] they reported majority of patients were adults (20–40) years. Male:female ratio was 2.1:1; male preponderance has been seen also in some earlier studies [21,22], while, others have showed female predominance, with females mainly having onychomycosis and tinea pedis because of household work and kitchen [23,24].

The dermatophytes distribution in addition to their causative agents showed different frequencies, with varied prevalence percent based on various countries or even regions in same country. Herein, *T. mentagrophytes* showed the utmost commonly isolated organism 20 (37%), followed by *T. rubrum* 16 (26%), *T.violacieum* 14 (23%), *T. schonlenii* 7 (11%), and *M. canis* 5 (8%). These results are in agreement with other previous studies [25, 26,27].

Although most infections are a result of dermatophytes, their rapid elimination can be attained when topical as well as systemic antifungals were applied. When dermatophytosis show no response to topical therapies, the only choice for treatment depends on oral antifungal therapy by means of new agents, including voriconazole, terbinafine, itraconazole in addition to fluconazole. Such drugs display varied activity spectrum, failing in treatment of about 25-40% of cases. This failure may be attributed to limited penetration of drug into the nail, poor compliance of patient, medication bioavailability or interactions and resistance [28].

Dermatophytosis has significantly increased during this decade by abuse of topical corticosteroids cream alone or in combination with topical antibacterial and antifungal agents. The rising tendency of resistance among dermatophytes leading to bad response and frequent reversals is of serious concern and has been due to improper treatments with steroid combination creams, improper dosages of antifungals and lifestyle changes. In this literature resistance of dermatophytic infections to all antifungals tested (except voriconazole) has been reported [29].

The E-test represent a promising technique with extensive uses in clinical laboratory practice and is reinforced by outcomes of widespread bacteria and yeasts testing. Nevertheless, only a few reports are found, which describe utilization of such approach for dermatophytes [16] .

In the current work, we examined MIC values of five antifungal drugs (fluconazole, ketoconazole itraconazole, terbinafine, and voriconazole) to various species of dermatophyte strains separated from clinical specimens by means of E-test technique.

Consistent with reported results from other works, fluconazole showed the least activity among examined antifungal drugs with high MIC range (2-256) µg/ml, MIC_{50} (16-128) µg/ml and MIC_{90} (24-256) µg/ml [30,31,32]. Fernandez-Torres *et al.* [16] study reported MIC values for fluconazole of >256 µg/ml for *T. rubrum* and *T. mentagrophytes*. Barros *et al.* [33] reported the same result. Minimum inhibitory concentration ≥64 µg/ml was considered resistant [34] . Accordingly, 24 strains (37%) were found to be resistant to fluconazole. In other previous studies, resistance to fluconazole in different dermatophyte strains is well documented [35,36,37].

Minimal inhibitory concentration of ketoconazole in this study was in the range of 0.04 -24 µg/ml. Seven strains (11%) were resistant to ketoconazole .In a study done by Satyendra *et al.*[29] in which they report MICs of ketoconazole 0.06 to >16 µg/ml with (14.6%) resistant strains out of 41 strains of *T. mentagrophyte*

tested .Magagnin *et al* [36] reported high incidence (53%) of resistance to ketoconazole.

Regarding MICs range of itraconazole in this study was 0.03-16 µg/ml. Ataides *et al* [38] and Sonyia *et al.* [26] reported Itraconazole MICs range 0.062–15 µg/ml, 0.03 to >16 µg/ml respectively. A narrower range of MIC (0.01– 4 µg/ml) was noted by other previous studies[36,39,40]. While, a wider range of MICs (0.06–32 µg/ml) was observed by Gupta *et al.*[41,42]. In this study five strains (8%) reported to be resistant to itraconazole which is similar to a study done by Mahajan *et al.*[43] which observed (6%) incidence of resistance to itraconazole. Magagnin *et al.* [36] observed itraconazole resistance in 42.3% of the strains they studied.

In this study, MIC of terbinafine ranged from 0.03 - 16 µg/ml with only 3 strains (5%) reported resistant, which is similar to other previous studies in which they reported MIC ranged from 0.003 to 16 µg/ml[26,40]. A high incidence of resistance to terbinafine was noted by Satyendra *et al.*[29] reported 33 strains (65.9%) of *T. mentagrophytes* and three strains (100%) of *T. rubrum* were resistant to terbinafine.

Voriconazole is a broad-spectrum triazole antifungal agent equivalent to fluconazole in structure and itraconazole in spectrum of action. Voriconazole has sturdy fungicidal activity towards most filamentous fungi, and this may be because of high affinity held by voriconazole towards fungal 14-alpha-demethylase. This kind of activity was confirmed using ultrastructural as well as biochemical analysis. The voriconazole pharmacokinetics in man formed persistent high blood and tissue levels after oral and intravenous applications of 50 to 200 mg/day [44].

In this study the voriconazole has the least MIC range (0.003-0.125 µg/ml), MIC_{50} (0.06 µg/ml), MIC_{90} (0.06 µg/ml). In other studies, MIC for voriconazole was found in the range of 0.03–16 µg/ml [26] . In this study no reported resistance to voriconazole. The excellent anti-dermatophyte activity of voriconazole encounter in this study was the same to a study done by Alfonso *et al.*[44].

The absence of clinical breakpoint for dermatophytes, because of limited pharmacokinetic/ pharmacodynamic studies as well as data with respect to epidemiological cutoff values, proposes that an upsurge in MICs may not be linked to a drug resistance mechanism, but instead with high antifungal dosage or a longer treatment period for achieving an optimal clinical response. Our findings can help understand local susceptibility patterns, despite the need of standard surveillance studies. Antifungal sensitivity analysis is a vital and energetic field in medical mycology. Both development and standardization of antifungal susceptibility test have revealed noticeable advancement in the medical mycology field [41].

CONCLUSION

Based on our results, highest incidence of cases falling in the 6–20 years old age group with male predominance. *Tinea corporis* was the most common clinical type followed by tinea capitis, tinea pedis, tinea ungums and tinea cruris. *T.mentagrophytes* was the most prevalent species isolated followed by *T.rubrum, T. violaceum, T. Schoenlinii, and M.canis*. Voriconazole showed notably low MIC values and was the most active azole against all dermatophytes isolates, followed by terbinafine, itraconazole ketoconazole .While fluconazole showed the least antifungal activity with high MICs values. Use of antifungal drugs irregularly and inadequately has led to the appearance of resistant strains, which cause bad treatment outcomes. So, it is necessary to test for antifungal susceptibility to check for resistance to antifungals.

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- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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