Original Research Article

Isolation and Identification of Dermatophytes and their Antifungal Susceptibility to Ketoconazole, Fluconazole, Clotrimazole, Miconazole and Terbinafine by Agar Diffusion Neo-Sensitabs Method

ABSTRACT

Dermatophytosis or tinea is a type of cutaneous infection caused by keratinophilic fungi, infecting the skin, nails and hair. A correct diagnosis is important for epidemiological purposes and initiating appropriate treatment. An increase in the prevalence of fungal infection worldwide is due to abuse of antibiotics, immunosuppressive treatments and numerous medical conditions.

Aim: To isolate, identify, and examine the in-vitro antifungal susceptibility of dermatophytes in clinically suspected cases of tinea infections.

Methodology: We worked on 65 specimens of tinea infection, after processing them according to standard operating procedures at Department of Microbiology, University of Health Sciences,Lahore, Pakistan. KOH wet mount was done for identification and culture was carried out on Sabouraud Dextrose Agar and Dermatophyte Test Medium. The cultures were incubated at 30ºC for up to 4 weeks in case of SDA and 2 weeks in case of DTM. Lacto phenol cotton blue stain was used to identify the species morphologically. Susceptibility test was done by agar diffusion method using antifungal disks and zones of inhibition were measured.

Results: More females (55.38%) than males (44.61%) were observed in the study. Most of the cases belonged to the age categories of 1-10 years and 21-30 years. Tinea corporis was the most common clinical type found (27.69%) followed by Tinea capitis (21.53%) and Tinea cruris (12.30%).*Trichophyton mentagrophytes* was the commonest species isolated (32%) followed by *Trichophyton violaceum* (28%) and *Trichophyton rubrum* (12%). Terbinafine was seen to be the most effective drug against the isolates, followed by clotrimazole. Fluconazole showed least activity.

Conclusion: Fungal culture remains the gold standard in identifying the causative species. Terbinafine promises to be a potent antifungal, whereas fluconazole has low efficacy against such organisms. Disk diffusion method adopted for antifungal susceptibility is cost effective and easily performable in small laboratories not having an established mycology bench.

Keywords: Dermatophytes, disk diffusion method, antifungal drugs.

1. INTRODUCTION

Dermatophytes are a group of filamentous fungi prone to infect keratin-rich tissues, i.e., skin, nail and hair; this feature leads them to be designated as keratinolytic fungi. Dermatophytosis ranks among the most common and rampant infectious diseases worldwide [1]. Living in close communities, poor personal hygiene, low socioeconomic status, humidity, contact with animals, unnecessary use of antibiotics, corticosteroids and tumour suppressing drugs are a few risk factors for such disease [2,3,4].

Research on fungal infections was started around 19th century by European physicians. Raimond Sabouraud, one of the most prominent medical

mycologists, started his scientific experiments on dermatophytes around 1890. The three genera of dermatophytes [5] are further differentiated by their classical spores (microconidia and macroconidia) into a total of 40 species [6,7].*Trichophyton* species infect all three body sites; *Epidermophyton* having the prefix epiderm focuses on skin and nails. *Microsporum* is the opposite of *Epidermophyton* and infects hair [8, 9].

Medically, the disease can be categorized on the basis of the site involved. The clinical manifestations include tinea barbae (beard), tinea capitis (scalp), tinea corporis (hairless body skin), tinea cruris (groin), tinea manuum (hand), tinea pedis (foot) and tinea unguium (nails) [10].

Dermatophytes can either opt for human (anthropophilic), animal (zoophilic) or soil (geophilic) as their host [11].

The laboratory diagnosis of dermatophytes is of prime importance since it is difficult to diagnose such infections simply based on clinical presentation. In routine, direct microscopy of a clinical specimen is done, followed by culture [12].

The antifungal therapy for such infections can sometimes be quite complicated because of the long duration of the treatment, high cost, and possible side effects [13]. This has led to the emergence of resistant strains, which consequently cause poor treatment outcomes. Thus, it is very important to check the drug sensitivity for resistance patterns.

The disk diffusion method is one of the procedures to carry out antifungal sensitivity test. This method is simple and doesn't require any specialized equipment. Therefore, it can be performed in a clinical laboratory on routine basis [14,15,16,17]. In 1996, Barry and Brown whereas in 2000, Meis *et al.,* found out the disk diffusion method as an accurate, economical, easily performable and reproducible test for antifungal sensitivity [18,19].

2. MATERIAL AND METHODS

The study was carried out at the Department of Microbiology, University of Health Sciences,Lahore, Pakistan. Sample collection was done from the patients of all age groups and of both sexes, attending outpatient/inpatient department of Dermatology at Mayo Hospital, Lahore, Pakistan. Colonies that were blue, black or green were regarded as non-dermatophytes [20].

2.1 Specimen Collection, Microscopic Examination and Fungal Culture

The specimens were taken from all three sites after cleaning with 70% ethyl alcohol. This was done to remove any surface bacterial contaminants. Skin scrapings were taken from the periphery of the erythematous inflamed margins of the wound with the help of a sterile blunt scalpel blade (size 22).

The nail clippings were taken from the affected part of the nail with the help of a nail clipper. A blunt scalpel blade was used to scrape the area, which could not be clipped.

Infected hair was epilated with the help of epilating forceps. Samples were collected from different sites if more than one area of the scalp was involved.

All the specimens obtained were sealed in sterile dry petri dishes. These samples were labelled with the name, sex, age and date of collection.

Direct microscopy under low power (10X) and high power (40X) was done on all the collected samples with 10% and 40% KOH to check for the presence of fungal elements. Culture of the specimens was done on Sabouraud Dextrose Agar (SDA) and Dermatophyte Test Medium (DTM), both incorporated with chloramphenicol and cycloheximide.

All the plates were incubated aerobically at 28-30ºC up to 4 weeks for SDA and 2 weeks for DTM after placing them in polythene zipper bags to prevent drying and contamination [7,1]. The plates containing SDA+CC were checked twice weekly for a maximum of 30 days. DTM plates were checked daily for 2 weeks. SDA plates showing no growth after 4 weeks were considered negative. DTM plates showing no growth after 14 days were considered negative. Plates showing any growth after above mentioned time period on any of the above mentioned culture media were also discarded.

2.2 Identification of Dermatophyte Species:

Dermatophyte species were identified by observing the colonial morphology. Colonies of dermatophytes were recognized as being light colored, often brown and white as shown in Fig.1.

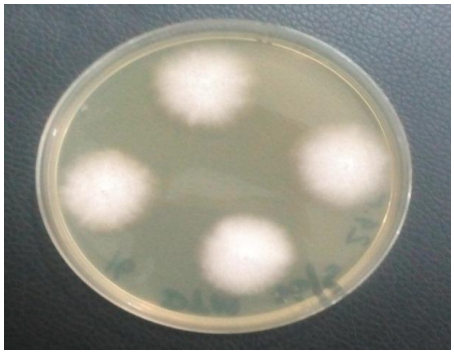

Fig.1 Macroscopic view of T. mentagrophytes (Forward View)

2.3 Antifungal Susceptibility

The samples were transferred to sterile distilled water in tubes and stocked at 25°C until required [14,21]. When needed, they were sub-cultured on PDA at 28ºC to augment sporulation. 7 days old cultures were mixed with 1ml distilled water. The colonies were explored with the tip of a sterile Pasteur pipette to get an assortment of mycelium and conidia. The suspensions were allowed to sediment for 30 minutes. Suspensions were adjusted by using 0.5 McFarland standard. [21,22]. The adjusted inocula was evenly streaked on the surface of petri dishes containing SDA [22]. Commercially available neo-sensitabs discs (Rosco) fluconazole (25µg), clotrimazole (10µg), ketoconazole (15 µg), miconazole (10µg) and terbinafine (30µg) were applied onto the inoculated agar plates and incubated at 28°C to 30°C. The zones of inhibition around the discs were measured after 3 and 7 days of incubation. The criteria of susceptibility and resistance to antifungal discs were measured, which is presented in tabular form below:

Antifungal Drugs	**Potency**	**Zone Diameter in mm**		
		S	**I**	**R**
Fluconazole	25µg	≥22	21-15	≤14
Ketoconazole	15µg	≥30	29-23	≤22
Clotrimazole	10µg	≥20	19-12	≤11
Miconazole	10µg	≥20	19-12	≤11
Terbinafine	30µg	≥20	19-12	≤11

Legends: S- Sensitive, I- Intermediate, R- Resistance

3. RESULTS

65 patients with clinically suspected disease were included in this study. Out of them, 29 were males and 36 females. Of the seven age groups made, the most commonly groups affected were 1-10 years and 21-30 years, both with 17 cases each.
In accordance with the clinical types, Tinea corporis was predominant, followed by Tinea capitis, Tinea cruris, Tinea facium, Tinea unguium and Tinea pedis. The males were seen to have high incident of Tinea corporis whereas, females showed more cases of Tinea capitis. The most common species isolated was *Trichophyton mentagrophytes* (Fig.2). It was followed by *Trichophyton violaceum, Trichophyton rubrum, Trichophyton tonsurans, Epidermophyton floccosum, Trichophyton verrucosum* and *Microsporum ferrugenium*.

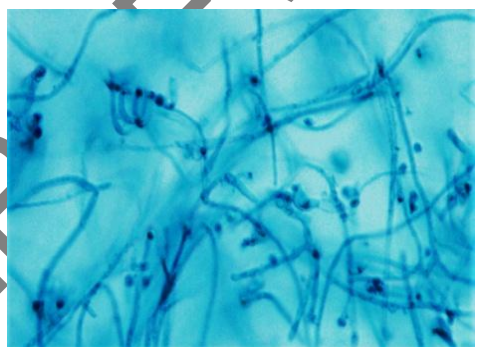

Fig.2 Microscopic view of T.mentagrophytes (LPCB, 40X)

The tests for the susceptibility to antifungal drugs showed following results; fluconazole: 4 (16%) sensitive, 21 (84%) resistant. ketoconazole: 14 (56%) sensitive, 2 (8%) intermediate and 5 (20%) resistant. clotrimazole: 24 (96%) sensitive and 1 (4%) resistant. miconazole: 20 (80%) sensitive and 4 (16%) resistant. terbinafine: 25 (100%) sensitive. Regarding the data, it was revealed that terbinafine and clotrimazole were the most effective antifungal drugs and fluconazole had the poorest activity (Fig.3).

Table 1: Table shows that Antifungal Susceptibility

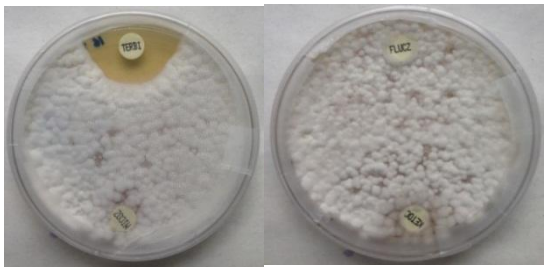

Fig.3 Susceptibility plates showing inhibitory zones of the drugs for T. mentagrophytes.

4. DISCUSSION

The fungus that causes tinea is very common all over the world and affects all. The increased risk of such infections in females as seen in this study may be attributed to the fact that more patients of this gender attended the outpatient department. Women, being more involved in house chores mostly tend to ignore their hygiene, leading to having tinea infections.

Similarly, children and young adults pose a greater risk to contract tinea infections by staying out for longer periods of time (schools, parks etc.) hence, increasing their chance of exposure. Having pets is another cause. Cats, dogs, goats and pigs in such cases, are infected with dermatophytes and transmit the condition to humans through saliva, infected urine/feces or close contact. A population whose immune function is compromised for any reason is most susceptible to the infection.

Tinea corporis and Tinea capitis were the most common clinical types found in patients under study. The risk factors include strenuous physical activity outside, warm climate, contact sports such as wrestling, use of communal baths or locker rooms, poor nutrition, sharing of infected materials among family members (combs, clothing, towels and bed linen) and occupational contact (gardeners

The Agar diffusion method, on the other hand, has a practical approach which assists in the determination of the activity of various antifungal drugs against various fungal genera and species.

For countries like Pakistan, the disk diffusion method is a good model to be used for investigation purposes to test other fungal genera and drugs as well. This method can be adapted for routine diagnosis in the laboratory and for assessment of dermatophyte resistance against antifungal drugs. There are studies which focused on the comparison of the disk diffusion method with the reference micro-dilution method. These studies suggest that disk diffusion is a reproducible method which in general shows good correlation with the reference method for micro-dilution antifungal susceptibility test [17,19].

This study showed that terbinafine and clotrimazole had large inhibition zones around the disks; terbinafine had the best activity against all the isolates. Fluconazole, on the other hand, had the least activity and in most isolates, no inhibition zone at all. Results of this study are in line with other studies conducted on dermatophytes [18,19].

5. CONCLUSION

Through this study, terbinafine and clotrimazole have been proved to be the most effective antifungal drugs against dermatophytic infection. Necessary changes in antifungal administration can be made according to the antifungal susceptibility tests to prevent recurrent tinea infections. Genetic basis of antifungal resistance can be explored in future work.

6. REFERENCES

1. Asticcioli, S., Di Silverio, A., Sacco, L., Fusi, I., Vincenti, L. and Romero, E., 2008. Dermatophyte infections in patients attending a tertiary care hospital in northern Italy. *New Microbiol*, *31*(4), pp.543-8.
2. Falahati, M., Akhlagi, L., Lari, A.R. and Alaghehbandan, R., 2003. Epidemiology

and farmers).

In recent years, there has been an astonishing development in the standardization of antifungal susceptibility tests in the field of medical mycology throughout the world. Unfortunately, in Pakistan, the field of mycology has been neglected for quite a long time. There is a lack of a reference mycology lab, skilled personnel, and resources that deprive us of making efforts in doing research in this field. Although, studies have been done on various fungi like Candida, Aspergillus, and non-dermatophytes, very less work has been done related to dermatophytes and little or no data is available on their antifungal susceptibility. Regardless of the many guidelines that NCCLS have published for susceptibility tests of molds (such as M-27A, M28A), there is no exact method and a routine test for the screening of dermatophyte antifungal activity [23].

Macro- and micro-dilution methods both can be used to determine antifungal susceptibility of dermatophytes, but these methods are costly and require specific media and equipment such as RPMI, MOPS buffer, and microplate trays.

4. Ekwealor, C.C. and Oyeka, C.A., 2013. Cutaneous mycoses among rice farmers in Anambra State, Nigeria. *Journal of Mycology, 2013.*
5. Martin, & Kobayashi, 1999
6. Starova, A., Balabanova-Stefanova, M. and V'lckova-Lasckoska, M. 2010. Dermatophytes in republic of macedonia. *Contributions, Sec Biol Med Sci MASA*, *31*, pp.31.
7. Hanif, F., Ikram, A., Abbasi, S.A. and Malik, N., 2012. Pattern of dermatophytes among dermatological specimens at AFIP, Rawalpindi. *Journal of Pakistan Association of Dermatologists*, **22**(2).
8. Krishan, Anand S,Ray R, Chatterjee M, Khandare M. ACross Sectional Descriptive Study On Clinical Type And Etiological Agent Of Superficial Dermatophytosis. JDA Indian Journal of Clinical Dermatology 2018;1:71-74.
9. Ghannoum MA, Isham NC (2009) Dermatophytes and dermatophytoses. 2nd ed. Clinical Mycology, pp. 375-384.
10. Weitzman, I. and Summerbell, R.C., 1995. The dermatophytes. *Clinical microbiology reviews*, *8*(2), pp.240-259.
11. Dhinakaran, A., Kalaiselvam, M., Sekar, V. and Sethubathi, G.V.B., 2012. Isolation of of dermatophytoses in an area of south of Tehran, Iran. *Mycopathologica*, **156**(4): 279-287.
3. Sen, S.S. and Rasul, E.S., 2006. Dermatophytosis in Assam. *Indian journal of medical microbiology*, **24**(1):77.
susceptibility testing of dermatophytes: establishing a medium for inducing conidial growth and evaluation of susceptibility of clinical isolates. *Journal of clinical microbiology*, **38**(1): 341-344.
16. Nweze, E.I., 2011. Dermatophytoses in domesticated animals. *Revista do Instituto de Medicina Tropical de São Paulo*, *53*(2), pp.94-99.
17. Macura, A.B., 1993. Dermatophyte infections. *International journal of dermatology*, **32**(5): 313-323.
18. Barry, A.L., Pfaller, M.A., Rennie, R.P., Fuchs, P.C. and Brown, S.D., 2002. Precision and accuracy of fluconazole susceptibility testing by broth microdilution, Etest and disk diffusion methods. *Antimicrobial agents and chemotherapy*, **46**(6): 1781-1784.
19. Meis, J., Petrou, M., Bille, J., Ellis, D., Gibbs, D. and Global Antifungal Surveillance Group, 2000. A global evaluation of the susceptibility of Candida species to fluconazole by disk diffusion. *Diagnostic Microbiology and Infectious Disease*, *36*(4), pp.215-223.
20. Shamim, S.u.m.b.u.l., Ahmed, S.W., Siddiqui, S.A. and Azhar, I.Q.B.A.L., 2005. Superficial Mycoses: A study Performed For the Isolation and Identification of Fungal Species from Infected Patients. *Pakistan Journal of Pharmacology*, **22**(1): 41-46.
21. Esteban, A., Abarca, M.L. and Cabanes, F.J., 2005. Comparison of disk diffusion method and broth microdilution method for antifungal susceptibility testing of dermatophytes. *Medical mycology*, **43**(1): 61-66.
22. Keyvan, P., Leila, B., Zahra, R., Manuchehr, S. and Kamiar, Z., 2009. In vitro activity of six antifungal drugs against clinically important dermatophytes. *Jundishapur Journal of Microbiology*, *2009*(4, Autumn), pp.158-163.
23. Ghannoum, M., 2001. Antifungal susceptibility testing of dermatophytes.*Dermatology Online Journal*, *7*(1).

Penicillium sp. and its antagonistic activity against dermatophytes from volcano soil of Baratang Island, Andaman. *International Journal of Pharmaceutical Sciences and Research*, *3*(2), p.564.
12. Côbo, E.C., Silva, J.C., Cota, U.A., Machado, J.R. and Castellano, L.R., 2010. Evaluation of a modified microscopic direct diagnosis of dermatophytosis. *Journal of microbiological methods*, *81*(2), pp.205-207.
13. Robert, R. and Pihet, M., 2008. Conventional methods for the diagnosis of dermatophytosis. *Mycopathologia*, *166*(5-6), pp.295-306.
14. Yadav, A., Urhekar, A.D., Mane, V., Danu, M.S., Goel, N. and Ajit, K.G., 2013. Optimization and isolation of dermatophytes from clinical samples and in vitro antifungal susceptibility testing by disc diffusion method. *RRJMB*,*2*(3), pp.19-34.
15. Jessup, C.J., Warner, J., Isham, N., Hasan, I. and Ghannoum, M.A., 2000. Antifungal