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COMPARATIVE ANALYSIS OF VARIOUS ANTI-FUNGAL AGENTS ON KERATINOPHILIC FUNGI ISOLATED FROM HABITATS OF JAIPUR

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ABSTRACT

Soil is the fundamental habitat for the growth of Keratinophiles and Dermatophytes. Among vast array of habitats, soil is major contributing factor of infections worldwide. Superficial infections have a major portion in infections resulting from dermatophytes. This fungal group degrades keratinous material present in soil in form of hairs, nails and skin scrapings etc. From past few years, vast studies have been done by using different keratinic baits on Indian as well as on foreign soil samples to detect the presence of these fungal groups. However, there is no documentation on comparative study among distinct habitats of a Jaipur city with physical and chemical parameter with particular interest of relative antifungal effectiveness. Therefore, this study was taken into consideration to isolate, identify and characterize keratinophiles in 100 soil samples of 9 different habitats of Jaipur city viz. sabji mandi, garden area, bird habitat, animal habitat, cropland, nursery, hospital area, bus stand and dumping area in relation to pH of soil sample. The To. Ka. Va. Hair baiting technique was applied isolation of Keratinophiles. A total six species of keratinophilic fungi *Chrysosporium, Microsporum, Trichophyton, Torula, Trichoderma* and *Gymnoascus* were isolated from different soil samples having pH in alkaline ranges (7.0-9.0). Out of these six species, 3 isolates *Chrysosporium tropicum* (70%), *Microsporum* sp. (40%), followed by *Trichophyton* (34%) were mainly found in the soils of Jaipur. Furthermore, among 10 antibiotics, a comparative analysis on antibiotics susceptibility was drawn. It was observed that fluconazole was the most effective antibiotic against tinea infections caused by *Chrysosporium* and *Microsporum* sp. while Terbinafine was found highly effective on infections raised by *Trichophyton*. Identification of these fungal species and analysis of various antifungal on infectious isolates provide an insight into keratinophiles diversity and antibiotic resistance leading to better selection of these antibiotics in the fungal infection.

Keywords: Keratinophilic fungi, soil habitats, physico-chemical parameters, antibiotics

INTRODUCTION

On everyday basis, many of keratinous baits like hair, nails, bird feathers etc. are disposed into the soil. These materials not only contaminate the soil environment and but also causes superficial infection "Tinea" among human and animals on global scale. Soil acts as the main reservoir for growth of various infectious species including keratinophilic fungi (Anane et al. 2015). These fungal species use various hydrolytic enzymes for breakdown of dead organic matter and keratinaceous debris into simpler form to acquire nourishment from it in the form of carbon and nitrogen (Mignon et al. 1998, Sharma & Rajak 2003).

India is the major contributor in production of keratinous waste material. Every year 350 million tons poultry scrap material from industries are disposed either directly into soil (land filling) or incinerated (Ashwathanarayana & Naika 2016, Prasanthi et al. 2016). In humans, these fungal species present in stratum corneum and inside the hair follicles are responsible for invading keratin baits like hair, nails and skin (Ghannoum et al. 2000). These fungal isolates having proteolytic and lipolytic activity play significant role in keratin denaturation because of tightly packed polypeptide chains of á- helix make this keratin's structure resistant from degradation.

These clinical isolates break disulfide bridges present in keratin protein through sulphitolysis makes this structure more susceptible for proteolytic activity (Marchisio 2000, Verma & Sharma 2017). This clinical criterion develops different entities of superficial infection according to the infected place either skin, nails or hair. For constraint non-extensive scraps engendered by dermatophytes topical treatments with different

antifungals of class imidazole, allylamines, tolnaftate, morpholine derivates etc. are commonly used (Vandeputte et al. 2012). These antifungals have been examined for their effective role on these fungal infections, as well as for cost-friendly and non-irritant nature. Several experiments were conducted to determine the antifungal effectiveness in which antibiotic disc method was found to be highly efficient (Esteban et al. 2005, Khadka et al. 2017). Therefore, this study was focused on isolation as well as determination of different antifungal susceptibilities pattern against these keratinolytic species.

MATERIALS AND METHODS

Collection of Soil Samples

100 soil samples from various keratin abundant habitats like road side, sabji mandi (vegetable market), garden area, bird habitat, bus stand, animal habitat, dumping area, nursery and cropland were collected individually from superficial layer of soil with the help of clean and sterilized spatula in sterile plastic bags. Approximately 500g of each soil sample was brought to the laboratory and their pH (in soil suspension) was examined.

Collection of Keratinic Baits

The keratin protein rich baits i.e. animal and human hair, nails, peacock and pigeon feathers were collected, cleaned and kept on the soil surface in form of small pieces for the growth of keratinophilic fungi.

Isolation of Fungi

The To.Ka.Va.Hair baiting method was applied for isolation of fungal group belonging to keratinolytic class. Firstly, soil samples were homogeneously spread in clean and sterilised petriplates and several pieces of 4 types of keratinic baits viz. pigeon feathers, peacock feathers, nails (Human) and hair {human and animal (Cow & Buffalo)}were uniformly distributed on the surface of the soil samples. Sterile distilled water was sprinkled over the soil surface to maintain moisture in baited plates. All these soil and baits containing plates were kept for incubation at 26±2°C in dim light for 30-45 days in the aerobic conditions and moistened timely if needed. After observation of fungal growth around the baits, fungal colonies were stained using lactophenol cotton blue for microscopic examination.

Identification, Purification and Maintenance of Fungi

The baited plates were observed for positive growth and plates with no growth were discarded. After primary screening of fungal growth, fungal colonies were transferred to Sabouraud's Dextrose

Agar (SDA) supplemented with streptomycin (0.05mg/mL) to examine pure fungal colonies. Additionally, another method i.e. Dilution plate technique/ Single Spore method was also applied for isolation of pure colonies. An Oculometery method was used for hyphal or spore measurement. These measurements like shape and arrangement of sporulating structure etc. were made for characterization of isolated species. The colour, texture and peculiar colony features were noticed.

Selection of Antibiotics

After consultation with dermatologists, we selected 10 different highly recommended antifungals viz. griseofulvin, clotrimazole, doxycyclin, fluconazole, amoxicillin, cephalotoxin, miconazole, cephlaxin, terbinaphine and itraconazole for tinea infection. The effectiveness of antifungals with respect to isolated fungal strain was determined by disc method. A wet disc (in antibiotic solution) was put carefully on plates with positive fungal growth. These plates were incubated at 37°C for 3-5 days for antibiotic susceptibility testing and inhibition zones around the fungal growth were measured.

RESULTS

Out of 100 soil samples collected from keratin rich areas of Jaipur, 90 samples were found positive and 6 species of keratinophilic fungi were isolated using 4 different keratinic baits (Fig.1). Compared to other keratinic baits, hair fragments were found most appropriate for growth of keratinophilic fungi because of less hardened structure than other baits, since these were easily degraded by the extracellular enzymes of fungal isolates.

Soil samples collected from different habitats of Jaipur were found alkaline with the exception of bird habitat found slightly acidic (pH=6.9). Alkaline soil promotes growth of keratinophilic fungi mainly *Chrysosporium tropicum* (pH-8.0), *Microsporum* sp. (pH-9.0) and *Trichophyton* sp. (pH-8.0) responsible for causing tinea infections (Sharma 2014). *Chrysosporium* sp., *Torula* sp., *Gymnoascus* sp., and

Fig.1. Baited plates with fungal growth.

Trichoderma sp. were also isolated in the soil samples of Jaipur because of their alkaline nature. *C. tropicum* was found to be dominant (70%) and most frequently observed group

Table 1. Fungal species isolated from soil samples of varied habitats of Jaipur with variable pH.

S.No.	Habitats	pHrange	*Chrysosporium* sp.	*Torula* sp.	*Gymnoascus*sp.	*Microsporum*sp.
1.	Vegetable market	7.92-8.24	-	-	-	+
2.	Garden areas	7.54-8.56	+	-	-	-
3.	Bird habitats	5.52-8.62	+	-	-	-
4.	Animal habitats	9.01-9.85	-	-	-	+
5.	Road Side	7.34-8.57	+	-	-	-
6.	Crop land	8.45-8.95	-	+	-	-
7.	Bus stand	5.52-8.38	-	-	+	-

from habitats such as sabji mandi, garden area, bird habitats and roadside soil samples. Secondly, maximum isolates (40%) of *Microsporum* sp. were recovered from bird habitats on feather bait. After it, *Trichophyton* sp. (34%) was found most frequently in roadside soil samples of Jaipur, India (Table 1).

Effect of Antifungal Agents

Antifungal agents affected growth of kertinolytic fungi. Based on size of inhibiting zone, fluconazole was found highly effective for *Microsporum* sp. while cephlatoxin and cephlaxin were the least effective (Table 2). Zone of inhibition of 3 antifungals are shown in Fig. 2. Standard drug i.e. griseofulvin was found least effective on *Chrysosporium* sp.(IZ-17mm) as compared to other species, for *Microsporum* sp.(IZ-21mm) and *Trichophyton* sp. (IZ-23mm) whereas fluconazole and terbinafine were the most effective on *Microsporum* sp. and *Trichophyton* sp. respectively (Table 2).

a) b) c)

Fig.2. Zone of Inhibitions (I.Z): a) Griseofulvin on *Chrysosporium* sp.; b) Fluconazole on *Microsporum* sp.; c) Terbinafine on *Trichophyton* sp.

Activity index (A.I) = Inhibition zone (I.Z.) of sample÷ Inhibition zone (I.Z.) of standard

Table 2. Activity index (A.I.) of different antifungal agents.

Antifungal agent	*Chrysosporium* sp.		*Microsporum* sp.		*Trichophyton* sp.	
	I.Z. (mm)	A.I	I.Z. (mm)	A.I	I.Z. (mm)	A.I
Griseofulvin (Standard)	17		21		23	
Clotrimazole	15	0.83	19	0.91	20	0.87
Doxycyclin	12	0.72	18	0.87	15	0.66
Fluconazole	18	0.84	23	1.12	19	0.84
Amoxicillin	12	0.68	17	0.80	16	0.69
Cephlatoxin	16	0.90	16	0.76	16	0.70
Cephlaxin	13	0.70	15	0.70	16	0.71
Miconazole	19	0.93	22	1.06	22	0.78
Terbinafine	14	0.78	18	0.86	26	0.97
Itraconazole	16	0.89	20	0.98	24	1.14

DISCUSSION

Keratinophilic fungus in or on soil degrades keratin material (Biodegradation) by breaking disulfide bonds present in keratin structure which could not be done by protease alone (Gupta & Ramnani 2006, Verma & Sharma 2017). This group of fungal species degrade complex molecule into simpler/low molecular weight compounds (Marchisio 2000). Soil is the major source of superficial fungal infection among humans

(anthropophiles) as well as in animals (zoophiles) (Adebiyi & Oluwayelu 2017, Peres et al. 2010).These infectious species are important ecologically as well as in study of superficial infection treatment (Sharma et al. 2015). From the past few years, number of cases related to superficial infections has increased. Some studies were also conducted to find out best medium for the growth of keratinophilic fungi. Mannitol Salt broth was found perfect for the growth of *Trichophyton* and *Microsporum* species (Kumar & Bhadauria 2017). On global scale, various studies have been done for understanding the role of soil which is a major contributing factor in causing infection and for treatment of these contagious infection on early basis (Randhawa & Sandhu 1964).

In present study, a total 6 species of keratinophiles isolated from 100 soil samples of keratin rich habitats of Jaipur were *Chrysosporium, Microsoprum, Torula, Gymnoascus, Trichophyton and Trichoderma*. We observed the prevalence of mainly 3 species *C. tropicum* (70%), *Microsporum* sp (40%) followed by *Trichophyton* sp. (34%) in pH range of 6.0-8.0. Where, *C.tropicum* was the dominant (70%) and most frequently observed group from sabji mandi, garden areas, bird habitats and from road side soil samples, followed by *Microsporum* sp.(40%). Maximum isolates of *Microsporum sp.* were recovered from bird habitats on feather bait and *Trichophyton* sp. (34%) found most frequently in roadside soil samples of Jaipur city. The similar studies were done by Deshmukh and Verekar (2006) on soil samples of Himachal Pradesh. They isolated five genera from 122 soil samples mainly *Chrysosporium queenslandicum* (25%), *Chrysosporium tropicum* (19%) and *Chrysosporium indicum* (11 %). Bhadauria & Kumar (2016) made similar studies on samples collected from Sawai Man Singh Hospital, Jaipur. They reported dominance of *Trichophyon* species. Many physico-chemical factors like pH, temperature and soil moisture were studied to determine the heterogeneous presence of keratinophiles. Bohme and Ziegler (1969) isolated keratinophilic fungi from 178 samples out of total 250 soil samples of Berlin with average 5.8 pH of soil, no fungal group was observed on highly acidic soil i.e pH < 5.0. Whereas, in present study pH of soil samples collected from Jaipur was found with acidic to alkaline range (5.0-9.0). The keratinophiles occur more frequentlyin weak acidic and weak alkaline soils. Sharma (2014) isolated most of the species at neutral and alkaline pH.They observed that *Trichophyton mentagrophytes* was the most predominant fungi reported from all sites at pH 6.8-7.3.*Microsporum gypseum* was the second most common reported fungi at 6.2 to 7.2 and *T. rubrum* was the third most common fungi at pH 7.0 to 7.7. Ramesh and Hilda (1999) reported 31 species of 15 genera with dominance of *C. tropicum* (62.2%) in 45 soil samples collected from Primary School and Public Parks. Jain and Sharma (2011) recovered new isolates *Trichophyton verrucosum, Microsporum audouinii* and *M. canis* in the soil samples from Jaipur. Marsella and Mercantini (1986) examined 161soil samples collected from Abruzzo National park with 2.5% presence of *Microsporum* sp. A same study report on garden soil of Tunisia determined the presence of *Chrysosporium keratophilum* (30.5%).

These keratinophilic fungi are pathogenic in nature causes superficial infections "ringworm" among human as well as in animals which is highly infectious. It is very important to treat these infections at early stages because their severity depend upon seasonal changes and life style (Bhadauria & Kumar 2015) Many researchers are working to find out the highly specific antifungals to treat these transmissible infections. According to Rahman and Nahata (1997), an antifungal Terbinafine is the first oral fungicide agent that blocks ergosterol formation at squlene epoxidation stage. Some studies have been conducted to check the maximum effectivity of natural antifungal agents obtained from plants to minimise the side effect caused by antifungal drugs (Bhadauria & Kumar 2012). Another study on antifungal drugs and their resistance was conducted to check the actual biochemical and molecualr effect of antifungals responsible for causing drug resistance (Nigam 2015).

Out of these 6 species, we had chosen 3 species on the basis of their dominance in Jaipur soil to test affectivity of of 10 antifungals to check their specificity for an infection. Guarro et al. (2000) found fluconazole and miconazole to be highly effective drugs for *Chrysosporium* sp. These workers also used miconazole containing shampoo against *Microsporum* sp. and *Trichophyton* sp. In present study, *Microsporum* sp. found highly susceptible to fluconazole and resistant to cephlaxin. Similarly terbinafine was more effective drug used for *Trichophyton* sp. compartive to others.

CONCLUSION

On routine basis, multiple cases of tinea infection have been reported because of regular contact of skin with infectious soils and therefore screening of polluted soils for kertinolytic fungi is important for controlling tinea infection. Amongst 100 soil samples of Jaipur, India *Chrysosporium* sp. was the most prevalent fungi. This step of isolation with comparative data of antifungals effectiveness provides a better insight in clinical treatment with highly effective and specific antifungal treatment.

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