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Microbiology

Isolation and identification of Malassezia species from patients of pityriasis versicolor and its comparison with normal subjects

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ABSTRACT

Background: Pityriasis versicolor is a superficial infection of the stratum corneum, caused by a group of yeasts formerly named pityrosporium. The taxonomy of these lipophilic yeasts has recently been modified and includes seven species referred as *Malassezia.*

Aims: The aim of this study is to compare the distribution of *Malassezia* species isolated from pityriasis versicolor lesions and those isolated from healthy skins.

Methods: This cross sectional epidemiological case-control study was carried out at LTMG Hospital, Mumbai. 116 clinically diagnosed patients of pityriasis versicolor and 50 healthy persons of all age group and both sexes were studied. Skin scrapping was inoculated on Leeming and Notman agar, followed by subculture on Sabouraud's dextrose agar with chloramphenicol and gentamicin with olive oil overlay as well as without olive oil. Biochemical tests and Tween assimilation test were performed. 10 per cent KOH mount and Grocott's Methenamine Silver (GMS) staining was also done. Statistical analysis was done using chi-square test.

Results: Out of the total 116 case, 104 were mycologically proved (direct examination or culture or both positive) only 38 were culture positive. Four species of Malassezia were isolated mainly from 38 culture positive cases. *M. sympodialis* 52.6 per cent (n=20), *M. globosa* 15.9 per cent (n=6), *M. slooffiae* 7.9 per cent (n=3) and *M. furfur* 2.6 per cent (n=1). All 50 healthy people were negative for fungus.

Conclusion: Although pityriasis versicolor has worldwide occurrence, its frequency is variable and depends on different climatic, occupational and socio-economic conditions. Both sexes and all age group are equally affected with slight predisposition to males. In our study most common species of Malassezia isolated was M. sympodialis (52.6 per cent).

KEYWORDS : Leeming Notman agar, Tween assimilation test, Catalase test, GMS staining.

Introduction: The incidence of pityriasis versicolor is particularly high in tropical countries than the temperate ones. The causative agent is the yeast of the genus *Malassezia*. Recently many new species of Malassezia have been identified on the basis of ribosomal RNA and characterized genetically, physiologically and biochemically.[1]

There is a variation in the susceptibility of various *Malassezia* spp. to different antifungal drugs like ketoconazole, voriconazole, itraconazole and terbinafine. Among the three azoles drugs, strains of all *Malassezia* spp. are most sensitive to ketoconazole and itraconazole. Strains of *M. sympodialis, M. slooffiae, M. pachydermatis* and *M. restricta* are most sensitive to terbinafine than those of *M. furfur, M. globosa* and *M. obtuse.* Hence correct identification of *Malassezia* spp. could facilitate selection of the appropriate antifungal therapy. [2]

There is a paucity of studies in India on the various species of Malassezia, associated with pityriasis versicolor as well as studies comparing in healthy individuals. Our study aiming to isolate, speciate various *Malassezia* spp. from clinically diagnosed patients of pityriasis versicolor and their comparison with the healthy individual as control.

Material and Methods:

This Cross sectional epidemiological Case-Control Study was carried out at LTMG Hospital, Mumbai, after granting approval by institutional ethics committee.

- **Test Group (Group-I)-**Total of 116 clinically diagnosed patients of pityriasis versicolor, attending the outpatient department of a general hospital during the study period. Patients of all age groups and both the sexes and were freshly diagnosed or without any antifungal medication for more than one month were included.
- **Control Group (Group-II) -** Comprised of 50 healthy persons, who were age matched with the test group**.** Healthy individuals of both the sexes and no history of antifungal therapy for any reason for past one month were included.

A detailed history of the patients was taken.

Collection of specimen:

- **Test Group:** The site of the lesion was properly disinfected with spirit and allowed to dry. Scrapings were collected with no. 15 Parker blade in a sterile Petri dish by gently scraping the lesion. The site which showed fluorescence on wood lamp examination was preferred for scraping. In patients with multiple site affection, specimens were collectively obtained from different sites.
- **Control Group:** Back was chosen as the preferred site. It was cleaned with spirit, allowed to dry. The site was scraped with no. 15 Parker blade and swabbed vigorously with a sterile saline soaked cotton swab (Since yield of scales from healthy skin is minimal, the area was swabbed to increase the yield.) which was directly inoculated on Leeming Notman agar (LNA).

As per other studies, [3,4] only culture was perform from swabs collected from the control group, direct examination was not done.

Processing of Specimen: (Table A)

- Direct Examination:

1. 10 per cent KOH mount was observed. (figure 1 and 2)
2. Simplified Grocott's Methenamine Silver (GMS) staining was done.[5] (figure 3)

- **Culture.**

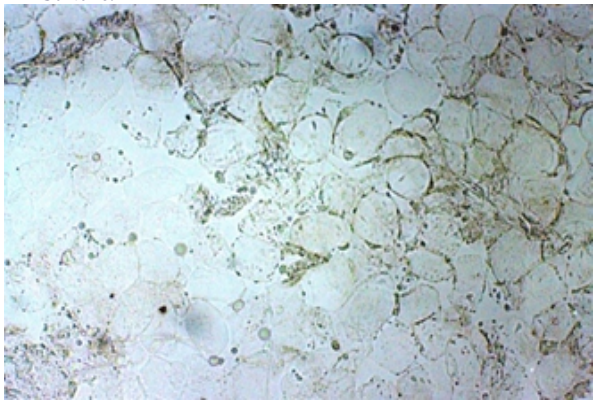


Figure 1: Skin scrapings from a patient of pityriasis versicolor showing group of yeast cells and occasional short hyphal elements.

KOH mount X 100.

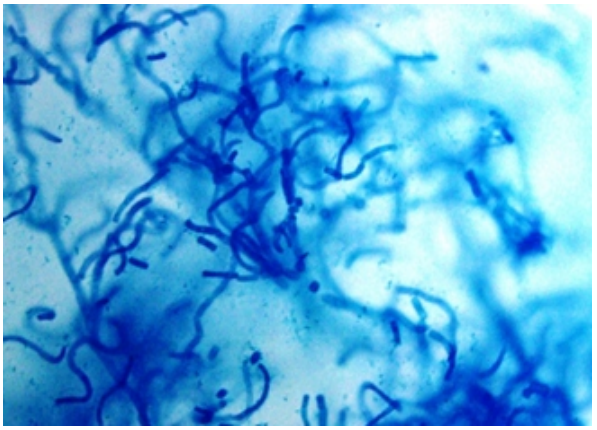


Figure 2: Skin scrapings from a patient of pityriasis versicolor showing occasional yeast cell and plenty of short hyphal elements. Methylene blue staining X 400

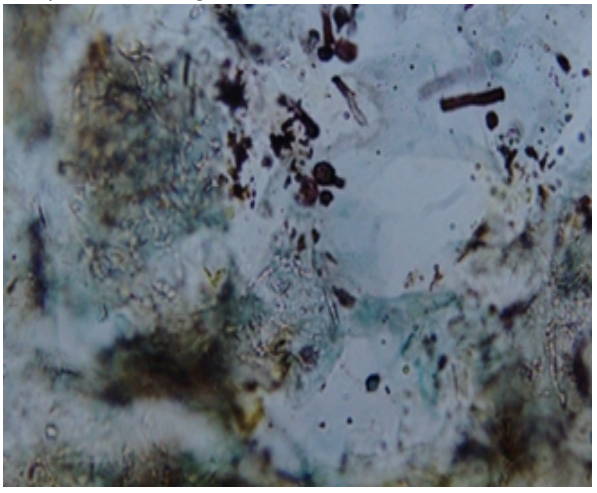
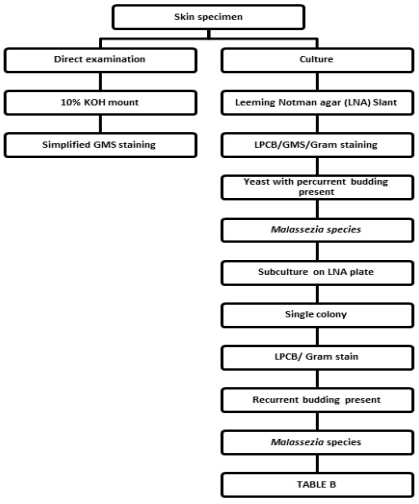


Figure 3: Yeast and short hyphal form of Malassezia seen in skin scrapings. Simplified Gomori's methenamine silver staining X 1000.

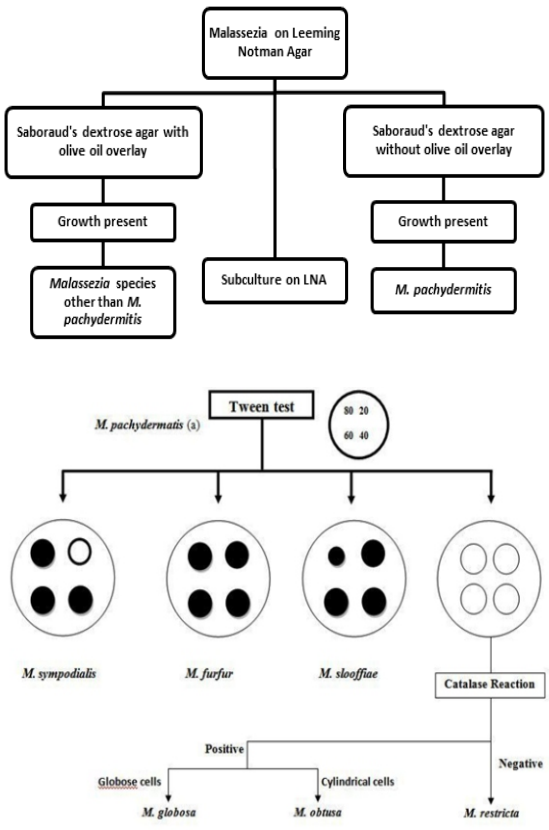
Table A
Processing of Specimen



Culture of Specimen:
The skin scraping was inoculated on LNA for primary isolation of Malassezia species (Table-A). Any growth which appeared on the agar was confirmed by staining. For species identification a single colony from LNA plate was processed for subculture on Sabouraud's Dextrose agar (SDA) with Chloramphenicol and gentamicin (Emmon's modification) with olive oil overlay as well as without olive oil. Biochemical tests and Tween assimilation test were performed. (Table –B, figure 4)

Figure 4: Culture of *Malassezia sympodialis* on Leeming Notman Agar. cream colored, rough textured, 1-2mm colonies seen on primary isolation.

Table B
Identification of various species of *Malassezia*



Leeming and Notman agar (LNA): used for primary isolation.[6]
Ingredients- Bacteriological peptone (10 gm), Dextrose (5 gm), Yeast extracts (0.1 gm), Ox bile* (4 gm), Glycerol (1 ml), Glycerol mono-strearate (0.5 gm), Tween 60 (0.5 ml), Whole fat cow milk (10 ml), Agar agar (17 mg), distilled water (1000 ml), Chloramphenicol (50 µg/ml) (By dissolving in 2 ml alcohol), Cycloheximide (200 µg/ml) (By dissolving in 2 ml acetone).

*We have used bacteriological Ox bile instead of Desiccated Ox bile.
Preparation- All components except Tween 60, Milk and antimicrobial agents were added to distilled water, gently heated to mix and allowed to stand for 10 mins. The Tween 60 was added at temperature of 60-70oC. Milk and antimicrobial agents were then added. The final pH was adjusted to 6.2. Autoclave at 121oC at 15 lbs for 20 mins, Mix well and dispensed in tubes/plates.

Inoculation & incubation- After inoculation, the culture was incubated at 32oC for 2 weeks. The culture was examined daily for first week and then alternate day in second week. Culture was reported negative after two weeks of incubation. Single isolated colony was obtained by platting on LNA which was then used for species identification as per flow chart given below. (Figure 5 and 6)

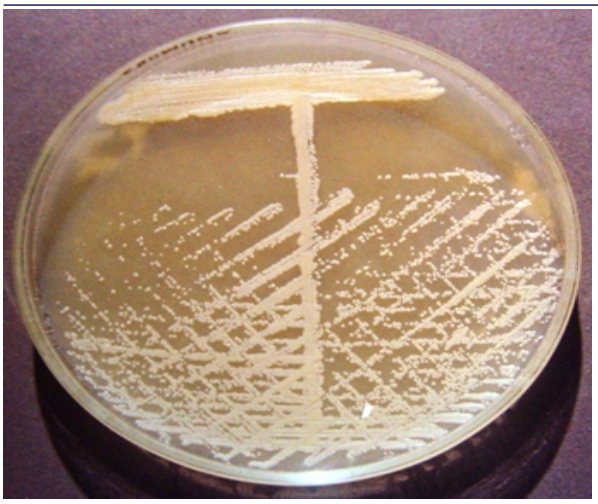


Figure 5: Isolated, small cream colored, convex, rough textured, colonies of Malassezia sympodialis on Leeming Notman Agar plate.

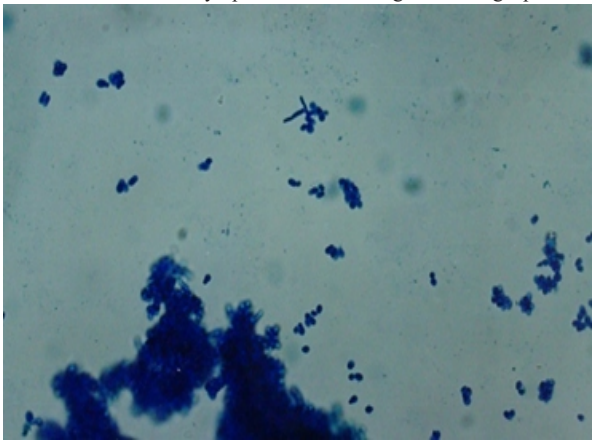


Figure 6: Lactophenol cotton blue mount prepared from a colony of *Malassezia sympodialis* showing yeast cells with characteristic percurrent budding X 400.

Sabouraud's Dextrose Agar (SDA) (Emmon's modification): To prepare SDA with oil overlay, sterile olive oil was poured over the surface of the agar and tube labeled according and the media inoculated. (Olive oil was sterilized in hot air oven at 160°C for one hour twice).

Respective subculture on LNA, SDA with oil and SDA without oil was incubated at 32°C for 2 weeks. Confluent growth was noted in LNA in one week which was used for yeast identification depicted on Table B. Growth on SDA was checked daily for two weeks. As growth appeared, it was noted.

Tween Assimilation test: With the help of a 1.3cm/0.8cm internal diameter punch four holes were made in the centre of each quadrant of SDA plate (Emmons modification) under sterile conditions. A lawn culture was made taking heavy inoculums from subcultured LNA using a sterile swab stick. 0.25 ml each of the following Tween compounds (Tween 20, Tween 40, Tween 60 and Tween 80) was poured using sterile syringe in the labelled wells of the SDA.

The plates were incubated upright at 32°C for 1 week. The plates were examined after 4 days and at the end of 1 week.

Interpretation: Growth of *Malassezia* spp. as concentric ring around the labelled Tween compound well indicates assimilation of the respective Tween. (Figure 7)

By Tween assimilation test *M. dermatis* cannot be differentiated by *M. furfur.* It can be differentiated by genotypic methods only. Since this species was described in year 2002 and *M. japonica* in year 2003, the identification of species in our study is limited to previously known seven species (not including *M. dermatis* and *M. japonica*).

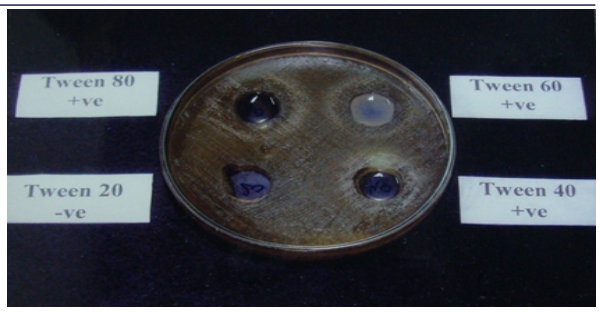


Figure 7: Tween assimilation test for identification of *Malassezia species*. Growth seen around the well containing Tween 40, 60 and 80 but no growth around 20, indicating *M. sympodialis*

Catalase test:

Principle: H_2O_2------$\xrightarrow{\text{CATALASE}}$----------> H_2O+ [O] gas

Procedure: Smear yeast on a clean grease free slide. Place a drop of 30 per cent H_2O_2 on the smear. Positive reaction is denoted by production of gas bubble with in 10 sec.

GMS staining: was performed to differentiate between *M. globosa* and *M. obtuse.*

Results:

Of the total 116 cases clinically diagnosed as Pityriasis versicolor, 104 were mycologically proved (Direct examination or culture or both positive). All the clinical specimens from patients of Pityriasis versicolor were positive on 10 per cent potassium hydroxide mounts were also positive on simplified GMS staining. GMS staining was used to fortify the presence of percurrent budding in primary and secondary smears which is a characteristic feature of *Malassezia genus.*

Table-1a

Direct examination and culture positivity for *Malassezia* species in patient group

Status of Direct Examination	Status of Culture	No.	Percentage
Positive	Positive	35	30.17
Positive	Negative	66	56.89
Negative	Positive	03	02.58
Negative	Negative	12	10.35
TOTAL	116	100	

Table 1b

Culture results of *Malassezia* in control group

Status of culture	No.	per cent
Negative	50	100

A total of 104 (89.6 per cent) patients showed evidence of *Malassezia* infection on 10 per cent KOH mount and GSM staining or on culture or both. Of these 104 patients, 101 patients were positive on direct examination and thirty-eight patients were culture positive. (Table 1a) Of these, three patients were positive on culture only but negative on direct examination. Thus the overall KOH positivity rate was 87.06 per cent and culture positivity rate was 32.75 per cent.

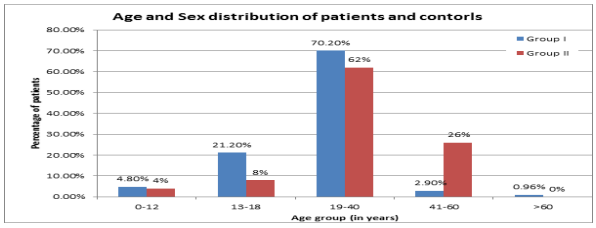
Further analysis was carried out in the 104 patients who were mycologically positive. Statistical analysis was done using chi-square test.

All the individuals belonging to the control group were negative on culture for *Malassezia.* (Table 1b)

Grouping of Patients:

Group I: constituted 104 mycologically positive patients of Pityriasis versicolor.

Group II: constituted 50 healthy aged matched controls.

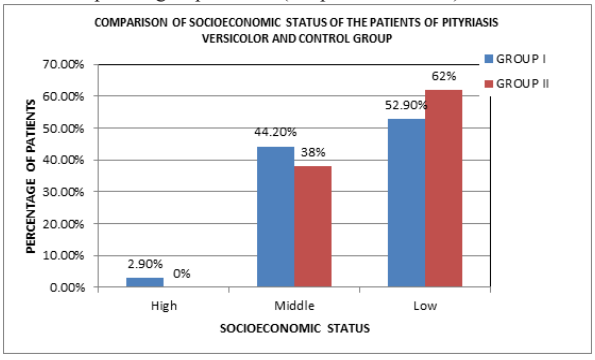


Graph-1 Age and sex distribution

Table 2
Age and Sex Distribution of Patients and Controls

Age Group	Group- I (Patients) N (%)			Group- II (Control) N (%)		
	Male	Female	Total	Male	Female	Total
0-12	01 (0.96)	04 (3.85)	05 (4.8)	01 (2)	01 (2)	02 (4)
13-18	16 (15.4)	06 (5.7)	22 (21.2)	03 (6)	01 (2)	04 (8)
19-40	52 (50)	21 (20)	73 (70.2)	25 (50)	06 (12)	31 (62)
41-60	02 (1.9)	01 (1)	03 (2.9)	11 (22)	02 (4)	13 (26)
>60	01 (0.98)	00 (0)	01 (0.96)	00 (0)	00 (0)	00 (0)
Total	72 (69.24)	32 (30.76)	104 (100)	40 (80)	10 (20)	50 (100)

The prevalence of pityriasis versicolor in patient group was found to be highest in the age group of 19-40 years (70.2 per cent), followed by the adolescent age group of 13-18 years (21.2 per cent). One patient was in the elderly age group of more than 60 years. The overall male to female ratio in the patient group was 3:1. (Graph 1 and Table 2)



Graph- 2 comparison of socioeconomic status of patients and control

Table 3
Comparison of socioeconomic status of the patients of pityriasis versicolor and control group

Socioeconomic Status	Group I		Group II	
	No.	%	No.	%
High	03	2.9	00	0
Middle	46	44.2	19	38
Low	55	52.9	31	62
Total	104	100	50	100

The highest prevalence of pityriasis versicolor was found to be in patients with low socio-economic status (52.9 per cent) followed by the middle socio-economic status (44.2 per cent). A very low prevalence of (2.9 per cent) was found to be in the higher socio-economic strata. The socio-economic status of the control group was comparable to the patient. (Graph 2 and Table 3)

Of the 104 patients, *Malassezia* species could be grown in culture in 38 (36.5 per cent) patients. The highest number of isolates (13) were from patients with neck and upper back affection (34.2 per cent), followed by chest and upper back affection (28.9 per cent). (Table 4A)

Table 4 A
Association of culture positivity of Malassezia with sites affected in patients with pityriasis

Site of lesion	Growth on culture	No growth on culture	Total
Upper back	07	14	21
Chest	02	02	04
Face	00	01	01
Neck	00	01	01
Chest and back	11	15	26
Neck and upper back	13	20	33
Back and face	02	01	03
Nack and chest	01	01	02
Back and axilla	01	00	01
Hand and back	01	10	11
Back and face and neck	00	01	01
Total	38 (36.5%)	66 (63.5%)	104 (100%)

Four species of *Malassezia* were isolated mainly, *M. sympodialis* in 20 patients (52.6 per cent). This was the commonest species isolated followed by *M. globosa in 6* patients (15.9 per cent), *M. slooffiae* in 3 patients (7.9 per cent) and *M. furfur* in one patient (2.6 per cent). In 8 patients though *Malassezia species* was isolated in culture as identified by typical percurrent budding, speciation was not possible as seven isolates were lost due to contamination. (Table 4B)

Table 4 B
Association of species of Malassezia with sites affected in patients with pityriasis versicolor

Site of lesion	Identification				Not identified	Total
	M. sympodialis	M. globosa	M. slooffiae	M. furfur		
Upper back	01	00	02	00	04	07
Chest	01	00	00	00	01	02
Face	00	00	00	00	00	00
Neck	00	00	00	00	00	00
Chest and back	08	00	00	00	03*	11
Neck and upper back	08	03	01	01	00	13
Back and face	01	01	00	00	00	02
Nack and chest	01	00	00	00	00	01
Back and axilla	00	01	00	00	00	01
Hand and back	00	01	00	00	00	01
Back and face and neck	00	00	00	00	00	00
Total	20 (52.6%)	6 (15.9%)	3 (7.9%)	1 (2.6%)	7+1* (21%)	38 (100%)

One isolate gave unusual pattern of tween assimilation.

From back and chest, one isolate showed a pattern of assimilation of Tweens which was different from the pattern shown by any of the known seven species and the two newly described species of *Malassezia* (*M. dermatis* and *M. japonica*) which have been very recently identified (year 2003). The said isolates from our study failed to assimilate Tween 20, 60 and 80 but assimilated only Tween 40.

Discussion:

Pityriasis versicolor is one of the most common disorders of cutaneous pigmentation in the world. [7] The prevalence of pityriasis versicolor in temperate climate is around one per cent but prevalence as high as 40-60 per cent has been reported in some tropical climates. [8, 9] The lesion may be more extensive in tropical climate. [10] The prevalence of pityriasis versicolor in a semi-urban community in Delhi was 0.34 per cent of the population as studied by Karanti K Bhalla. [11] The incidence of pityriasis versicolor in Mumbai has reported in various public hospitals as 1.4 per cent to 8.3 per cent of all Dermatomycosis. [12, 13] Bhushan Kumar *et al* [14] from Chandigarh reported that 71.5 per cent of the patients first noticed the appearance of lesion in summer months while 14 per cent first noticed the appearance of lesion in winter months. It can be explained on the basis of high ambient temperature and a high relative humidity predisposing to pityriasis versicolor. [15]

In our study, no seasonal variation was apparent probably because of a near constant range of temperature and humidity in Mumbai.

A Nakabayashi *et al* [4], from Japan have reported the prevalence of *Malassezia species* from 35 normal subjects. *M. globosa* was the maximum 22 per cent, followed by *M. sympodialis* 10 per cent, *M. furfur* 3 per cent, *M. slooffiae, M. pachydermatis; M. restricta* and *M. obtuse* were infrequently isolated from the normal skin. No Indian reports on carriage of *Malassezia* in healthy controls were available for comparison of our finding that 50 healthy controls studied were all negative in culture of *Malassezia.* A similar finding was noted by Abraham Z (1987) [16] in 60 healthy children who yielded no positive

specimen for *Malassezia.*

Pityriasis versicolor is common in young adults probably due to increased sebaceous gland activity during that age but a wide age variation is noted.[10,17] Miskeen A K *et al*[18] reported a prevalence of 6.7 per cent of pityriasis versicolor in children over a period of six years. The majority of patients were in the age group of 1-5 years (54.5 per cent) followed by 28.9 per cent in the age group of 5-10 year and least in the age of infancy (<1 year) i.e. 16.6 per cent.

Young adults are more commonly affected as was seen in our study in which 70.2 per cent of patients were in the age group of 19-40 years. Bhushan Kumar *et al*[14] have reported 73.7 per cent of patients in the age group of 16-30 years, 8 per cent were below 15 years and 18.3 per cent were above 30 years. The incidence of pityriasis versicolor decreases in later life,[19] this decreases may related to alteration to sebum production that decreases with age.[20]

Bhushan Kumar *et al* [14] and Roberts S O B [21] reported Male to Female (M: F) ratio of 2:1 and 1.5:1 respectively. Mohanty J *et al* from Department of Dermatology, Cuttack, India reported a sex ratio (M: F) of 3:1 in patients with extensive pityriasis versicolor.[22]

Our report of M: F ratio of pityriasis versicolor as 3:1 is in conformity with report of Mohanty J *et al*[22] and some other reports from India.

In a study, it was found that all socio-economic classes of population were equally affected by pityriasis versicolor.[14] In our study of the 116 clinically diagnosed patients of pityriasis versicolor, 101 (87.07 per cent) were positive on KOH as well as GMS staining. Gupta *et al*[23] has cited a microscopic positivity rate of 68.1 per cent of *Malassezia* element.

Mohanty J *et al* in 1999[24] noted a KOH positivity rate of 100 per cent in 22 patients of Pityriasis versicolor studied. Mohanty J *et al*[22] in their other study in 2001 it was 75 per cent.

Gupta A K *et al* have reported from Canada, a culture positivity rate of 43.8 per cent out of the total KOH mount positive samples using LNA for primary isolation, but the recovery of *Malassezia* species in pure culture from the lesion was successful in less than 50 per cent of cases despite the use of media and techniques well known to be optimal cultivation of organism.[23] Nakabyashi A *et al* [4] were able to speciate 17 isolate from 22 isolates (77.27 per cent) using modified Dixon media for culture and Tween assimilation test for speciation, from the isolates obtained from trunk. Three (14 per cent) were culture negative and 2 (9 per cent) were either unknown or contaminated. In our study, the culture positivity rate was 36.5 per cent.

Limitation: Gupta A K *et al* cited a loss of 45 (28.8 per cent) cultures of *Malassezia* mainly due to contamination, out of the total 156 growths of *Malassezia* obtained. Thus, only 111 subcultures were further analyzed by them.[23] The loss of *Malassezia* cultures in our study was 7 out of 38 (i.e. 18.7 per cent). *Malassezia* spp. is notorious for the low viability and difficult maintenance *in vitro*. These are the one of the main difficulties that affects the study of this genus.[25]

Gupta A K *et al* [23] have reported isolation of all *Malassezia species* except *M. pachydermatis* from pityriasis versicolor lesions. *M. sympodialis and M. globosa* were more common in 59.5 per cent and 25.2 per cent of the isolate respectively, which is in conformity with our study. In our study, the isolation rate of *M. sympodialis* was 52.6 per cent as that of *M. globosa* was 15.9 per cent.

Authors[4] have reported Tween assimilation test for speciation of *Malassezia species* that does not corresponds with any of the known pattern. 2 out of 22 have been reported by Nakabayshi *et al* as unknown/contamination. In our study also, one isolate fail to assimilate Tween 20, 60, and 80 but assimilated Tween 40, which does not correspond to any of the unknown patterns and has been categorized as not identified.

Results similar to our study were reported in a recent work in 2004 by Kindo A J *et al* 103 from south India.[26] The authors have reported from 70 cases, 48 (68.75 per cent) positive culture obtained on modified Dixon media. The commonest isolate was *M. sympodialis* (58 per cent) followed by *M. globosa* (40 per cent) and *M. restricta* (2 per cent) this was not a case control study. Thus *M. sympodialis* appears to be the commonest species affecting Indian population.[26]

Conclusion: *Malassezia* is an important etiological agent of Cutaneous and systemic disease. It has been associated with a wide range of superficial infections. Though it commonly causes a seemingly innocuous disease like pityriasis versicolor, it is capable of serious life threatening systemic infection also.

The recently identified species *M. dermatis* can be differentiated from *M. furfur* by only genotypic methods. Thus genotypic characterization of phenotypically identified species is another challenge for the scientist.

Variation in the susceptibility of the *Malassezia* species to various antifungal drugs *in vitro* emphasizes the need of studied on drugs susceptibility *in vitro* with reference to the various species of *Malassezia.*

A proper understanding of the epidemiology of the *Malassezia* which help in building up data to perform advanced studies**.**

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