“ISOLATION OF *MALASSEZIA* SPECIES FROM CLINICALLY DIAGNOSED CASES OF PITYRIASIS VERSICOLOR AND SEBORRHEIC DERMATITIS AND DETERMINATION OF ANTIFUNGAL SUSCEPTIBILITY OF THE ISOLATES’’

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DOCTOR OF MEDICINE

IN

MICROBIOLOGY

INSTITUTE OF MICRBIOLOGY
MADRAS MEDICAL COLLEGE
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APRIL 2015

CERTIFICATE

This is to certify that this dissertation titled **"ISOLATION OF *MALASSEZIA* SPECIES FROM CLINICALLY DIAGNOSED CASES OF PITYRIASIS VERSICOLOR AND SEBORRHEIC DERMATITIS AND DETERMINATION OF ANTIFUNGAL SUSCEPTIBILITY OF THE ISOLATES"** is a bonafide record of work done by **DR.M.JERSEY GAYATHIRI**, during the period of her Post Graduate study from 2012 to 2015 under guidance and supervision in the Institute of Microbiology, Madras Medical College and Rajiv Gandhi Government General Hospital, Chennai- 600003, in partial fulfilment of the requirement of **M.D MICROBIOLOGY** degree Examination of The TamilnaduDr. M.G.R Medical University to be held in April 2015.

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DECLARATION

I declare that the dissertation entitled **"ISOLATION OF *MALASSEZIA* SPECIES FROM CLINICALLY DIAGNOSED CASES OF PITYRIASIS VERSICOLOR AND SEBORRHEIC DERMATITIS AND DETERMINATION OF ANTIFUNGAL SUSCEPTIBILITY OF THE ISOLATES"** submitted by me for the degree of M.D. is the record work carried out by me during the period of **October 2013 – September2014** under the guidance of **Dr.T.Sheila Doris, M.D.,** Professor, Institute of Microbiology, Madras Medical College, Chennai. This dissertation is submitted to the TamilnaduDr.M.G.R. Medical Unversity, Chennai, in partial fulfilment of the University regulations for the award of degree of M.D., Branch IV (Microbiology) examination to be held in April 2015.

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INTRODUCTION

Fungi are widely distributed in environment, but hardly 400 species are recognized as primary human and animal pathogens, of which less than 100 are encountered in routine clinical practice.[8] Most of the fungi which were considered non-pathogenic or mere laboratory contaminants are now being encountered as significant emerging agents of fungal diseases. Depending on the site of primary involvement, fungal infections may be classified as Superficial, Cutaneous, Subcutaneous and Systemic mycoses.[8]

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PAGE: 1 OF 106

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TABLE OF CONTENTS

S.NO.	TOPIC	PAGE NO.
1.	INTRODUCTION	1
2.	AIMS & OBJECTIVES	4
3.	REVIEW OF LITERATURE	5
4.	MATERIALS AND METHODS	41
5.	RESULTS	58
6.	DISCUSSION	86
7.	SUMMARY	98
8.	CONCLUSION	102
9.	APPENDIX -1- ABBREVIATIONS	
	APPENDIX - 2 – MEDIA, STAINS AND REAGENTS	
10.	ANNEXURES	
11.	BIBLIOGRAPHY	

ISOLATION OF*MALASSEZIA* SPECIES FROM CLINICALLY DIAGNOSED CASES OF PITYRIASIS VERSICOLOR AND SEBORRHEIC DERMATITIS AND DETERMINATION OF ANTIFUNGAL SUSCEPTIBILITY OF THE ISOLATES

ABSTRACT

INTRODUCTION:

Ever since its inception, the genus *Malassezia* has been a topic of intense basic research in various aspects including taxonomy, physiology, epidemiology, metabolomics and immunology. The etiopathologic links between these yeasts and many superficial and systemic infections have been unveiled by clinical research. Pityriaisvericolor (PV) and seborrheic dermatitis (SD) are superficial skin infections that have an established causal association with *Malassezia* species.

STUDY BACKGROUND AND OBJECTIVES:

The objective of this study is to make use of the biochemical and physiologic differences among the various species of *Malassezia* to gain an insight into the epidemiology and the risk factor associations of these yeasts with pityriasisversicolor and seborrheic dermatitis in this part of our country, known for its hot and humid climate, which favours the growth of these yeasts and also to determine the antifungal susceptibility of the isolates to the commonly used azoles and amphotericin B.

MATERIALS AND METHODS:

This study was done at Madras Medical College, Chennai for a period of one year involving 100 clinically diagnosed cases of *Malassezia* infections, of whom 73 had pityriasisversicolor and 27 had seborrheic dermatitis. Skin scrapings from the lesional sites were collected using strict aseptic precautions and processed bystandard microbiological methods. Christensen's urease medium with the addition of Tween compounds was used for antifungal susceptibility testing of the *Malassezia* isolates to fluconazole, itraconazole, ketoconazole, voriconazole and amphotericin B.

RESULTS:

Pityriasisversicolor commonly occurred in the 20 – 29 years age group (42.5%) and seborrheic dermatitis in the 40 -49 years age group (44.5%).

Males were affected more commonly than females with both PV (72.6% males) and SD (74% males). Majority of the PV patients presented with lesions on the back (50.7%). Face and scalp (63%) were the common sites involved in SD. Majority of PV patients had hypopigmented lesions (63.1%). The overall culture positivity rate was 70%, the yield being higher with PV lesions. Among the PV cases, 92.6% of the hyperpigmented lesions were culture positive. *M. globosa* was the most common species isolated in PV and SD. Low MIC ranges and low MIC_{50} values of < 1 μg/ml were noted with amphotericin B, ketoconazole and itraconazole for most of the *Malassezia* species.

CONCLUSION:

Culture is an important step in the diagnosis of *Malassezia*infections due to interspecies variability exhibited by *Malassezia* species in the etiological role in different geographical areas and differences in their susceptibility patterns to antifungal agents. Culture and antifungal susceptibility testing in the management of *Malassezia* infections will lead to a better targeted approach, reducing the cosmetic concerns, chronicity and recurrences associated with these infections.

INTRODUCTION

Fungi are widely distributed in environment, but hardly 400 species are recognized as primary human and animal pathogens, of which less than 100 are encountered in routine clinical practice.[8] Most of the fungi which were considered non-pathogenic or mere laboratory contaminants are now being encountered as significant emerging agents of fungal diseases. Depending on the site of primary involvement, fungal infections may be classified as Superficial, Cutaneous, Subcutaneous and Systemic mycoses.[8]

Infection limited to the outermost layers of the skin and its appendages with little or no tissue reaction is said to be superficial mycoses, which is by far the commonest type of fungal involvement.[8] Pityriasis versicolor and seborrheic dermatitis are such superficial mycoses caused by fungi belonging to the genus *Malassezia. Malassezia* species are part of the indigenous human flora found in the stratum corneum of the back, chest, scalp and face, which are rich in sebaceous glands.[9] Though considered as commensal yeasts of human and animal skin, these fungi have been implicated in inducing or aggravating the following conditions:

- superficial skin infections like pityriasis versicolor, seborrheic dermatitis, atopic dermatitis, folliculitis, papillomatosis, onychomycosis and external ear infections,[8]
- pneumonia,[8]
- peritonitis[8, 28] and also

- fungemia in patients receiving parenteral lipid supplementation, especially preterm neonates.[8, 28, 89]

The net outcome of the fungus – host relationship determines the establishment of the disease process.[11] This is particularly significant in the present era of advanced medical facilities, where the number of immunocompromised hosts is on the rise.

The recognition of the significance of *Malassezia* had been progressing slowly due to the difficulty in the isolation of these lipophilic yeasts in the routine laboratory setting. In the recent years, research on this genus has been expedited by the introduction of molecular methods, which have contributed to the understanding of its epidemiology, pathogenesis and management by robust interventional strategies, given that these yeasts are not only involved in superficial infections, but also disseminated infections and that different species exhibit varying sensitivity patterns[18] to the antifungal agents. To date, there are 14 species of *Malassezia* that have been isolated from human and animal skin.[29]

Pityriasis versicolor and seborrheic dermatitis, the two most common manifestations of *Malassezia,* though not alarming infections, are conditions known for their chronicity and recurrence and also for raising cosmetic concerns in those affected by these conditions.[8,11]

This part of our country is known for its hot and humid climate that favours the growth of *Malassezia* yeasts, and hence an understanding of the distribution of

these yeasts and the dominant species prevalent, will serve to complement previous studies conducted in India and elsewhere in determining the varying trends in distribution that may exist in different geographical locations.

This study purports to analyze the clinicoepidemiological profile of patients clinically diagnosed to have pityriasis versicolor and seborrheic dermatitis by making use of the physiological and biochemical differences among the various species of *Malassezia*. Also, an understanding of the locally prevalent antifungal susceptibility patterns of these yeasts would serve as a guide in the choice of appropriate antifungal agents, as *Malassezia* species exhibit variable susceptibility patterns. Though molecular methods are considered accurate and less time consuming for the confirmation of *Malassezia* species, simple methods based on evaluation of morphological, physiological and biochemical characteristics are more economical and adoptable by almost any laboratory and are hence employed in this study.

AIMS & OBJECTIVES

AIMS AND OBJECTIVES

- To identify and isolate *Malassezia* yeasts from patients clinically diagnosed with **Pityriasis versicolor.**

- To identify and isolate *Malassezia* yeasts from patients clinically diagnosed with **Seborrheic dermatitis.**

- To speciate the isolated *Malassezia* yeasts from pityriasis versicolor and seborrheic dermatitis using physiological and biochemical tests.

- To analyze the clinical patterns of pityriasis versicolor and seborrheic dermatitis infections with respect to epidemiological attributes and other risk factors.

- To determine the antifungal susceptibility of the *Malassezia* isolates obtained from pityriasis versicolor and seborrheic dermatitis lesions.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

HISTORY

Malassezia was first identified as the causative agent of pityriasis versicolor by Eichstedt in 1846 and by Sluyter in 1847.[1] In 1953, the yeast was named *Microsporum furfur* by Robin, as it was closely related to the dermatophyte *Microsporum audouinii*. Baillon recognized the error and created the genus *Malassezia*.[11] The description of *Malassezia* was given in 1874 by the French scientist Louis Charles Malassez, who identified budding 'spores' in the epidermis of patients with seborrheic dermatitis. [1]

Spherical and oval budding cells were observed by Bizzozero, who named them *Saccharomyces sphaericus* and *Saccharomyces ovalis*, respectively. Baillon in 1889, used the designation *Malassezia furfur* in honour of Malassez.[8] Sabouraud in 1904, proposed the genus *Pityrosporum* to describe budding yeast cells without hyphal elements. Castellani and Chalmers in 1913 were the first to isolate the lipophilic oval budding cells from normal skin and patients with seborrheic dermatitis and to coin the term *Pityrosporum ovale*.[8]

Previously, the mycelial phase of the organism was designated *Malassezia* and the yeast phase was called *Pityropsorum orbiculare* (round cells with budding from narrow neck) or *Pityrosporum ovale* (oval yeast cells with budding from wide neck). On the basis of the distinct cell surface antigens, Cunningham and

colleagues in 1990 defined the serovars A, B and C. These serovars are now identified as *M. sympodialis, M. globosa* and *M. restricta*, respectively.[44]

The lipophilic nature of the genus *Malassezia* was discovered in 1939 by Rhoda Benham.[11] Von Abbe in 1964 described the Dixon medium for the isolation of *Malassezia* yeasts which was subsequently modified for better yield.[28]

TAXONOMY

Taxonomically, the genus *Malassezia* is classified under the Family Cryptococcaceae of form Class Blastomycetes in the order Malasseziales in Phylum Basidiomycota, based on its morphologocial and biochemical characteristics like

- laminar ultrastructure of cell wall
- ability to hydrolyse urea
- positive staining with Diazonium Blue B (DBB)[8]

The taxonomy of the genus *Malassezia* has been controversial since 1889, when Baillon first created it on identifying *Malassezia furfur*. *Malassezia furfur,* the first species to be identified has been known by various other names including *Pityrosporum orbiculare, Microsporum furfur, Sporotrichum furfur, Oidium furfur, Malassezia tropica, Malassezia macfadyeni.* [11]

The genus has been revised based on morphology, physiology, cellular characteristics, temperature requirements, biochemical reactions, dependence on

long chain fatty acids, guanine and cytosine content of cellular DNA and ribosomal RNA sequences to include fourteen species.[2,3,4,6]

The fourteen species are enumerated below along with the name of the scientist who first reported the species and the year of reporting:

***Malassezia*spp.**	**Authors who first reported**	**Year**
M. furfur	Baillon	1889
M.pachydermatis	Dodge	1925
M. sympodialis	Simmons and Gueho	1990
M. globosa	Midgley, Gueho and Guillot	1996
M. obtusa	Midgley, Guillot and Gueho	1996
M.restricta	Gueho, Guillot and Midgley	1996
M. slooffiae	Guillot, Midgley and Gueho	1996
M. dermatis	Sugita, Takashima, Nishikawa, Shinoda	2002
M. japonica	Sugita, Takashima, Kodama, Tsuboi and Nishikawa	2003
M.yamatoensis	Sugita, Tajima, Takashima *et al*	2004
M. nana	Hirai, Kano, Makimura, Yamaguchi, Hasegawa	2004
M. caprae	Cabanes and Boekhout	2007
M. equi	Cabanes and Boekhout	2007
M. cuniculi	F. J. Cabanes *et al*	2011

ECOLOGY:

Malassezia species are mesophilic and have an absolute requirement of lipids for growth and survival. Hence, they are known to inhabit the skin of only warm-blooded animals.[11] *M. pachydermatis* is known to cause canine ear infections. *M. furfur* and *M. obtusa* have also been isolated form otits externa in dogs and *M. sympodialis* from cats. Many lipophilic species of *Malassezi*a have also been isolated from pigs, monkeys, rhinoceros, bears and birds. [11]

Crespo Erchiga *et al* by their studies on healthy human skin have concluded *M. sympodialis* to be the most common species isolated from normal human skin, especially the trunk.[64] It can be found alone or in combination with *M. globosa* in the skin of face.[64] Midgley has reported a higher incidence of *M. globosa* in the human scalp.[2]

Japanese authors and others have obtained varied results probably due to differences in the sampling techniques, culture media used and also due to geographic and ethnic factors.[4, 5]

Sugita *et al* found *M. furfur, M. sympodialis, M. globosa*and*M. restrcia*to be the four common species in the scalp and nape of healthy individuals, using a nested polymerase chain reaction technique.[5]

PATHOGENICITY OF MALASSEZIA:

Pityriasis versicolor (PV):

PV is the most common condition associated with *Malassezia* fungi. It is a chronic, recurrent superficial skin infection involving the stratum corneum, resulting in fine scaly hypopigmented to hyperpigmented lesions of various shades.[10, 28] It most commonly involves upper trunk, neck, face and upper arms. Recent studies by culture identification and characterization of physiological and morphological features have suggested *M. globosa* to be the most common species associated with PV.[63, 68]

Seborrheic dermatitis (SD):

SD is a chronic inflammatory dermatitis, where erythematous skin is covered with greasy, white, furfuraceous flakes. In its mildest form involving the scalp, it is called dandruff.[8] The areas commonly affected are scalp, face, chest, back, nasolabial folds and flexures.[28]

M. globosa, M. sympodialis and *M. restricta* are the common species isolated from SD lesions.[8] SD is recognized as an early indicator of AIDS in immunocompromised individuals.[27]

Atopic dermatitis (AD):

Atopic dermatitis is a chronic dermatosis that is intensely pruritic. It occurs in individuals with a genetic predisposition to atopy. Erythema, papules and pruritis are common in neck, face and scalp of the affected. A vicious itch-

scratch-itch cycle occurs. Itching results in secondary excoriations and lichenification of the skin. [8]

Malassezia yeasts and fungal componenets from *Cryptococcus diffluens* and *Cryptococcus liquefaciens* may play a role in the pathogenesis of AD by acting as allergens. Yellowish desquamation of the scalp in the neonates, known as 'cradle cap' is a presentation of atopic dermatitis.[8]

Folliculitits:

Earlier known as *Pityrosporum* folliculitis, *Malassezia* folliculitis is a chronic inflammatory condition characterized by acneform, pruritic eruptions in the form of pustules and erythematous papules. The areas commonly involved are upper back, anterior chest, arms and shoulders. [8]

The yeast is present in the ostium and central and deep segments of hair follicle. This condition commonly affects young women. Acne vulgaris and staphylococcal folliculitis are among the conditions included in the differential diagnosis of *Malassezia* folliculitis.[28]

Systemic infections:

Systemic infections like pneumonia, peritonitis, fungemia and other disseminated infections due to *Malassezia* have been reported in immunocompromised individuals and neonates with cardiovascular diseases and those receiving lipid hyperalimentation.[9]

The portal of entry has been found to be intravenous catheters, the usual manifestation being lipid deposition in the pulmonary arteries. *M. furfur* has been recovered from the yeast-containing cutaneous pustules and positive blood cultures in these conditions.[51, 72]

Other conditions associated with *Malassezia*:

Malassezia may play a role in onychomycosis, confluent reticulated papillomatosis of Gougerot and Carteaud and obstructive dacryocystitis.[11]

M. pachydermatis, previously thought to be a zoophilic organism, has been linked to a series of neonatal infections in a nursery, that spread from a healthcare worker whose pet dog was colonized with the fungus.[89]

A recent study has also linked neonatal cephalic pustulosis, also known as neonatal acne to *M. sympodialis*.[62]*Malassezia* species have also been isolated from cases of onychomycosis and hyperproliferative lesions of psoriasis.[28, 55]

Recently, based on a metabolomics research, a hypothesis has been proposed, which links radiation induced basal cell carcinoma to aryl hydrocarbon receptors synthesized by *Malassezia* yeasts**.**[97]

PITYRIASIS VERSICOLOR

SYNONYMS:

Tinea versicolor, dermatomycosis furfuracea, chromophytosis, tinea flava, liver spots are the various names given to pityriasis versicolor.[11]

INTRODUCTION:

PV is a scaling, superficial, pigmentary dermatoses that occurs when *Malassezia* overgrowth extends to adjacent skin from the follicles. The name 'tinea versicolor', by which it was known earlier is a misnomer and 'tinea' is reserved for dermatophytic infections.[8]

The term 'pityriasis versicolor' is particularly apt in that the color of the lesion varies according to the skin colour of the patient, sun exposure and severity of the disease (pityriasis is derived from a Greek word 'ptyra' meaning abnormal proliferation and versicolor is derived from a Latin word meaning many colours).[11]

In the past, PV was considered as a mark of beauty in some rural areas of Sri Lanka and hence treatment was not sought. In local terms it was referred to as 'Gomora', meaning 'tears of liquid gold'.[8]

CLINICAL PRESENTATION:

PV may present in various forms as follows:

- Flat, fine scaly, hypopigmented or hyperpigmented 0.5 – 1 cm macules on the trunk, that may coalesce to form larger plaques or gyrating areas of intermittent scaling of different colors and shades in the skin of neck, arms, chest and back.[11]
- Inflammatory pustular lesions that may be scaling or non-scaling [29]
- Lesions affecting face, extremities and flexural areas that is commonly seen in immunocompromised individuals.[29]

Lesions on dark or tanned skin tend to be hypopigmented (achromic PV). In white skinned individuals, the lesions are hyperpigmented (chromic PV), appearing darker than the surrounding skin.[29]

Both hypopigmented and hyperpigmented lesions may occur in the same individual in different locations.[10] Various factors influence the colour variation, including the thickness of the horny layer, severity of infection and the metabolic products released by the organism.[30]

Early lesions are perifollicular in origin.The lesions may be aymptomatic or pruritic. Irritation or inflammatory response is rare, but some may present with reddening and pruritus especially after sun exposure.[10] The main complaint is cosmetic, the affected areas failing to tan normally.[11]

The scaly nature, unique to PV can be demonstrated by light scratching with a scalpel or microscopic slide or fingernail, the so called *coup d' angle* of Besnier or scratch sign or chip sign or fingernail sign.[11] This sign is absent in early lesions and in previously treated patients.[31, 32]

PATHOGENESIS:

As the causative fungus is universally present as normal flora of skin, it may be considered as an opportunist that elicits disease under special situations.[11] Lee *et al*, by scanning electron microscopic studies showed that a morphological change from the saprophytic yeast form to filamentous mycelial form is important in the pathogenesis of *Malassezia* infections.[33] A reduction in the turnover of squamous cells by endogenous or exogenous corticosteroids may lead to development of PV.

Malassezia are dependant on exogenous lipids, especially 12 to 14 carbon fatty acids for their growth. Human and animal skin, rich in free fatty acids, serve as media for their growth and survival. Metabolism of skin surface lipids by lipoxygenase enzymes of *Malassezia*, yields fatty acids that are toxic to the melanocytes. [13]

Many theories have been postulated to explain the pigment alterations associated with PV:

1. Studies by Nazzaro – Porro in 1993 showed that the metabolism of oleic and vaccenic acids by *Malassezia* yielded lipid fractions like C_9 to C_{11} dicarboxylic acids. One such metabolite is azelaic acid, a dicarboxylic acid which competitively inhibits tyrosinase in the melanin synthesis pathway leading to smaller and fewer melanosomes and also inhibits the mitochondrial enzymes, leading to vacuolation and degeneration of the melanocytes, thus resulting in hypopigmentation.[34]

2. In studies by Allen et al and Charles et al, hyperpigmented lesions were found to show an increased turnover rate of stratum corneum, i.e, 8 days compared to 15 days for normal areas and also the presence of singly distributed, large melanosomes in contrast to multiple – packaged, smaller melanosomes in normal areas.[11]

3. Mayser and coworkers, by their studies on the metabolism and nutritional requirements of *Malassezia* species in 1998, have concluded the colour change of the affected skin in PV to reflect the exclusive property of *M. furfur* to assimilate L- tryptophan as a nitrogen source.[15, 16]

4. Raabe *et al* in 2000, have confirmed the pigmentogenesis of *M. furfur*, based on their observations of non-lipid-dependent *M. pachydermatis* that produces lower pigment. The indole pigment pityriacitrin produced

exclusively by *M. furfur* acts as a UV filter, preventing normal tanning.[17,84]

5. Borgers *et al,* by scanning and transmission electron microscopic studies, have confirmed the penetration of *Malassezia* into the keratinocytes, causing degradation of the keratin into an amorphous lipid–like substance, that acts as a potential ultraviolet light blocker and results in hypopigmentation.[35]

6. The hyperpigmentation (chromic type of PV) is said to be multifactorial, caused by the presence of large number of the causative fungi, increased thickness of the keratin layer, increased turnover rate of cells in the stratum corneum, increased perivascular infiltration of lymphocytes and increased inflammatory responses that stimulate the melanocytes. Hyperpimented lesions have been found to contain more hyphae and spores than the hypopigmented ones.[36]

PATHOLOGY:

The budding yeast cells and hyphae are confined to the outermost layers of stratum corneum. If very large numbers are present, they may be seen cemented together as a continuous layer, replacing the outer cells of the stratum corneum. The organism may be seen as a mass near the hair follicle and extending into the follicular opening. The yeast cells are 2 - 8 μ in diameter and hyphae about 3 – 4 μ in width. [11]

IMMUNOLOGY:

Normal epithelial turnover in the skin precludes infection. In chronic cases, antibodies are detectable. Specific IgG, Ig A and IgM antibodies are induced by *M. furfur.Malassezia* may induce psoriasis-like lesions by activating the alternate complement pathway.[12]

EPIDEMIOLOGY:

Pityriasis versicolor is worldwide in distribution. Hot and humid environmental conditions favour the infections and so it is more common in tropical and subtropical climates than in temperate ones, where the infections may occur only in summer.[25]

In tropical countries, the incidence of PV is about 35 – 40%, while in the temperate climates it may be as low as 1 – 4 %. The true prevalence however, is very difficult to estimate, as the affected individuals seek medical attention only for cosmetic reasons.[21]

PV occurs commonly in adolescents and young adults, in whom sebum production is higher than in other age groups.[11] A severe form of disease occurring in infants as a depigmenting diaper rash (achromia parasitica) may be seen in some climates.[10]

The amount of colonizing yeasts in different age groups seems to correlate with variations in sebum secretion that are age-dependent.[10]

AGE GROUP	PERCENTAGE OF SKIN COLONISATION WITH MALASSEZIA YEASTS
0 – 10 years	5 – 15 %
11 – 20 years	56 – 90 %

Predisposing factors for the infection include heredity, glucocorticoid medications, chronic infections, ill health, hyperhidrosis, greasy skin and exogenous oil application.[25]

DIFFERENTIAL DIAGNOSIS:

Pityriasis rosea, other pigmentary disorders like vitiligo and chloasma, erythrasma, secondary syphilis and pinta[11] have to be differentiated from pityriasis versicolor.

SEBORRHEIC DERMATITIS (SD)

SYNONYM: Seborrheic eczema

INTRODUCTION:

Seborrheic dermatitis is a chronic papulosquamous disorder, affecting 3 – 5 % of the general population, predominantly children and young adults. Seborrhoea is derived from the Latin word 'sebum' meaning 'grease'and the Greek word 'rhoea' meaning 'flow'.[8]

Cradle cap in neonates can also occur as a part of a generalized disorder called Leiner disease, associated with diarrhea and failure to thrive.[22]

CLINICAL FEATURES:

SD manifests as erythematous, pruritic scaly lesions in the scalp and face, particularly, forehead (especially glabella), external auditory canal, nasolabial folds, retroauricular area, eyebrows and moustache and also the presternal area.[8,25]

The individual lesions may be dull-red, sharply marginated papules or macules on a greasy, erythematous base with extensive scaling and crusting with variable pruritis.[9]

In patients with advanced Acquired Immuno Deficiency Syndrome (AIDS) with low CD4 counts, seborrheic dermatitis is often severe and difficult to treat.[9]

RISK FACTORS FOR SD:

Immunosuppression, prematurity, Human Immunodeficiency Virus (HIV) infection, Parkinson's disease, dark skinned races, alcoholism, emotional stress, endocrine disorders associated with obesity, facial Psoralen Ultraviolet A therapy (PUVA), all predispose to seborrheic dermatitis.[26]

A higher incidence of SD has also been seen in patients with pityrosporum folliculitis and pityriasis versicolor.[26]

Drugs that can trigger SD-like eruptions include griseofulvin, cimetidine, methyldopa, buspirone, chlorpromazine, phenothiazine, methoxsalen, interferon – α, ethionamide, etc.[25]

PATHOGENESIS:

Therapeutic studies with the antifungal agent ketoconazole, have suggested the causative role of *Malassezia* in seborrheic dermatitis.[23] The observation that people with Parkinson's disease who typically show increased sebum secretion on account of dopamine deficiency have a markedly higher incidence of SD, supports the role of sebum in the causation of SD.[24]

Higher skin surface levels of squalenes and free fatty acids (FFA) have been found in patients with SD. The resident flora of the skin, i.e, *Propionibacterium acnes* and *Malassezia* have lipase activity, that transforms triglycerides to free fatty acids. The FFA and the reactive oxygen species (ROS) thus produced, have antibacterial activity that alters the normal skin flora. This disturbance in flora, lipase activity and ROS may be linked to SD.[25]

Recent studies have shown an increased production of aryl hydrocarbon receptor (AhR) ligands by *Malassezia* species isolated from patients with seborrheic dermatitis.[97] An interplay of the AhR and EGFR (Epidermal Growth Factor Receptor) has also been suggested to play a causative role in SD.[97]

PATHOLOGY:

Malassezia may grow in large numbers in and around the hair follicles and sebaceous glands. Histopathologic studies have shown their presence in follicular ostia, infundibulum of pilosebacous canal and the surrounding dermis. The follicles are dilated and plugged with keratinous debris. Parakeratosis containing

sebum and neutrophils are seen at the ostia (called ‘follicular lipping’). Inflammatory infiltrates of lymphocytes and neutrophils are seen in follicular epithelium.[22]

In SD associated with HIV infection, plasma cells and apoptotic keratinocytes may also be observed. These changes contribute to folliculitis and seborrheic dermatitis.[11]

IMMUNOLOGY:

Abnormal immune response to normal levels of *Malassezia* species on the skin, resulting in depressed T- helper cell response and lower levels of phytohaemagglutinin and concanavalin may be observed.[12]

An increase in the number of CD16 and NK -1 cells in seborrheic dermatitis have been demonstrated by Faergemann *et al*[87]. A disturbed lymphocytic cellular immune response and the resulting elevated IL-10 and reduced IL-2 and IFN- γ can lead to complement activation via the direct and alternative pathways.[25]

SD IN HIV AND AIDS:

Extensive SD is seen in patients with HIV and AIDS, that is refractory to standard therapy. On histopathological examination, much more necrosis, lymphocytosis, focal leukocytosis and parakeratosis are common compared to the seronegative controls. SD usually occurs with CD4 counts of 200 – 500 cells / cu.mm as one of the earliest skin manifestations in HIV patients.[27]

EPIDEMIOLOGY:

The incidence of seborrheic dermatitis has been estimated to be 3 – 5 % of the general population. A male predominance is seen in all ages for SD.[25] In the United States, the reported prevalence of SD is high, reaching 11.6%.[25]

A generalized form of seborrheic dermatitis is rarely seen in infants and may be linked to immunodeficiencies.[22]In individuals with Parkinson's disease and those with neutropenia, the incidence and severity of SD have been found to be higher.[8]

Flare ups are common with seasonal fluctuations in humidity and temperature, particularly with low humidity in the winter and early spring.[25]

Though a family history of SD has often been reported, it was only recently that a mutation in ZNF750 encoding a Zinc finger protein C2H2 had been identified, resulting in autosomal dominant seborrhea-like dermatitis.[25]

DIFFERENTIAL DIAGNOSIS:

Acne vulgaris, dermatophytosis, psoriasis, candidiasis, atopic dermatitis,acne rosacea, systemic lupus erythematosus, Langerhans cell hsitiocytosis are among the conditions that should be differentiated from seborrrheic dermatitis.[8]

DIAGNOSIS OF MALASSEZIA INFECTIONS

The diagnosis of superficial skin infections caused by *Malassezia* is mainly clinical. Laboratory diagnosis in the form of potassium hydroxide mount can be done to differentiate from other similar conditions.[25]

Culture is rarely done for diagnostic purposes, but carries importance in research and epidemiological settings and for confirmation in case of systemic infections.

Pityriasis vesicolor can be detected by the characteristic fluorescence on Wood's lamp examination.[8, 25]

WOOD'S LAMP EXAMINATION:

Wood's lamp was invented by Robert Woods, a Baltimore physicist. It was initially used by Margaret and Devez for detection of dermatophyte infections of hair.[37]

Wood's lamp consists of a high pressure mercury arc that emits long wavelength ultraviolet light and a filter made of 9% nickel oxide and barium silicate (called 'Wood's filter'), that transmits light of wavelength between 320 – 400 nm, being opaque to all other wavelengths of light. Fluorescence of the illuminated tissue results when this UV light is absorbed and a visible light of longer wavelength is emitted.[38] Skin is fluorescent due to its constituents like

aromatic amino acids, melanin and its precursors and elastin. Normal skin is very faintly fluorescent or not at all.[39]

Before using a Wood's lamp, it should be ensured that

- The light source is at a distance of 4 inches from the object to be illuminated
- The lamp is allowed to warm up for 1 – 2 min
- The room should be dark and the examiner must get adapted to the dark to be able to appreciate the contrast clearly.
- Topical creams and ointments and other cosmetics should be wiped off thoroughly from the area to be visualized in order to avoid false positive fluorescence due to petroleum jelly or salicylic acid.[40, 41, 42]

Advantages of Wood's lamp:

Wood's lamp is small, inexpensive, easy to handle requiring no expertise. It can be used in the outpatient department for instant diagnosis.[38]

In the case of pityriasis versicolor, yellowish orange or golden yellow fluorescence is produced due to coproporphyrin.[24] It is also useful in detecting sub-clinical lesions and the extent of skin involvement.[43]

SPECIMEN COLLECTION:

The nature of the specimen to be collected in infections caused by *Malassezia,* depends on the type of involvement. For superficial skin infections, skin scrapings and punch biopsy of the lesional skin may be sampled. Scrapings

or clippings from the involved nail in case of onychomycosis, hair sample in case of folliculitis, peritoneal fluid in case of peritonitis, respiratory secretions in case of pneumonia, catheter tip and venous blood in case of fungemia are to be examined.[9, 28, 11, 30]

In superficial skin infections like PV and SD, skin scrapings have proven to be simple, minimally invasive and informative samples.

I. Collection of skin scrapings:

The skin in the affected area is disinfected with 70% isopropyl alcohol to remove surface contaminants. The area is allowed to dry and scrapings are obtained on to a sterile dark paper or an alcohol cleansed, flame-sterilized glass slide, from the active edges of the lesion using the blunt end of a no. 15 flame–sterilized scalpel blade or the edge of a glass slide.[8]

The specimens from scalp may best be obtained by scraping with the blunt end of the scalpel so that the hair stubs, scales and the contents of the plugged follicles are included.[8]

Alternative methods:

Alternative methods adopted for specimen collection are the following:

- **Scotch tape method:**

  A Vinyl Adhesive Tape (also called VAT or scotch tape) can be pressed firmly over the affected area of the skin and the scales can be collected for preparation of potassium hydroxide mount. The tape is placed on a glass

slide with a few drops of 10% KOH in the centre .[44] This method is especially useful when scales are minimal. The disadvantage of this method is that culture cannot be done.

❖ **Contact plate method:**

A culture plate containing suitable agar can be pressed directly on the affected skin for culture purpose. The disadvantage of this method is that direct KOH examination cannot be done.[71]

❖ **Wood's lamp method:**

When the lesional area is indistinct, an initial Wood's lamp examination to delineate the infected area may be done, followed by sampling by any of the above mentioned methods.[8]

DIRECT MICROSCOPY:

A) Potassium hydroxide (KOH) mount:

The scales obtained by scraping or scotch tape method from suspected superficial skin lesions are examined with 10 – 20% KOH, which digests the keratin and cellular debris and enables visualization of fungal elements.[8, 28]

Modifications of KOH mount:

- Dimethyl sulfoxide (DMSO) may be added to the preparation, which allows better visualization by acting as a clearing agent.[8]
- Methylene blue may be added to delineate fungal elements[28]

- Albert's stain stains the yeast cells and hyphae purple, delineating them from the background of keratinocytes.[8]
- Parker Quink's blue or black ink can be used with KOH to enhance the contrast[28]

B) Calcoflour white stain(CFW):

CFW is a water-soluble, non-toxic, colourless fluorescent whitener and textile dye that selectively binds to cellulose and chitin of the fungal cell wall. When viewed under fluorescent microscope, it fluoresces light blue on exposure to UV light (340 – 365 nm).[8]

CFW stain is superior to conventional KOH mount in that it provides a better differentiation of hyphal elements and collagen fibres.

Appearance in direct microscopy:

On direct microscopic examination of the KOH, modified KOH or CFW preparation, *Malassezia* species exhibit the characteristic 'banana and grapes' or 'spaghetti and meat ball' appearance, showing clusters of round or oval 2 – 7 μ yeast cells and short (about 20 – 25 μ), stout, septate, sometimes phaeoid appearing hyphal elements.

II. Punch biopsy:

Punch biopsy of skin obtained from the lesional area may be subjected to histopathological examination after staining with Gomorri's methanamine silver, Geimsa, haematoxylin and eosin or periodic acid Schiff stain.[28]

Presence of yeast cells and hyphal elements may be noted in the stratum corneum, which is thickened, compared to normal skin. The thickening is more pronounced in the hyperpigmented type of PV.[46]

CULTURE:

All *Malassezia* species are lipophilic (with the exception of *M.pachydermatis*), requiring 7 – 10 days for growth on lipid supplemented media.[11] Nonetheless, culture may be essential to establish the diagnosis in *Malassezia* infections.

Growth requirements:

Fatty acids are essential for the *in vitro* growth of all *Malassezia* species except *M. pachydermatis.* Replication of the organism is supported by saturated or unsaturated fatty acids of C_{12} to C_{24} series. Conversion of yeasts to mycelial form is supported on media containing glycine, glucose, mineral oils, glycerol monostearate or Tween 80.[14] Glycine has a unique positive effect on the growth of *M. furfur.*[11]

Malassezia yeasts grow ideally at a temperature of 30 - 35˚C. Temperatures above this range are inhibitory to most species. Preservation of *Malassezia* species is best done by freezing at -80˚C, as the cultures do not survive temperatures of 4 - 8˚C.[8]

CULTURE MEDIA:

The following media support the growth of *Malassezia* species:

1. Sabouraud Dextrose Agar with an overlay of olive oil[8, 28]

2. Modified Dixon's agar with 3.6% malt extract, 0.6% peptone, 1% Tween 40, 2% desiccated ox bile, 0.2% glycerol, 0.2% oleic acid, that provides substantial growth of the yeast with distinctive morphholological patterns for identification.[8, 28]

3. Leeming and Notman medium with whole fat cow's milk, that has a longer shelf-life and a higher recovery rate.[8,28]

4. GYP-S agar with peptone, glucose, yeast extract, olive oil, Tween 80, glycerol monostearate to provide quicker growth and quantitation of colonies.[8]

5. A minimal medium with lipid source and L- typtophan to selectively induce a diffusible brown pigmentation specific for *M. furfur*. This medium does not support the growth of some strains of *M.pachydermatis* and *M.sympodialis*.[8]

It is recommended that all the above media be supplemented with cycloheximide and chloramphenicol, as all *Malassezia* species are part of commensal flora of human and animal skin.[28]

Modified Dixon's medium and Leeming and Notman agar are the two most widely used media for culture. The former is preferred over the latter by many authors as its dark coloration makes recognition of colonies easier when more than one species is isolated and also enables better counting of colonies.[28]

All inoculated culture media should ideally be incubated at a temperature of 30 - 35˚C (preferably at 32˚C), for a period of 7 – 10 days and examined daily for growth.[25]

COLONY MORPHOLOGY ON MODIFIED DIXON'S AGAR:

The morphological features of the colonies of commonly isolated *Malassezia* species on modified Dixon's agar are as follows:

***Malassezia furfur* :**

M. furfur generates cream coloured, thick, convex, umbonate, 4 – 5 mm colonies with a smooth to rough surface.[8, 28]

***Malassezia pachydermatis*:**

M. pachydermatis forms cream coloured, thick, convex colonies with a matte surface and brittle texture, making it difficult to emulsify.[8, 28]

***Malassezia sympodialis*:**

M. sympodialis forms cream to buff coloured, flat, easily emulsifiable colonies with a slight central elevation and a smooth shiny surface.[8, 28]

Malassezia globosa:

M. globosa produces 4 mm cream to buff colonies with a rough and deeply folded surface and a brittle texture and hence are difficult to emulsify. [8, 28]

***Malassezia obtusa*:**

M. obtusa is a slow growing species and forms small colonies with a sticky texture. [8, 28]

***Malassezia restricta*:**

M. restricta is slow growing and forms irregular, small, 2 mm sized, cream coloured colonies with a hard texture.[8, 28]

***Malassezia slooffiae*:**

M. slooffiae forms 3 mm colonies, with finely folded margins and a brittle texture. [8, 28]

GRAM STAINING:

Gram staining of the colonies of *Malassezia* species show gram positive budding yeast cells, with cellular morphology characterisitic of each species. They are as follows:

M. furfur:

The gram positive yeast cells of *M. furfur* are morphologically heterogeneous, showing globose, cylindrical or oval yeast cells 4 – 5 µ long, with some filamentous forms (pseudohyphae without dolipores).[28]

M. pachydermatis:

The yeast cells are 2 – 4 μ oval to cylindrical with broad based budding.[28]

M. sympodialis:

The yeast cells are 2- 5 μ ovoid cells, with narrow based sympodial budding, giving a 'clover leaf' appearance.[28]

M. globosa:

The yeast cells are spherical, 6 – 8 μ diameter with narrow based buds and pseudofilaments.[28]

M. obtusa:

The yeast cells are among the largest in the genus, being up to 10 μ long and with broad based budding.[28]

M. restricta:

The yeast cells are 2 – 4 μ spherical to oval, with narrow based buds.[28]

M. slooffiae:

The yeast cells are short cylindrical and never globose or elongated. Buds are broad based.[28]

BIOCHEMICAL CHARACTERIZATION:

Conventional assimilation and fermentation tests employed in the speciation of other yeasts cannot be used for these non–fermentative, lipophilic species.[8, 28] The various tests used to identify *Malassezia* species are as follows:

Urease test:

All *Malassezia* species are capable of producing urease enzyme that hydrolyzes urea in the Christensen's urea agar or broth medium modified by the addition of 0.5% Tween 40, turning the colour of the medium pink.[8, 73]

Catalase test:

20 – 30% hydrogen peroxide can be used to detect the catalase activity of *Malassezia* species. *M. restricta* is the only species that is catalase negative.[67, 68]

Esculin hydrolysis test:

Beta glucosidase enzyme present in some species of *Malassezia* hydrolyzes esculin in esculin agar medium, releasing esculetin that reacts with iron in the medium to cause blackening of the agar.[68, 77]

This enzyme activity is positive with *M. sympodialis, M. obtusa, M.cuniculi* and *M. caprae*, weakly positive or absent with *M. furfur, and variable with M. pachydermatis*.[28]

Growth at 42˚C:

M. sympodialis, M. furfur, M. pachydermatis, *M. dermatis, M.japonica, M.slooffiae* and *M. cuniculi* are the species capable of growth at 42˚C.[28, 77]

Tween assimilaton test:

The ability of the individual *Malassezia* species to utilize different Tween compounds, i.e, 10% Tween 20, 0.5% Tween 40, 0.5% Tween 60 and 0.1% Tween 80 as the sole source of lipid is tested by tween assimilation test.[28]

Tween compounds are water – soluble, nonionic surfactants, which are polymers of ethylene oxide linked to sorbitan and a lipophilic group.[28]

Tween 20 - polyoxyethylene (20) sorbitan monolaureate

Tween 40 - polyoxyethylene (20) sorbitan monopalmitate

Tween 60 - polyoxyethylene (20) sorbitan monostearate

Tween 80 - polyoxyethylene (20) sorbitan monooleate

Cremophor EL assimilation test:

Cremophor EL is polyoxyethylated ricinoleate, a non – ionic surfactant. Growth of certain strains of *M. furfur* and *M. pachydermatis* is promoted by 1 – 10% cremophor EL.[28, 77]

CHARACTERISATION OF MALASSEZIA SPECIES:

Based on the results of the above biochemical tests, the 14 species of *Malassezia* can be identified as follows according to the scheme suggested by Guillot *et al* and later modified by Mayser *et al* and Gueho *et al*.[2, 3, 4, 5]

Species	**Catalase**	**Esculin hydrolysis**	**Tween 20**	**Tween 40**	**Tween 60**	**Tween 80**	**Growth at 42˚C**
M. furfur	+	-/W+	+	+	+	+	+
M.pachy dermatis	+/V	V	+/V	V	V	V	+
M. sympodialis	+	+	+/-	+	+	+	+
M. globosa	+	-	+	-	-	-	-
M. obtusa	+	+	-	-	-	-	-
M. restricta	-	-	-	-	-	-	-
M. dermatis	+	V	+	+	+	+	+
M. japonica	+	V	-	+	+	-	+
M. yamatoensis	+	V	+	-	-	-	-
M. sloofiae	+	-	-	-	-	W+	+
M. nana	+	-	+	+	V	V	V
M. caprae	+	+	V	V	V	V	-
M. cuniculi	+	+	-	-	-	-	+
M. equina	+	-	V	V	V	-	-

V – Variable; W+ Weakly positive

All *Malassezia* species produce urease enzyme and hence are urease test positive.

ANIMAL PATHOGENICITY:

Experimental studies to infect common laboratory animals like guinea pigs and Swiss white mice have resulted in the development of experimental dermatitis showing hyperkeratosis at pilous bulbs and follicular ostia.[8, 28]

IMMUNODIAGNOSIS:

Cell mediated immunity can be assessed by lymphocyte blastogenesis in response to specific fungal antigens. Antibody titers specific to *Malassezia* species can be determined by solid phase ELISA.[8] Indirect immunofluorescence staining of *Malassezia* yeasts in scales and cultures is possible by.[11]

MOLECULAR DIAGNOSIS:

Molecular methods provide definitive identification of the fungus for diagnostic and epidemiological purposes. They lead to a better understanding of the epidemiology and ecology of *Malassezia* species and aid in rapid screening of large number of isolates in epidemiological surveys.[28]

Estimation of the G+C content of chromosomal DNA and 25S rRNA sequencing were the methods initially employed. Other methods include karyotyping, restriction fragment length polymorphism (RFLP), multilocus enzyme electrophoresis (MLEE), PCR fingerprinting, randomly amplified polymorphic DNA analysis (RAPD) and PFGE.[8, 28]

Heterogeneity within the species has made RAPD unreliable for speciation. Karyotyping, though robust, is labour intensive and time consuming. At present, PFGE is the only method that allows reliable differentiation of various strains of *Malassezia* species.[8, 28]

ANTIFUNGAL SUSCEPTIBILITY TESTING:

Determination of minimal inhibitory concentration (MIC) by broth microdilution method :

The Clinical Laboratory Standards Institute (CLSI) has recommended a broth microdilution method for testing the antifungal susceptibility of yeasts (Document M27-A3). However, this method is not applicable for the susceptibility testing of *Malassezia* species because they are lipophilic, except for *M. pachydermatis*. Hence standardized assays are not available for the determination of the *in vitro* susceptibilities of these yeasts to any of the antifungal agents.

As all *Malassezia* species are urease positive,[19] a modification of the CLSI M27-A3 document method using Christensen's urea broth with the addition of 0.5% Tween 40 and 0.1% Tween 80, has been suggested by some authors[20, 86] for evaluation of the optimal antifungal susceptibility patterns of the isolates. Various other media have been evaluated in different studies, notable among which are

- RPMI 1640 with 1% glycerol, 1% peptone, 1.8% glucose, and 0.05% Tween 80[86]
- Modified Leeming and Notman medium[88]

(The above media measure growth inhibition)

- Modified Leeming and Notman broth with alamar blue, a calorimetric indicator[88](measures metabolic activity)

Though numerous antifungal susceptibility profiles have been reported for yeasts in general, very few are available for the different *Malassezia* species. Earlier studies were mostly done on *M. furfur*. The *in vitro* susceptibility of *Malassezia* species to various antifungal agents has been performed by Gupta and Colleagues[8] and other authors.[88, 91 – 94] Studies have shown that the susceptibility patterns to azole antifungals have been species dependent, with low MICs (<1 μg/ml) for most *Malassezia* spp. and high endpoints (MIC_{90} values 1 to ≥8 μg/ml) for *M. globosa* and *M. restricta.*[88, 91 – 94]

TREATMENT OF *MALASSEZIA* INFECTIONS:

The three major groups of drugs in clinical use for the treatment of superficial infections caused by *Malassezia* yeasts are azoles, polyenes and pyrimidines. The azole and polyene antifungal agents are directed against ergosterol, that plays a key role in maintaining the fluidity and integrity of the fungal plasma membrane and in the proper functioning of membrane bound enzymes. Inhibition of14 α-demethylase by azoles results in ergosterol depletion and accumulation of sterol precursors, leading to the formation of an ineffective plasma membrane with altered structure and function. [90]

Topical creams, lotions and shampoos of 2% ketoconazole, 2% miconazole, 1% clotrimazole, whitfield's ointment, 2.5% selenium sulphide, 10% sulphur, 1% terbinafine, naftifine, 1% zinc pyrithione, 50%propylene glycol, 25%

sodium hyposulfite, 6% benzoyl peroxide and 1% ciclopirox oleamine are effective in treating *Malassezia* infections. In addition, mild topical steroid creams may be used in seborrheic dermatitis.[9]

Treatment has to be given for 2 – 4 weeks in case of mild PV and longer in extensive involvement. For SD, medicated shampoos are used once or twice weekly for 4 weeks and thereafter once or twice monthly for maintenance.[29]

Fluconazole or itraconazole in a dose of 200 mg/ day or ketoconazole 400 mg/ day may be used for 5-7 days for extensive disease. A single dose of Fluconazole 400 mg may be used as an alternative for PV. Pulse therapy of fluconazole 150 mg once or twice weekly for 2 – 3 months is being practiced.[9, 10]

Ketoconazole is to be taken with breakfast, including an acidic juice, and the patient should be advised to avoid bathing for the next 12 hours, as the primary route of drug delivery to the skin is through sweat.[11]

A significant proportion of patients however, have experienced failure with azole treatment due to the development of drug resistance in *Malassezia* species. The drug resistance may be attributed to the variable genotypes of azole – metabolizing CYP51 enzyme in *Malassezia* yeasts.[97]

Twice daily intermittent application of 50% propylene glycol for 2 weeks has been used for the prevention of relapse.

New topical agents, calcineurin inhibitors like tacrolimus 0.03% for children and 0.1%for adults and pimecrolimus 1% are used for their immunomodulatory effects.[8]

Fungemia may be treated with amphotericin B or fluconazole, prompt removal of the intravascular catheter and discontinuance of parenteral lipid infusions.[9]

Griseofulvin and flucytosine are not effective in the treatment of *Malassezia* infections.[8, 89]

PROGNOSIS:

Malassezia skin infections are benign and self-limited, but recurrences are common because they are caused by endogenous flora.[9] 60 – 80% of the infections relapse or recur on account of genetic factors, hormonal factors or immunosuppression.[9]

PV is known for its chronicity, though long intervening periods of no lesions may be seen. Pigment alterations in PV may take months to resolve, especially with the hypopigmented variety.[11]

SD in infants is self-limited with a good prognosis while it is relapsing and chronic in adults. Generalised flares and erythroderma also occur.[25]

MATERIALS &
METHODS

MATERIALS AND METHODS

This study was carried out in the Mycology section of the Institute of Microbiology, Madras Medical College, Chennai, for a period of one year, enrolling patients from the Dermatology outpatient department (OPD) in Rajiv Gandhi Government General Hospital, Chennai.

Study design and study period:

This study was a Cross-sectional study, done for a period of one year from October 2013 to September 2014.

Study population:

The study population consisted of one hundred patients, of both sexes and all age groups, attending the Dermatology OPD of Rajiv Gandhi Government General Hospital, Chennai, who satisfied the following criteria:

Inclusion criteria:

1. Patients with superficial skin lesions clinically diagnosed as pityriasis versicolor or
2. Patients with superficial skin lesions cinically diagnosed as seborrheic dermatitis
3. Only those patients whose skin scrapings were positive for the characteristic ‘spaghetti and meat ball’ appearance on potassium hydroxide mount were included in the study.

Exclusion criteria:

1. Patients on topical antifungals within the preceding 2 weeks of visit,
2. Patients on systemic antifungals within the preceding 4 weeks of visit and
3. Patients whose skin scrapings were negative on KOH mount

were excluded from the study.

Ethical clearance [Annexure 1] was obtained for the study from the Institutional Ethics Committee, and informed consent [Annexure 3] was obtained from all the patients who participated in the study. Patients belonging to study population were interviewed with a structured questionnaire [Annexure 2].

COLLECTION OF DATA:

Patients who satisfied the inclusion criteria were interviewed using a preformed structured questionnaire for collection of demographic details like name, age, sex, address, clinical data like presenting complaints, personal history, past medical history, history suggestive of associated immunocompromised state and family history.

SPECIMEN COLLECTION:

The patients were made to sit comfortably in a well - lit area and the lesional sites were inspected. The sites were cleansed with 70% isopropyl alcohol, allowed to dry and scrapings were obtained from

1. hypopigmented or hyperpigmented scaly lesions in case of pityriasis versicolor, and

2. erythematous, greasy-looking areas of skin covered with loose flakes in the case of seborrheic dermatitis

on an alcohol cleansed, flame-sterilized glass slide (7.5 x 2.5 cm) using the blunt end of a flame-sterilized no.15 size scalpel.[8]

DIRECT EXAMINATION:

POTASSIUM HYDROXIDE (KOH) MOUNT:

The scrapings were placed on a clean microscopic glass slide and a drop of a prepared mixture of 10% KOH with an equal volume of Parker Quink's permanent blue ink [Appendix II - 1]was added.[28] A coverslip was placed on the preparation and left aside for 10 to 20 minutes for digestion of keratin and other debris that might otherwise preclude better visualization of fungal elements. Parker Quink's ink was added, so that the fungal elements take up the ink and stain bright blue against a background of digested keratinocytes.[1, 8, 28]

Examination under low power and high power objectives of the microscope revealed the characterisitic 'banana and grapes' or 'spaghetti and meatball' appearance of the short hyphal forms and yeast cells of *Malassezia*.[21]

CULTURE:

The skin scrapings that showed the characteristic 'banana and grapes' appearance suggestive of *Malassezia* on KOH mount were inoculated under strict aseptic precautions onto two Sabouraud Dextrose Agar (SDA) slants [Appendix II - 5] containing 0.05% chloramphenicol and 0.05% cycloheximide in 6 x ¾ inches

size test tubes, and an overlay of sterile olive oil was made using a sterile disposable syringe on one of the slants. The inoculated slants were incubated at 32˚C for 7 – 10 days and examined daily for cream coloured colonies with flat or raised surface. Tubes that showed no growth after two weeks of incubation were discarded and considered culture negative.

GRAM STAINING:

Gram staining [Appendix II - 2] of the above colonies showed Gram positive spherical or oval yeast cells with unipolar narrow–based or broad–based budding.[1, 8]

BIOCHEMICAL TESTS:

The following biochemical tests were done to identify *Malassezia* species:

1. Urease test
2. Catalase test
3. Esculin hydrolysis test
4. Growth at 42˚ C
5. Tween assimilation test

UREASE TEST:

All*Malassezia* speices are capable of hydrolyzing urea and this test, though not useful in speciation, is useful to eliminate cultures other than *Malassezia* like Ascomycetes and *Candida,* which commonly occur in the skin.[28]

A loopful of growth from a 5 to 7 day old culture was suspended in urea broth [Appendix II - 4] and incubated at 37˚C. The results were read in 3 to 4 hours and in case of doubtful reactions, the results were again read after 24 hours.[28, 86] All cultures that showed a colour change from yellow to bright pink in urea broth indicated the presence of urease enzyme and hence considered positive for urease test. Any isolate that showed no colour change was considered negative for urease activity and those cultures were discarded as negative for *Malassezia* species.

CATALASE TEST:

The enzyme catalase present in the members of the genus *Malassezia* breaks down hydrogen peroxide to produce nascent oxygen, which is evident by the production of effervescence. Using a sterile glass rod, a colony of *Malassezia* species was taken and immersed in a sterile test tube containing 3% hydrogen peroxide.[2] Immediate effervescence suggested presence of catalase enzyme. Lack of catalase enzyme activity is characteristic of *M. restricta*.[8, 28, 78]

ESCULIN HYDROLYSIS TEST:

Beta glucosidase enzyme activity of *Malassezia* yeasts was assayed using esculin agar [Appendix II – 6]. A loopful of colony was inoculated deep into the esculin agar and incubated at 37˚C for 24 hours. A brownish black discoloration of the medium indicated the presence of beta glucosidase activity.*M. sympodialis* and *M. obtusa* are positive for beta glucosidase acitivity, while the other species are negative and hence show no discoloration of the esculin agar medium.[8, 28, 48]

GROWTH AT 42 ˚**C:**

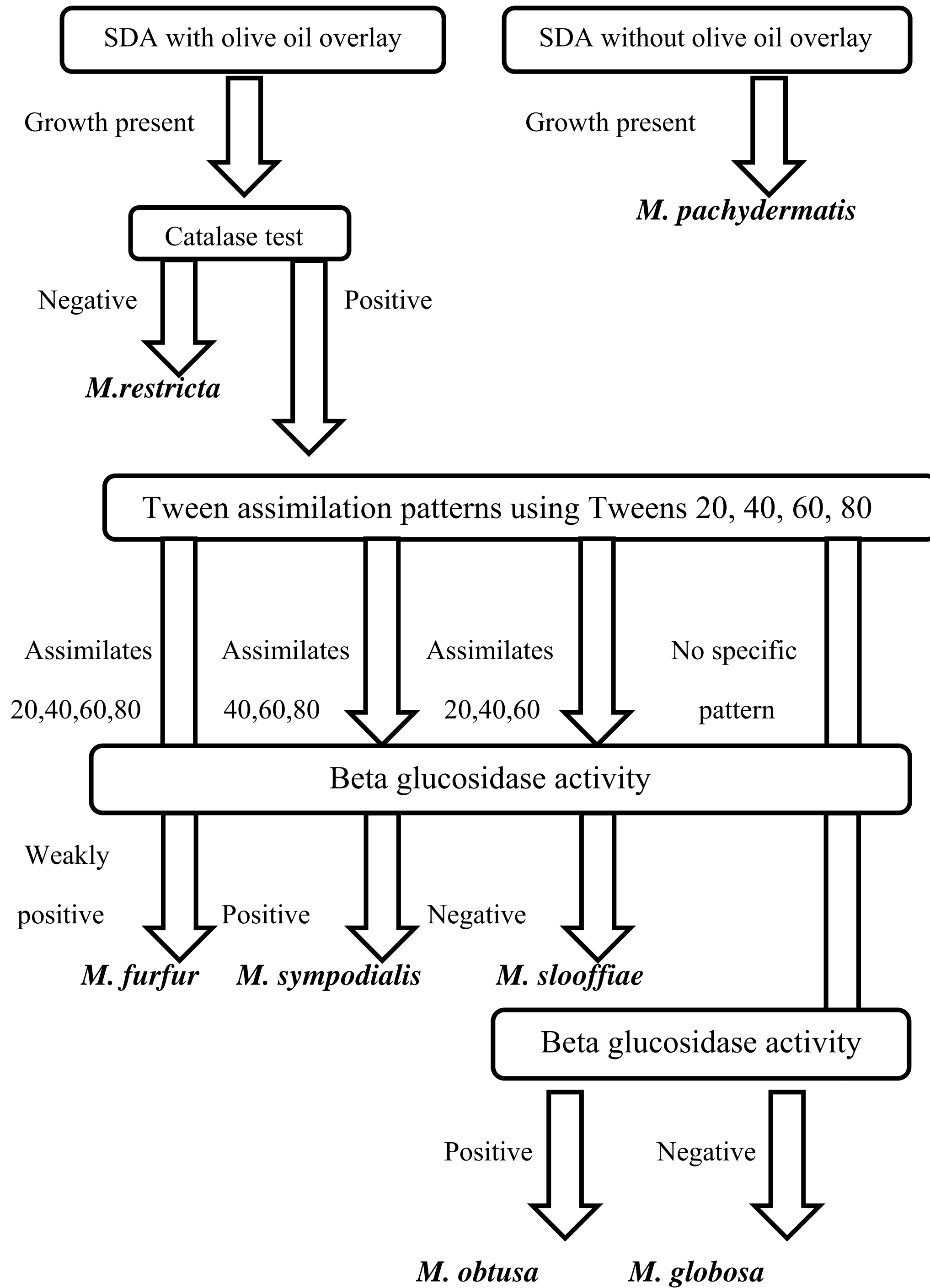
Ability to grow at 42˚Cis a physiological characteristic used to differentiate *Malassezia* species. All cultures were inoculated onto another SDA slope and incubated at 42˚C for one week. *M. pachydermatis, M. furfur, M. sympodialis, M.slooffiae, M. dermatis, M. japonica and M. cuniculi* are the species that are capable of growth at 42˚C within 4 – 5 days.[2, 8, 77]

TWEEN ASSIMILATION TEST:

This test was done according to the method proposed by Guillot *et al.*[2] 10^7 CFU/ml of yeast suspension was made in 2 ml of distilled water and poured into a plate containing 16 ml SDA with 0.05% chloramphenicol and 0.05% cycloheximide at 45 - 50˚C. After evenly spreading the inoculum, the plates were allowed to solidify and four wells each of 2 mm diameter were made and filled with 30 µl of the Tween compounds [Appendix II – 5], namely, 10% Tween 20, 0.5% Tween 40, 0.5% Tween 60 and 0.1%Tween 80 and the plates were incubated for upto a week at 31˚C. The plates were observed for the presence of growth around the individual wells on days 2, 4 and 7.[8, 28, 2]

As the Tween compounds are water–soluble, a concentration gradient was formed around each well and the utilization of the Tweens was assessed by the degree of precipitation of the lipophilic yeasts around individual wells.[78]

Based on the results obtained in the above tests, *Malassezia* species were identified according to the scheme suggested by Gueho *et al*[2] as follows:


CULTURE ON
SDA with olive oil overlay
SDA without olive oil overlay
Growth present
Growth present
M. pachydermatis
Catalase test
Negative
Positive
M.restricta
Tween assimilation patterns using Tweens 20, 40, 60, 80
Assimilates
20,40,60,80
Assimilates
40,60,80
Assimilates
20,40,60
No specific
pattern
Beta glucosidase activity
Weakly
positive
Positive
Negative
M. furfur
M. sympodialis
M. slooffiae
Beta glucosidase activity
Positive
Negative
M. obtusa
M. globosa

ANTIFUNGAL SUSCEPTIBILITY TESTING:

The *Malassezia* species identified by the above physiological and biochemical tests were subjected to antifungal susceptibility testing by broth microdilution method.

DETECTION OF MINIMAL INHIBITORY CONCENTRATION BY BROTH MICRODILUTION METHOD :

A modification of the CLSI recommended (Document M27-A3) broth microdilution method for testing the antifungal susceptibility of yeasts using Christensen's urea broth with the addition of Tween 40 (0.5%) and Tween 80 (0.1%), as suggested by some authors was used for antifungal susceptibility testing of the isolates. [20, 86]

REFERENCE STRAIN:

Cryptococcus neoformans – ATCC® 90112

ANTIFUNGAL AGENTS USED:

Fluconazole, Itraconazole, Voriconazole, Ketoconazole and Amphotericin B were the antifungal agents used for antifungal susceptibility testing of *Malassezia* species.

The antifungal agents were procured from Global Pharmaceuticals Private Limited, Chennai.

RANGE OF CONCENTERATIONS TESTED:

For each drug, the following range of concentrations were prepared:

Itraconazole	-	0.0313 to 16 µg/ml
Fluconazole	-	0.125 to 64 µg/ml
Ketoconazole	-	0.0313 to 16 µg/ml
Voriconazole	-	0.0313 to 16 µg/ml
Amphotericin B	-	0.0313 to 16 µg/ml

WEIGHING OF ANTIFUNGAL DRUGS:

$$\text{Weight (mg)} = \frac{\text{Volume (ml)} \times \text{Concentration } (\mu\text{g/ml})}{\text{Assay Potency } (\mu\text{g/mg})}$$

$$\text{Volume (ml)} = \frac{\text{Weight (mg)} \times \text{Assay Potency } (\mu\text{g/mg})}{\text{Concentration } (\mu\text{g/ml})}$$

REAGENTS REQUIRED:

- Antifungal agents
- A 7 day old culture for inoculum preparation
- 0.85% Normal saline
- Urease broth medium
- TWEEN 40

- TWEEN 80
- DMSO (Dimethyl Sulfoxide)
- Sterile 96-well microtiter plate
- Distilled water

MEDIUM

Urease broth with phenol red as indicator (HiMedia) was prepared as per manufacturer's instructions and supplemented with 0.5% Tween 40 and 0.1% Tween 80 to enable the medium to support the growth of lipophilic *Malassezia* species.[86]

DMSO was used for dissolving water insoluble drugs like itraconazole, ketoconazole, voriconazole and amphotericin B.

DISTILLED WATER was used for dissolving the water soluble drug fluconazole.

PREPARATION OF STOCK SOLUTION OF DRUGS:

All drugs were purchased as powders and were stored in the dark at 4°C. Stock solutions of ketoconazole, itraconazole, voriconazole and amphotericin B were prepared in dimethyl sulfoxide at concentrations of 1,600 µg/ml.

Stock solution of fluconazole was prepared in distilled water at a concentration of 5,120 µg/ml.

All the stock solutions were stored in the dark at −70°C for use within 3 months in case of azoles and one month in the case of amphotericin B.[94]

INOCULUM PREPARATION

1. Four to Five similar-looking *Malassezia* colonies grown on SDA were taken and suspended in 0.04% Tween 80 in distilled water at pH 7.4.

2. The suspension was then vortexed for 15 seconds.

3. The resulting suspension was adjusted to an absorbance of 0.5 Mc Farland Standard at 530 nm wavelength using a spectrophotometer, which will correspond to a yeast suspension of 1×10^6 to 5×10^6cells/ml.

4. The resulting suspension was further diluted 1:50 and then a further 1:20 with the standard medium so that the test inoculum is 1×10^3 to 5×10^3 CFU/ml.

PREPARATION OF SERIES OF DILUTIONS OF WATER INSOLUBLE DRUGS

The water insoluble drugs Amphotericin B, Itraconazole, Ketoconazole and Voriconazole were dissolved in DMSO. Initial drug concentration of 1600 µg/ml (STOCK) was kept in Tube 1. Intermediate drug concenterations were prepared as in the following table:

Tubes in Row I	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Amount of DMSO	-	0.5 ml	1.5 ml	3.5 ml	0.5 ml	1.5 ml	3.5 ml	0.5 ml	1.5 ml	3.5 ml
Amount of drug added (ml)	-	0.5 From tube 1	0.5 From tube 1	0.5 from tube 1	0.5 From tube 4	0.5 From tube 4	0.5 From tube 4	0.5 From tube 7	0.5 From tube 7	0.5 From tube 7
Intermediate drug concenteration (µg/ml) in Row I	1600	800	400	200	100	50	25	12.5	6.25	3.13

Then the final drug concentrations were prepared by diluting the prepared intermediate drug concentrations in urea broth as follows:

PREPARATION OF FINAL CONCENTRATION OF WATER INSOLUBLE ANTIFUNGAL AGENTS

Tubes in row II	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Amount of urea broth (ml)	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9	4.9
Amount of drug added from corresponding row I tubes (1-10) (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final drug concenteration in row II tubes (µg/ml)	32	16	8	4	2	1	0.5	0.25	0.125	0.0625

Then, the finally diluted variable concentration of drugs were added to the diluted inoculum.

ADDITION OF INOCULUM TO MICROTITER PLATE

Microtiter plate wells	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Amount of drug added from row II tubes (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Amount of inoculum added (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final drug concentration in each well (µg/ml)	16	8	4	2	1	0.5	0.25	0.125	0.0625	0.0313

CONTROLS:

Growth control containing 0.1 ml urea broth and 0.1 ml of the inoculum, and drug control containing 0.1 ml of the stock solution of the corresponding drug were included.

INCUBATION:

The seeded microtiter plates were incubated at 32°C for 72 hours.

PREPARATION OF STOCK SOLUTION AND SERIES OF DILUTIONS OF WATER SOLUBLE DRUGS

Stock solution in a concentration of 5120 μg/ml was prepared by dissolving the water soluble drug Fluconazole in distilled water (initial concentration of the drug in tube 1). Then, a series of intermediate drug concentrations were prepared with urea broth as follows:

Tubes in row I	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Amount of urea broth (ml)	7	1	3	1	1.5	3.5	1	1.5	3.5	1
Amount of drug (ml)	From tube 1	From tube 1	From tube 1	From tube 3	From tube 3	From tube 3	From tube 6	From tube 6	From tube 6	From tube 9
	1	1	1	1	0.5	0.5	1	0.5	0.5	1
Intermediate drug concentration (μg/ml) in row I	640	320	160	80	40	20	10	5	2.5	1.25

Then the final concentration of drug was prepared from the intermediate drug concentration by diluting in urea broth medium.

PREPARATION OF FINAL CONCENTERATION OF WATER SOLUBLE ANTIFUNGAL DRUGS:

Tubes in row II	1	2	3	4	5	6	7	8	9	10
Amount of urea broth (ml)	4	4	4	4	4	4	4	4	4	4
Drug added from corresponding row I tubes (1-10) (ml)	1	1	1	1	1	1	1	1	1	1
Final drug concentration(μg/ml) in row II tubes	128	64	32	16	8	4	2	1	0.5	0.25

Then the finally diluted variable concentration of drugs were added to the diluted inoculum.

ADDITION OF INOCULUM TO MICROTITER PLATE:

Microtiter plate wells	1	2	3	4	5	6	7	8	9	10
Amount of drug added to microtiter plate from row II tubes (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Amount of inoculum added (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Final drug concentration in each well (μg/ml)	64	32	16	8	4	2	1	0.5	0.25	0.125

CONTROLS:

Growth control containing 0.1 ml urea broth and 0.1 ml of the inoculum, and drug control containing 0.1 ml of the stock solution of the corresponding drug were included.

INCUBATION:

The seeded microtiter plates were incubated at 32°C for 72 hours.

READNG OF RESULTS FOR MIC BY BROTH MICRODILUTION METHOD:

Minimal Inhibitory Concentration was determined by comparing the amount of growth in the growth control well with the amount of growth in the wells containing the antifungal agent and a numerical score was given as follows:

Numerical Score :

0 - Optically clear or absence of growth

1 - Approximately 25% of the growth control or slight growth

2 - Approximately 50% of the growth control or prominent reduction in growth

4 - Approximately as of the growth medium or slight reduction in the growth (75% of growth control)

5 - No reduction in the growth

INTERPRETATION:

80% growth inhibition corresponding to a numerical score of 3 was taken as the end point for azoles. Complete absence of growth, corresponding to a numerical score of 0 was taken as the end point for amphotericin B.[86, 88]

MIC_{50} and MIC_{90} were determined for the isolates. MIC of ATCC® control strain was confirmed to be within the expected range, for the validity of the interpretation for the test strains.

To date, no standardized breakpoints are available for the MIC of various antifungal agents in the case of *Malassezia* species.

STATISTICAL ANALYSIS:

Statistical analysis was done using Statistical Package for Social Sciences 16 (SPSS 16) and Epi-info softwares. The results were analyzed by Pearson's Chi square test. P value less than 0.05 is considered to correlate with statistical significance.

RESULTS

RESULTS

This study was done for a period of one year from October 2013 to September 2014, at the Institute of Microbiology, Madras Medical College and the Department of Dermatology, Madras Medical College and Rajiv Gandhi Government General Hospital, Chennai.

A total of 100 patients who were clinically diagnosed to have either pityriasis versicolor (PV) or seborrheic dermatitis (SD), and whose skin scrapings showed the characteristic 'spaghetti and meat ball appearance' on 10% potassium hydroxide – Paker Quink's blue ink mount were chosen for the study, from those who attended the Dermatology outpatient department (OPD). The study population consisted of patients belonging to all age groups and both sexes.

Table 1: Distribution of pityriasis versicolor and seborrheic dermatitis cases in the study population (n = 100)

Infection	No. of cases	Percentage (%)
Pityriasis versicolor	73	73
Seborrheic dermatitis	27	27
TOTAL	100	100

Of the 100 cases chosen for the study, 73 were clinically diagnosed to have pityriasis versicolor (PV) and 27 were clinically diagnosed to have seborrheic dermatitis (SD).

PITYRIASIS VERSICOLOR (PV):

Table 2: Age and gender distribution of Pityriasis versicolor cases in the study population (n=73)

Age in years	Total no. of cases		Male patients		Female patients	
	Number	%	Number	%	Number	%
10 - 19	13	17.8	9	12.3	4	5.5
20 - 29	31	42.5	24	32.9	7	9.6
30 - 39	16	21.9	9	12.3	7	9.6
40 – 49	11	15.1	9	12.3	2	2.7
50 – 59	0	0	0	0	0	0
60 - 69	2	2.7	2	2.7	0	0
TOTAL	73	100	53	72.6	20	27.4

Majority of the patients with pityriasis versicolor belonged to the 20 – 29 years age group in both sexes, with an overall male predominance (53 males out of the total 73 PV cases) (72.6%). The median age of occurrence of PV was found to be 24 years.

Figure 1: Gender distribution of Pityriasis versicolor cases in the study population (n = 73)

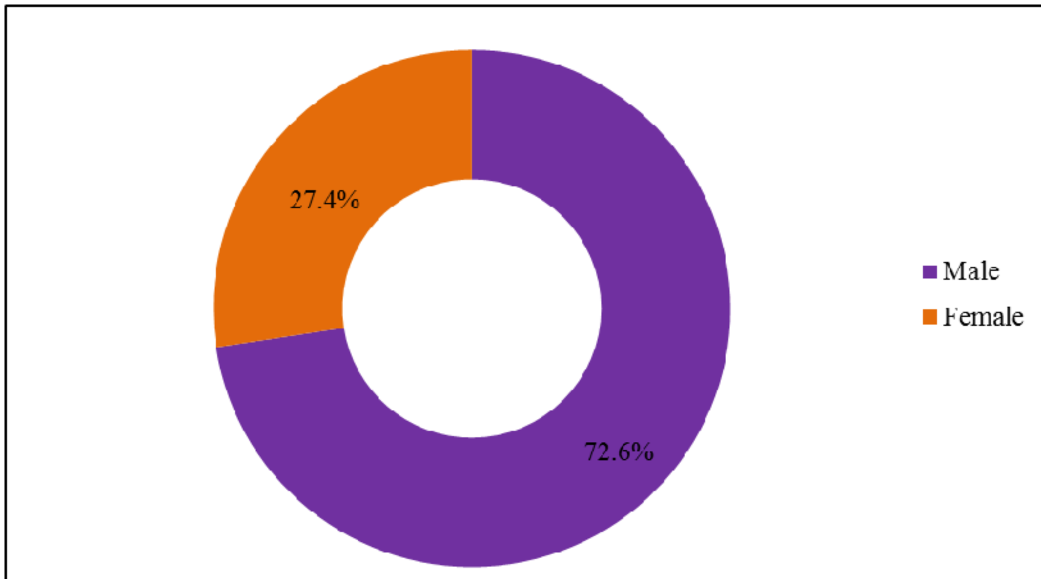


Figure 2: Age and gender distribution of Pityriasis versicolor cases (n = 73)

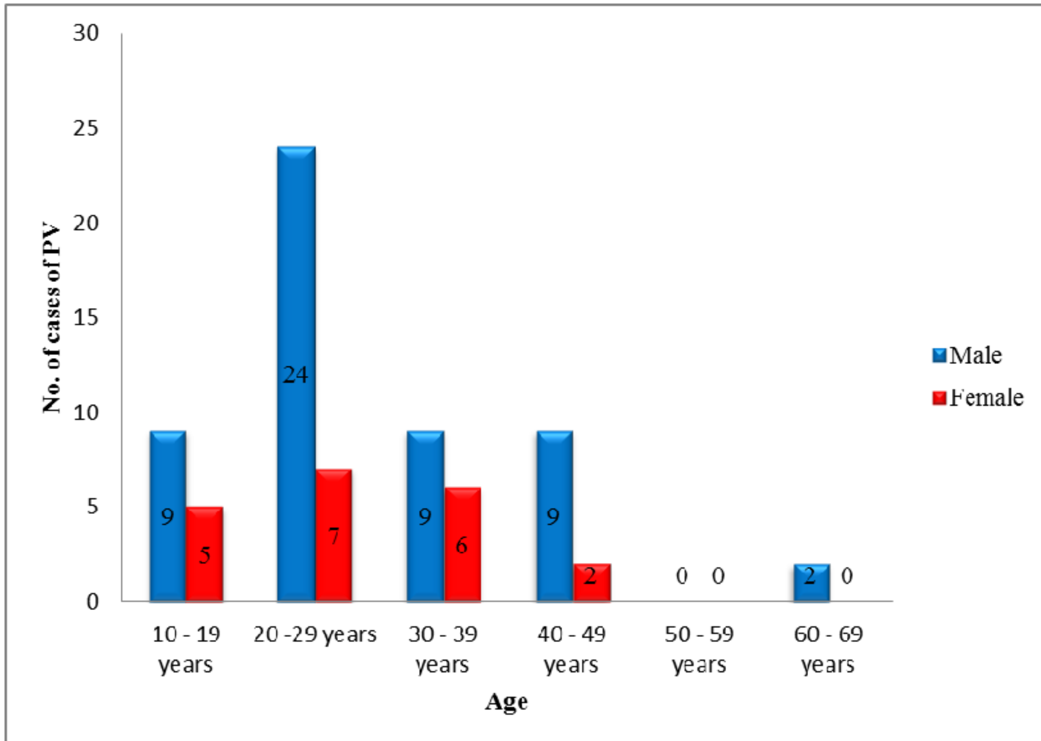


Table 3: Distribution of PV lesions in various body sites in the study group (n = 73)

Major body site involved	No. of cases of PV	Percentage (%)
Back	37	50.7
Chest	11	15
Face and neck	18	24.7
Arms	7	9.6
TOTAL	73	100

Majority of the patients with pityriasis versicolor presented with lesions on the back (50.7%), followed by face and neck (24.7%). Of the total 73 cases, there were 35 patients with multiple site involvement, in whom the predominant site involved was taken into account.

Figure 3: Distribution of PV lesions in various body sites (n = 73)

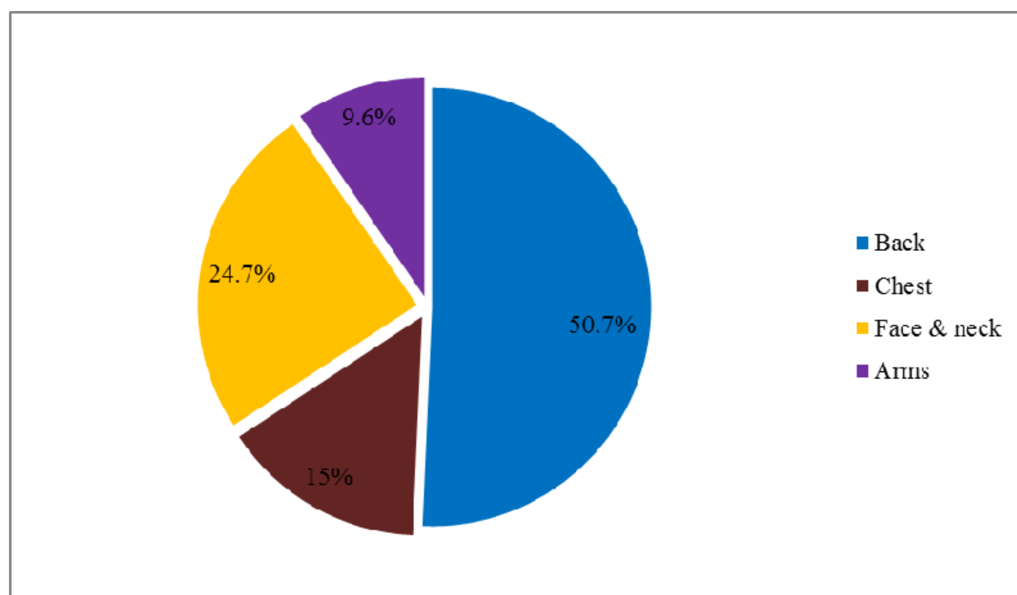


Table 4: Type of Pigmentation of PV lesions in the study population (n=73)

Type of Pigmentation	No. of cases	Percentage
Hypopigmented PV	46	63.1 %
Hyperpigmented PV	27	36.9 %
TOTAL	73	100 %

The hypopigmented type of pityriasis versicolor was more common (63.1%) than the hyperpigmented type..

Table 5: Frequency of pruritus in patients with pityriasis versicolor in the study population (n = 73)

Pruritus	No. of cases with PV	Percentage
Present	21	28.8 %
Absent	52	71.2 %
TOTAL	73	100 %

Pruritus was present only in 28.8%of pityriasis versicolor cases.

Table 6: Family history of pityriasis versicolor in the study group (n = 73)

Family history of PV	No. of PV cases	Percentage
Present	20	27.4 %
Absent	53	72.6 %
TOTAL	73	100 %

History of similar hypopigmented or hyperpigmented scaly lesions in other family members was present in 27.4% of pityriasis versicolor cases.

Table 7: Frequency of occurrence of pityriasis versicolor lesions in the study population (n = 73)

Presentation of PV	No. of PV cases	Percentage
First episode	49	67.1 %
Recurrent lesions	24	32.9 %
TOTAL	73	100 %

Recurrent lesions were found in 24 cases (32.9%) and the remaining 49 cases (67.1%) presented for the first time with lesions suggestive of PV.

Figure 4: Distribution of patients with pruritus, family history and history of recurrence of PV in the study population (n = 73)

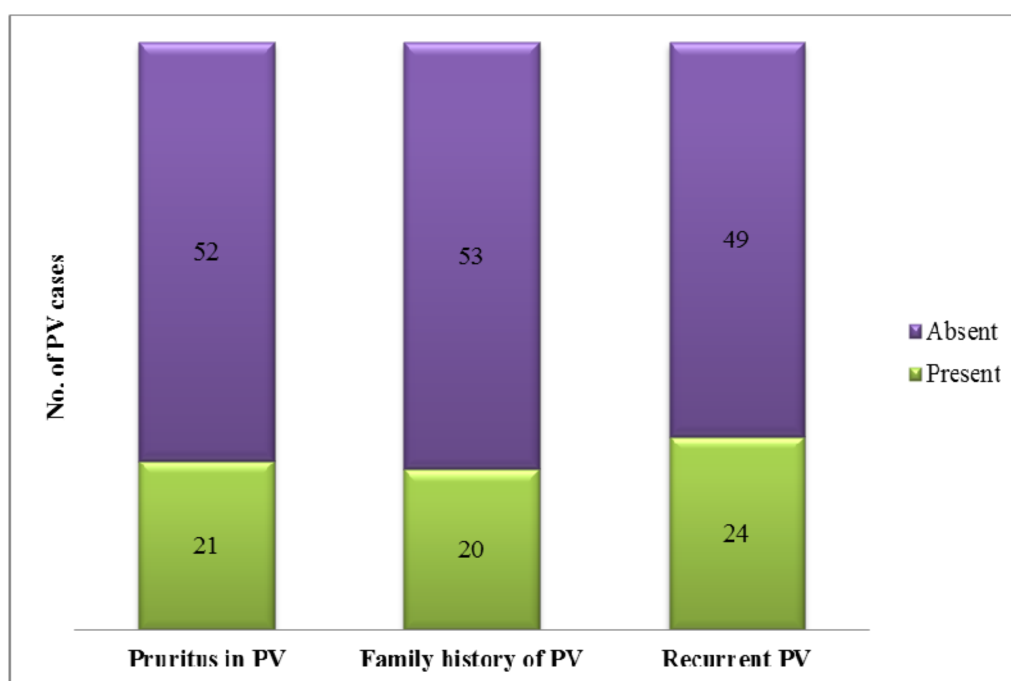


Table 8: Degree of scaling in PV lesions in the study population (n = 73)

Degree of Scaling	No. of cases with PV		
	Total (%)	Hypopigmented (%)	Hyperpigmented (%)
Mild	21 (28.8%)	14 (19.2 %)	7 (9.6 %)
Moderate	40 (54.8%)	31 (42.5 %)	9 (12.3 %)
Severe	12 (16.4%)	1 (1.3 %)	11 (15.1 %)
TOTAL	73 (100%)	46 (63 %)	27 (37 %)

Majority (54.8%) of the patients with pityriasis versicolor presented with moderate amount of scaling in the lesions. 28.8% had mild scales and 16.4% had severe degree of scaling in the lesions. Severe scaling was very common in the hyperpigmented type of PV (15.1%) compared to hypopigmented PV (1.3%).

Figure 5: Degree of scaling in PV lesions in the study population(n = 73)

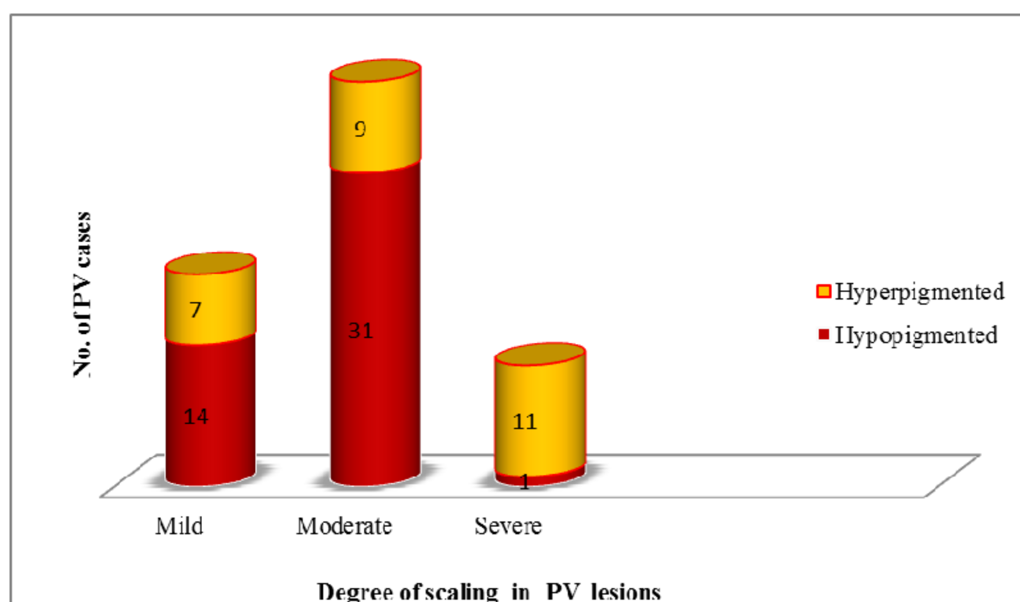


Table 9: Isolation rate of *Malassezia* spp. from pityriasis versicolor cases in the study population (n = 73)

Culture for *Malassezia* spp.	No. of PV cases	Percentage
Culture positive	56	76.7 %
Culture negative	17	23.3 %
TOTAL	73	100 %

The isolation rate of *Malassezia* species from PV cases was 76.7%, that is, out of the 73 samples of skin scrapings collected from clinically diagnosed PV cases who showed the characteristic 'spaghetti and meatball' appearance on KOH mount, 56 (76.7 %) yielded growth on Sabouraud dextrose agar with olive oil overlay and the remaining 17 (23.3%) were culture negative.

Figure 6: Isolation rate of *Malassezia* spp. from pityriasis versicolor cases in the study population (n = 73)

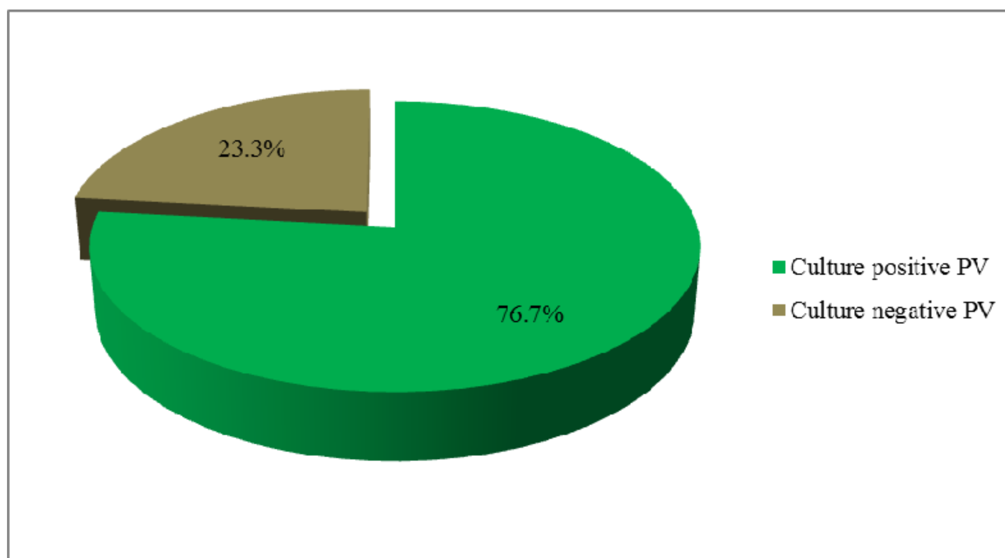


Table 10: Comparison of culture positivity in the hypopigmented and hyperpigmented types of PV in the study population (n = 73)

Type of PV	Total	Culture result in PV		
		Positive	Negative	% of culture positivity
Hypopigmented PV	46	31	15	67.4 %
Hyperpigmented PV	27	25	2	92.6 %

Isolation rate of *Malassezia* species from PV was more with the hyperpigmented type, (25 cases out of 27; 92.6%), compared to the hypopigmented type (31 out of total 46; 67.4%). This result is statistically significant (p value 0.013).

Figure 7: Comparison of culture positivity in the hypopigmented and hyperpigmented types of PV in the study population (n = 73)

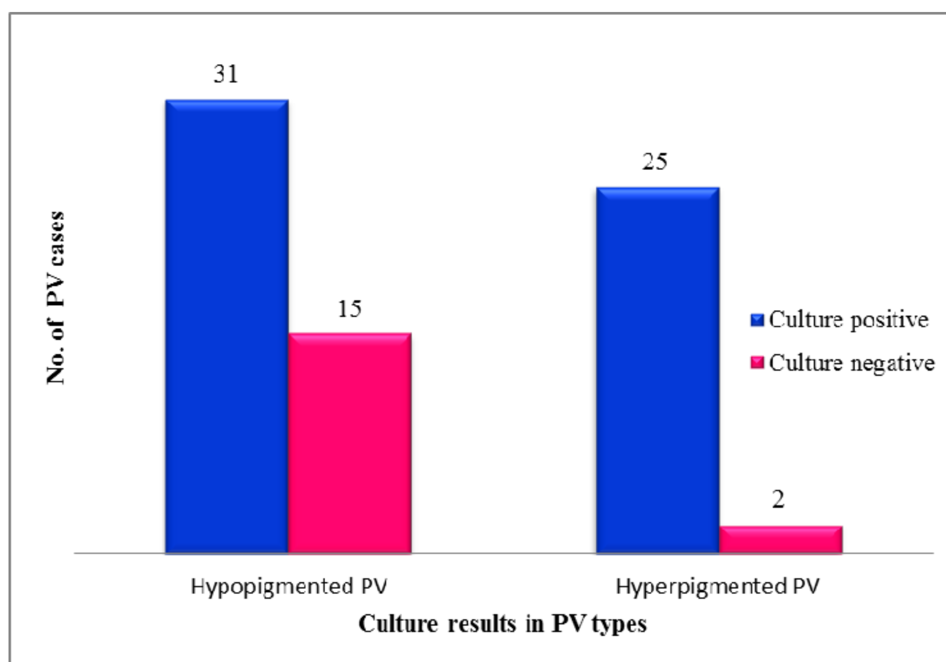


Table 11: Correlation between the duration of lesions and isolation rate of *Malassezia* species from PV in the study population (n =73)

Duration of PV lesions	No. of PVcases (%)	Culture positive PV cases	
		Number	Percentage of culture positivity in each group
1 week to 1 month	16 (21.9%)	12	75 %
>1 month to 6 months	44 (60.3%)	38	86.4 %
>6 months to 1year	12 (16.4%)	6	50 %
>1 year	1 (1.4%)	nil	nil

Majority (60.3%) of the PV cases had skin lesions for a duration of 1 to 6 months. Culture positivity was high in those patients (86.4%). This result is statistically significant (P value 0.015).

Figure 8: Correlation between the duration of PV lesions and culture positivity (n = 73)

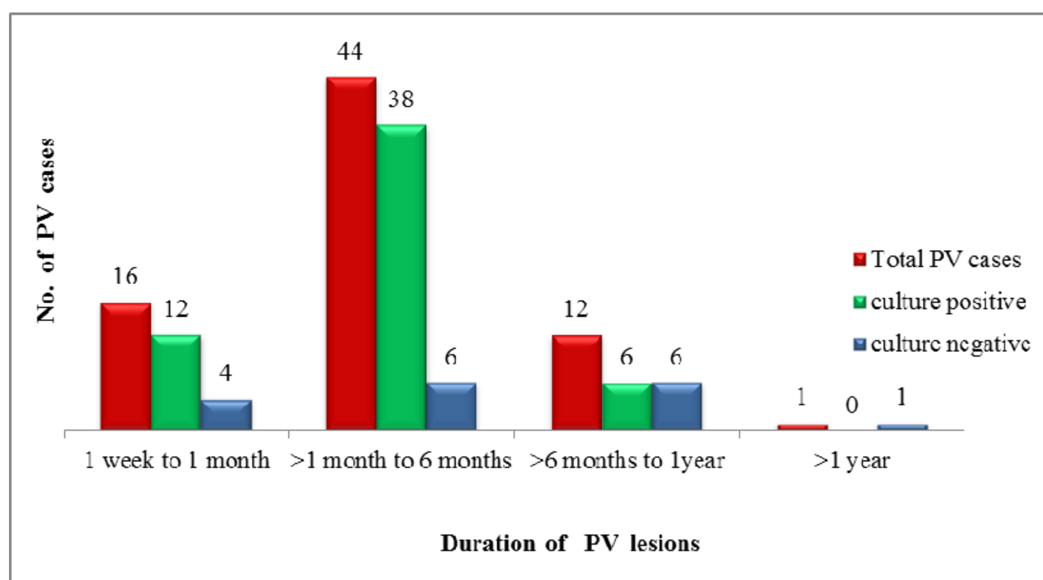


Table 12: Distribution of *Malassezia* species among the culture positive PV cases in the study population (n = 56)

Malassezia species	No. of isolates in PV	Percentage
M. globosa	30	53.6 %
M. sympodialis	12	21.4 %
M. furfur	7	12.5 %
M. restricta	5	8.9 %
M. obtusa	2	3.6 %
TOTAL	56	100 %

M. globosa was the most common species isolated (53.6%) from PV cases, followed by *M. sympodialis, M. furfur, M. restricta* and *M. obtusa*.

Figure 9: Distribution of *Malassezia* species among the culture positive PV cases (n = 56)

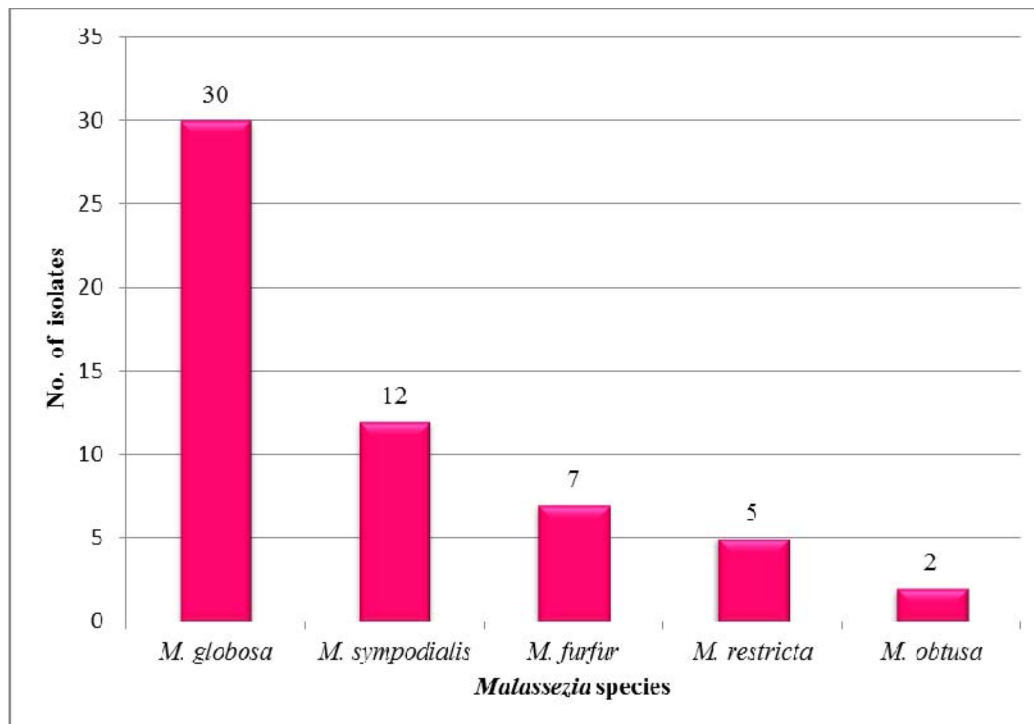


Table 13: Correlation between the site of PV lesion and *Malassezia* spp. isolated in the culture positive study population(n = 56)

***Malassezia* species (n = 56)**	**Site of PV lesion and the number of *Malassezia* isolates**							
	BACK(n =32)		**FACE (n = 12)**		**CHEST (n =9)**		**ARM (n = 3)**	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
M. globosa	19	59.4 %	7	58.4 %	3	33.3 %	1	33.3 %
M. sympodialis	5	15.6 %	3	25 %	4	44.5 %	0	0 %
M. furfur	6	18.8 %	1	8.3 %	0	0 %	0	0 %
M. restricta	2	6.2 %	1	8.3 %	1	11.1 %	1	33.3 %
M. obtusa	0	0%	0	0 %	1	11.1 %	1	33.3 %
TOTAL	32	100 %	12	100 %	9	100 %	3	100 %

M. globosa was the most common species isolated form back (19 cases) (59.4%) and face (58.4%) lesions. *M. sympodialis* was the predominant species isolated from chest (4 cases) (44.5%).This result is not statistically significant (p value = 0.066).

Figure 10: Correlation between the site of PV lesion and the *Malassezia* spp. isolated(n = 56)

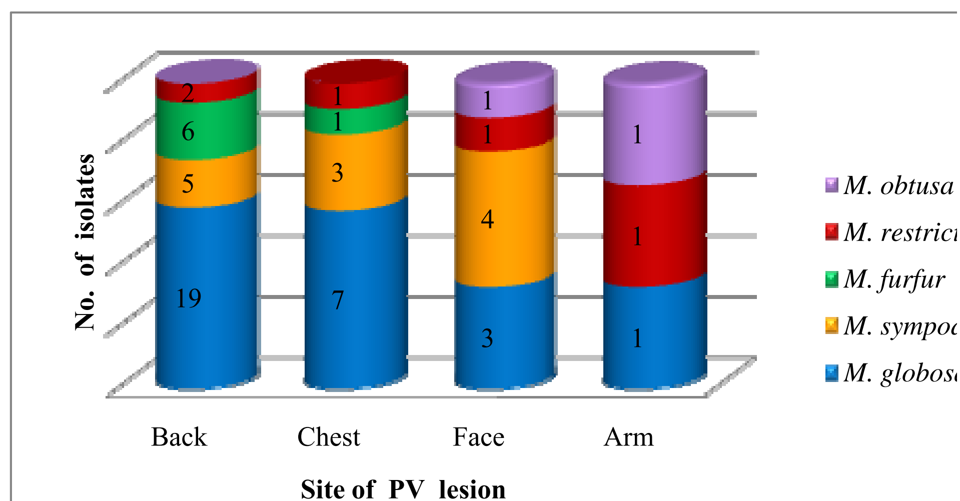


Table 14: Correlation between type of PV and isolation rate of various *Malassezia* spp. in culture positive PV cases (n = 56)

Malassezia spp.	Total no. of isolates	Type of Culture positive PV lesions			
		Hyperpigmented (n = 25)		Hypopigmented (n = 31)	
		No. of isolates	%	No. of isolates	%
M. globosa	30	9	36%	21	67.7%
M. sympodialis	12	7	28%	5	16.1%
M. furfur	7	5	20%	2	6.5%
M. restricta	5	2	8%	3	9.7%
M. obtusa	2	2	8%	0	0%
TOTAL	56	25	100%	31	100%

M. globosa was the most common isolate in both the hypopigmented and hyperpigmented types of PV.

Figure 11: Distribution of various *Malassezia* species in the PV types (n=56)

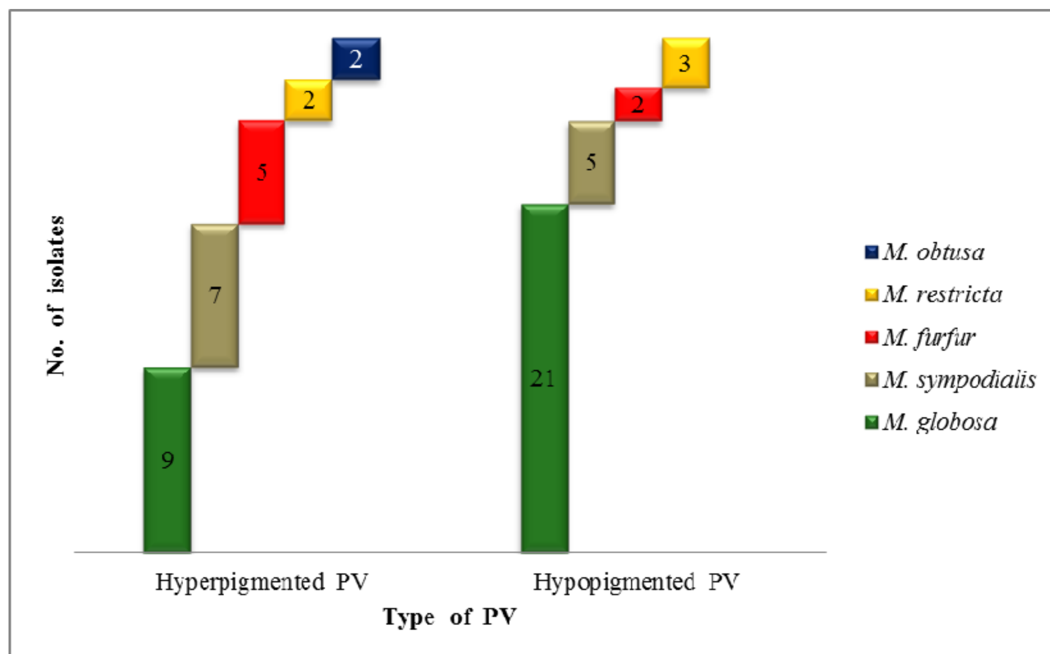


Table 15: Correlation between the PV episode and *Malassezia* spp. isolated from culture positive PV cases in the study population (n = 56)

***Malassezia* spp.**	**No. of isolates**	**Culture positive PV lesions**	
		First episode (%)	**Recurrent lesions (%)**
M. globosa	30	22 (39.3%)	8 (14.2%)
M. sympodialis	12	8 (14.3%)	4 (7.1%)
M. furfur	7	5 (8.9%)	2 (3.6%)
M. restricta	5	3 (5.4%)	2(3.6%)
M. obtusa	2	Nil (0 %)	2 (3.6%)
TOTAL	56	38 (67.9%)	18 (32.1%)

M. globosa was the most common species isolated from both fresh cases (39.3%) and recurrent cases (14.2%) of PV, followed by *M. sympodialis* in both groups.

Figure 12: Correlation between the episode of PV lesion and *Malassezia* species isolated (n = 56)

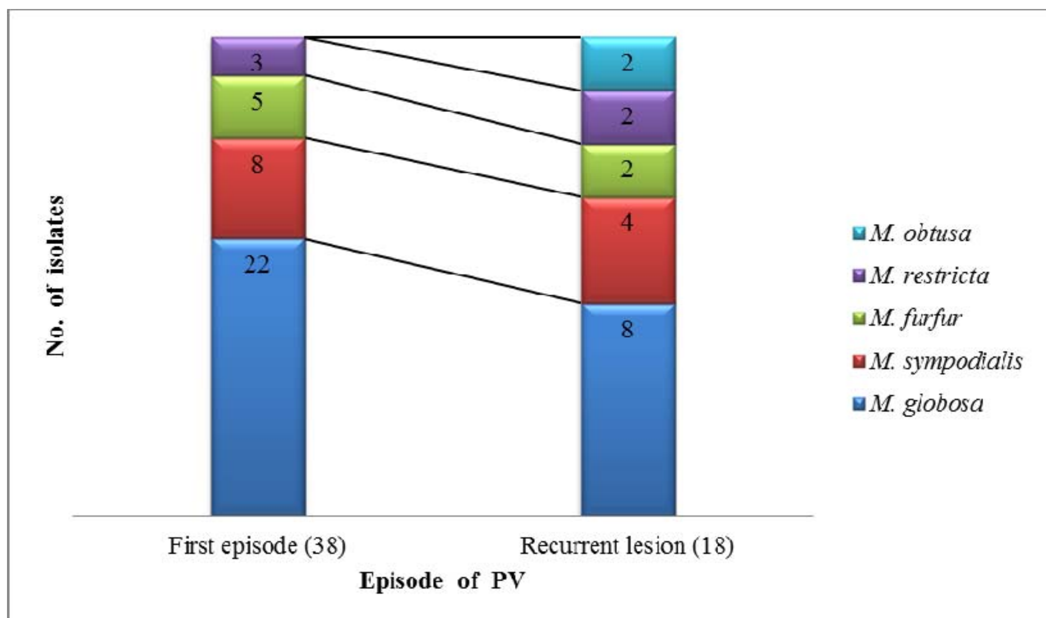


Table 16: Frequency of various risk factors associated with pityriasis versicolor in the study population (n = 73)

Risk factor	No. of PV cases	Percentage
Diabetes mellitus	4	5.5 %
Immunocompromised status	9	12.3 %
Intake of steroids or other immunosuppressive drugs	5	6.9 %
Use of bath oils	23	31.5 %
Excessive sweating	28	38.4 %

(Rheumatoid arthritis, tuberculosis, post renal transplant state, HIV infection were the immunocompromised states encountered in PV patients in the study population)

Risk factors like excessive sweating, use of bath oils and immunocompromised status were present in 28 cases (38.4%), 23 cases (31.5%) and 9 cases (12.3%) of PV respectively. But excessive sweating as a risk factor for PV was found to be statistically insignificant in this study (p value 0.39).

SEBORRHEIC DERMATITIS:

In this study done for a period of one year from October 2013 to September 2014,27 clinically diagnosed seborrheic dermatitis cases with the characteristic 'spaghetti and meat ball appearance' on direct KOH mount of skin scrapings from seborrheic dermatitis lesions were included.

Table 17: Age and gender distribution of seborrheic dermatitis in the study population (n = 27)

Age in Years	Total patients		Male Patients		Female Patients	
	No.	%	No.	%	No.	%
20 - 29	5	18.5 %	4	14.8 %	1	3.7 %
30 - 39	9	33.3 %	7	25.9 %	2	7.4 %
40 - 49	12	44.5 %	8	29.6 %	4	14.9 %
50 - 59	1	3.7 %	1	3.7 %	0	0 %
TOTAL	27	100 %	20	74 %	7	26 %

In all age groups, males (20 cases; 74%) were found to be affected more commonly than females (7 cases; 25.9%).

Majority of those affected with SD belonged to the 40 – 49 years age group in both sexes (12 cases; 44.44%), followed by the 30 – 39 years age group (9 cases; 33.33%).

Figure 13: Gender distribution of seborrheic dermatitis in the study population (n = 27)

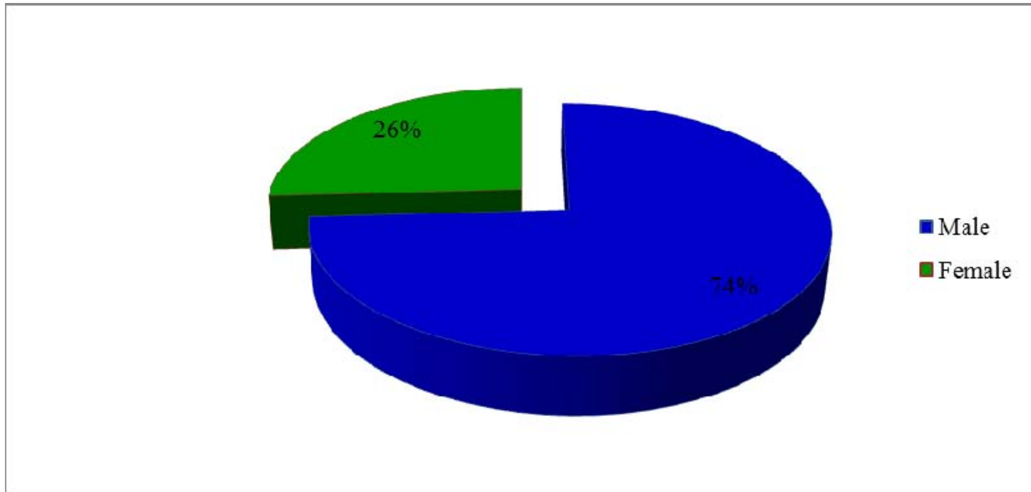


Figure 14: Age and Sex distribution of patients with seborrheic dermatitis in the study population (n = 27)

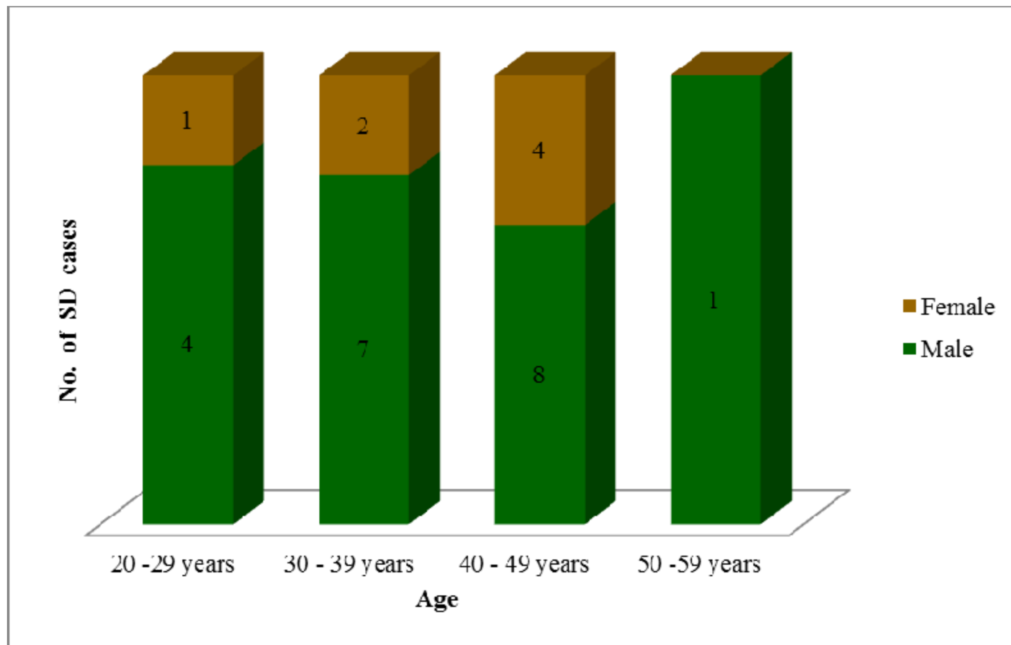


Table 18: Distribution of SD lesions in various body sites in the study population (n = 27)

Major body site involved	No. of SD cases	Percentage
Face	9	33.33 %
Scalp	8	29.63 %
Chest & back	5	18.52 %
Neck	4	14.81 %
Arm	1	3.71 %
TOTAL	27	100 %

Among the body sites involved in SD, face was the most common site (9 cases; 33.33%) , followed by the scalp (8 cases; 29.63%) .

Figure 18: Distribution of SD lesions in various body sites in the study population (n = 27)

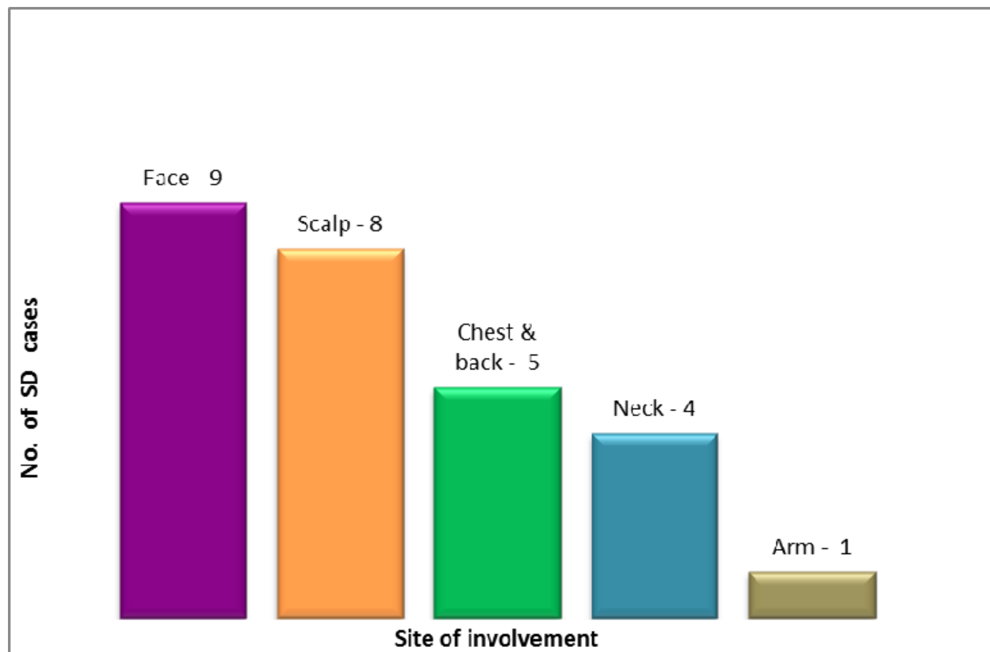


Table 19: Frequency of occurrence of SD in the study population (n=27)

Presentation of SD	No. of SD cases	Percentage
First episode	18	67 %
Recurrent lesions	9	33 %
TOTAL	27	100 %

33% of the patients with SD had recurrent lesions. SD involving scalp was found to recur commonly, compared to lesions at the other sites.

Table 20: Frequency of culture positivity for *Malassezia* species in SD cases in the study population (n = 27)

Culture for *Malassezia* spp.	No. of SD cases	Percentage
Positive	14	52 %
Negative	13	48 %
TOTAL	27	100 %

The isolation rate of *Malassezia* species from SD lesions was 52%.

Figure 16: Frequency of culture positivity in SD cases (n = 27)

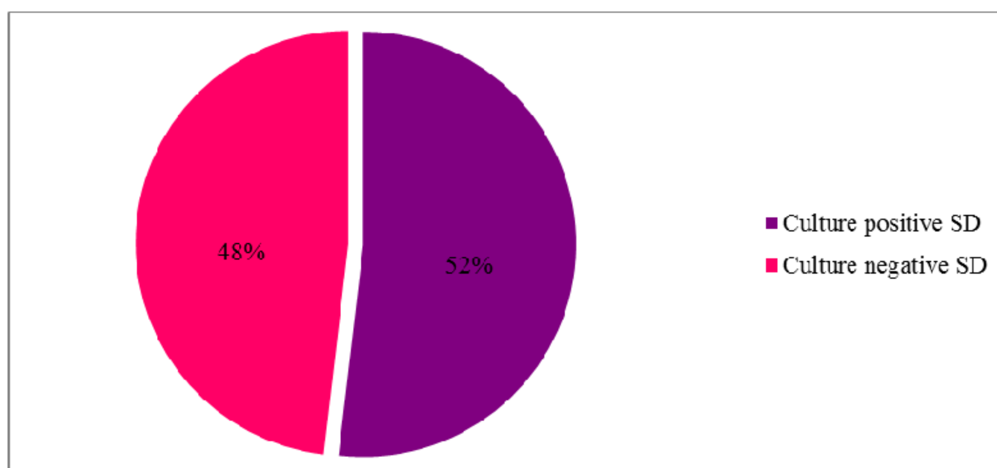


Table 21: Correlation between the site of SD lesion and culture positivity for *Malassezia* spp. in the study population (n = 27)

Site of SD lesion	No. of SD cases					
	Total (n=27)	Culture positive (n=14)		Culture negative(n=13)		
		No.	%	No.	%
Face	9	4	44.4 %	5	55.6 %
Scalp	8	6	75 %	2	25 %
Chest & back	5	3	60 %	2	40 %
Neck	4	1	25 %	3	75 %
Arm	1	0	0 %	1	100 %

Culture positivity for *Malassezia* spp. was high with scalp lesions(75%), followed by chest and back lesions (60%). But this correlation was statistically insignificant (p value = 0.36).

Figure 17: Correlation between site of lesion and culture positivity in SD (n = 27)

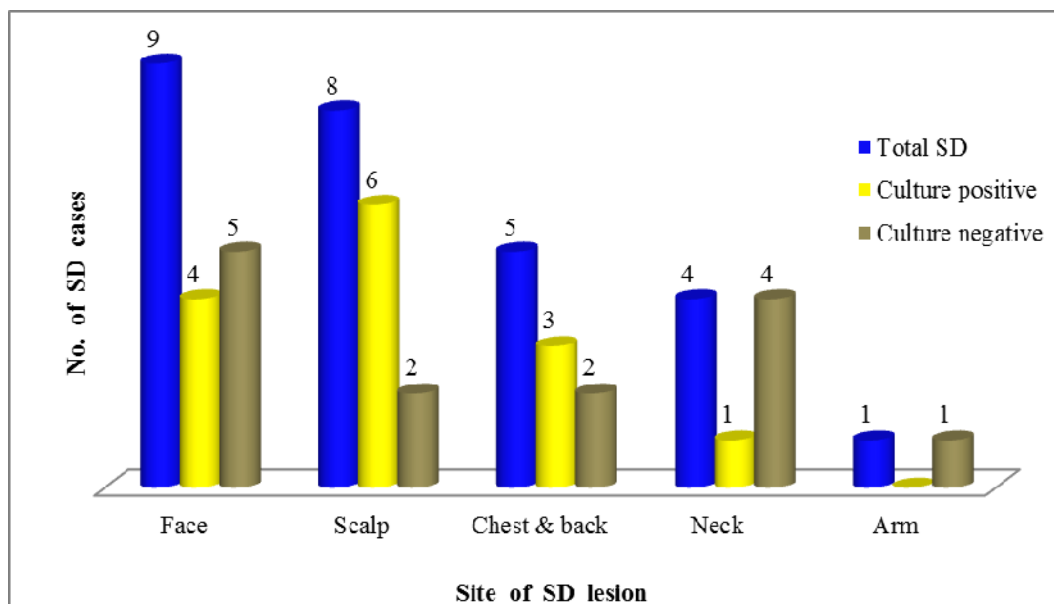


Table 22: Distribution of *Malassezia* isolates in culture positive seborrheic dermatitis cases in the study population (n = 14)

Malassezia species	No. of culture positive SD cases	Percentage
M. globosa	7	50 %
M. furfur	4	28.6 %
M. sympodialis	2	14.3 %
M. restricta	1	7.1 %
TOTAL	14	100 %

M. globosa was the most frequently isolated species (50%) in SD, followed by *M. furfur (*28.6%), *M. sympodialis* (14.3%) *and M. restricta* (7.1%).

Figure 18: Distribution of *Malassezia* species in culture positive seborrheic dermatitis cases in the study population (n = 14)

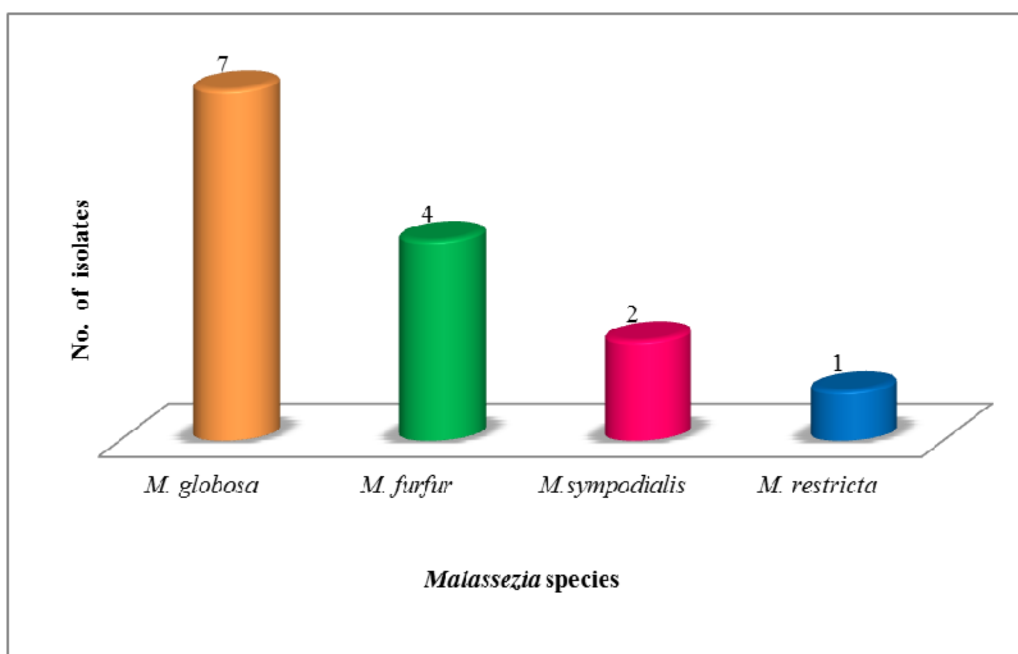


Table 23: Correlation between site of lesion and the *Malassezia* species isolated from culture positive SD cases (n = 14)

Malassezia species	Site of SD lesion and no. of *Malassezia* isolates							
	Face (n= 4)		Scalp (n = 6)		Chest & back (n = 3)		Neck (n = 1)	
	No.	%	No.	%	No.	%	No.	%
M. globosa	2	50%	3	50%	2	66.7%	0	0%
M. furfur	1	25%	2	33%	1	33.3%	0	0%
M. sympodialis	0	0%	1	17%	0	0%	1	100%
M. restricta	1	25%	0	0%	0	0%	0	0%

M. globosa was the most common species isolated from SD lesions on chest & back (66.7%), face (50%) and scalp (50%). *M. furfur* was the second common species form these three sites.

Figure 19: Correlation between site of lesion and the *Malassezia* spp. isolated from culture positive SD cases (n = 14)

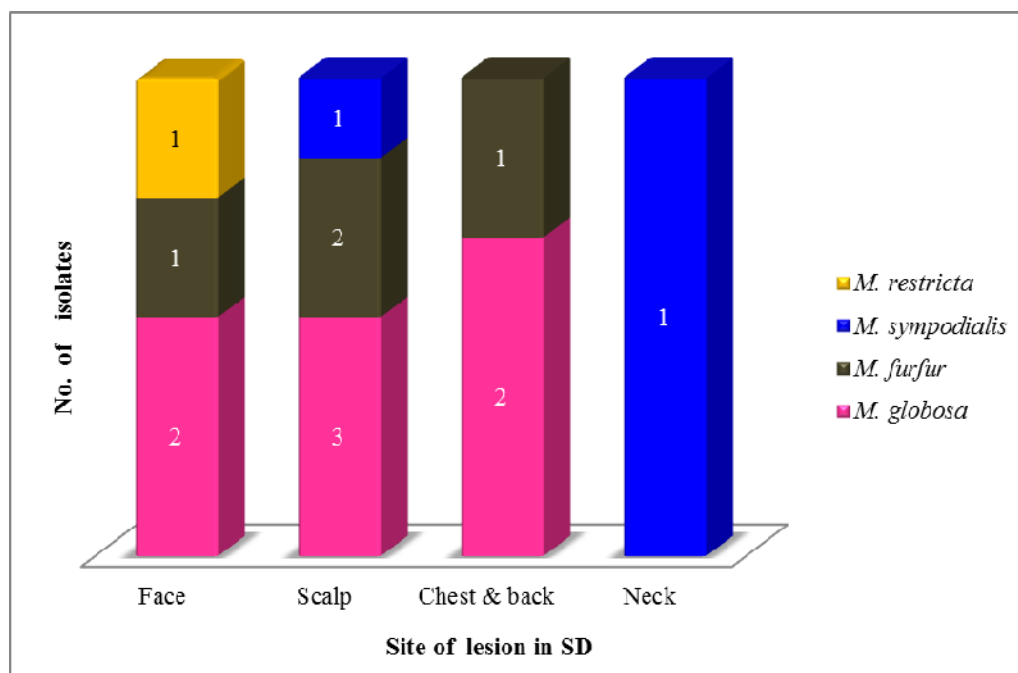


Table 24: Correlation between duration of lesions and isolation rate of *Malassezia* species from SD lesions in the study population (n = 27)

Duration of lesions in SD	Total cases (n=27)		Culture positive cases (n=14)	
	No.	%	No.	%
<3months	9	33.33%	3	33 %
3 months to 1 year	12	44.44%	8	66 %
>1 year	6	22.22%	3	50 %

The duration of lesions in majority (44.44%) of the SD cases was 3 months to 1 year, and 66% (8 cases out of 12) of those in this duration were culture positive for *Malassezia* species. But this correlation was statistically insignificant (p value 0.39).

Figure 20: Correlation between the duration of lesions and isolation rate of *Malassezia* species from SD lesions(n = 27)

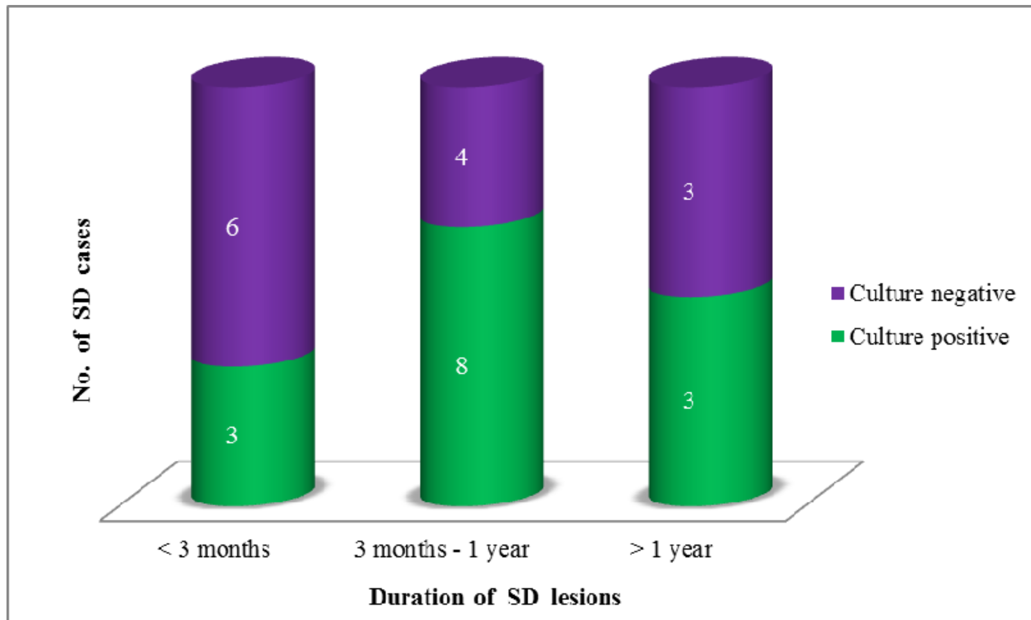


Table 25: Association of SD lesions with immunocompromised status (n = 27)

Immunocompromised status	No. of SD cases	Percentage
Diabetes	3	11 %
Steroid intake	1	3.7 %
HIV	2	7.4 %
Total	6	22.1 %

Immunocompromised status was present in 6 out of the total 27 cases of seborrheic dermatitis. 11% of the SD patients were diabetics (3 cases) and 7.4% of them were HIV positive (2 cases). The remaining 21 cases of SD did not have any associated immunocompromised status.

Table 26: Overall isolation rate of *Malassezia* species in PV and SD cases in the study population (n = 100)

Culture for *Malassezia* spp.	Total cases	PV cases		SD cases	
		No.	%	No.	%
Positive	70	56	76.7%	14	52%
Negative	30	17	23.3%	13	48%
TOTAL	100	73	100%	27	100%

In this study, the isolation rate of *Malassezia species* from PV and SD lesions put together was 70%, the yield being higher in pityriasis versicolor lesions (56 out of 73; 76.7%), than seborrheic dermatitis lesions (14 out of 27; 52%).

Table 27: Overall isolation rate of various *Malassezia* species in culture positive PV and SD cases in the study population (n = 70)

Malassezia species isolated	No. of isolates in culture positive cases			
	PV	SD	Total	Percentage
M. globosa	30	7	37	52.8 %
M. furfur	7	4	11	15.7 %
M. sympodialis	12	2	14	20 %
M. restricta	5	1	6	8.6 %
M. obtusa	2	0	2	2.9 %
TOTAL	56	14	70	100 %

M. globosa was the most commonly isolated species from both PV and SD lesions, whereas the second common species was *M.sympodialis* in PV and *M. furfur* in SD.

Figure 21: Overall isolation rate of *Malassezia* species in PV and SD in the study population (n = 100) (Table 26)

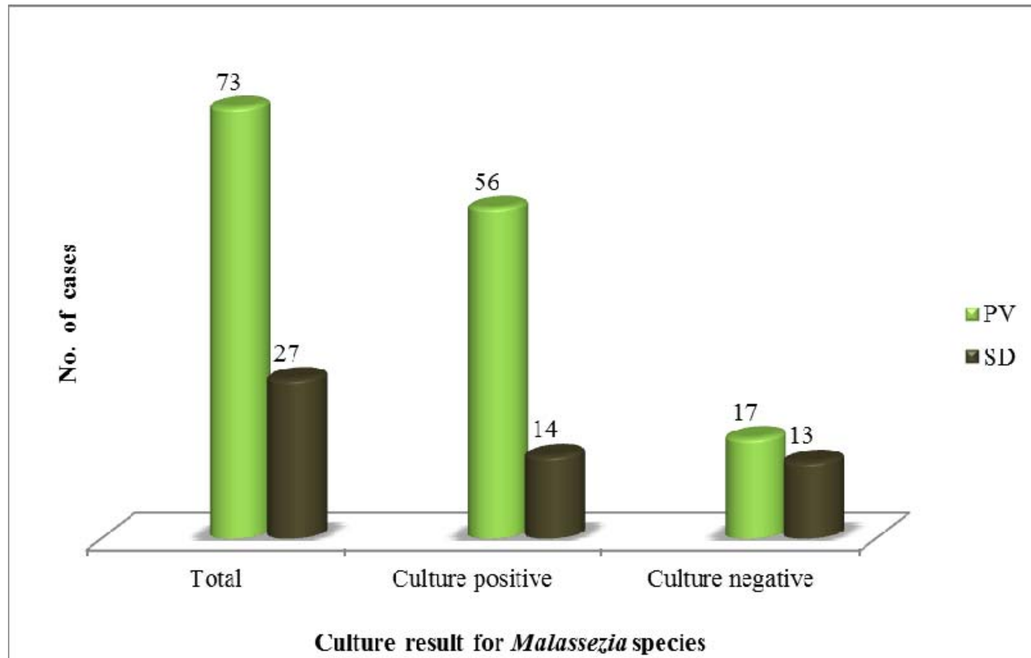


Figure 22: Overall distribution of *Malassezia* species in culture positive PV and SD cases (n = 70) (Table 27)

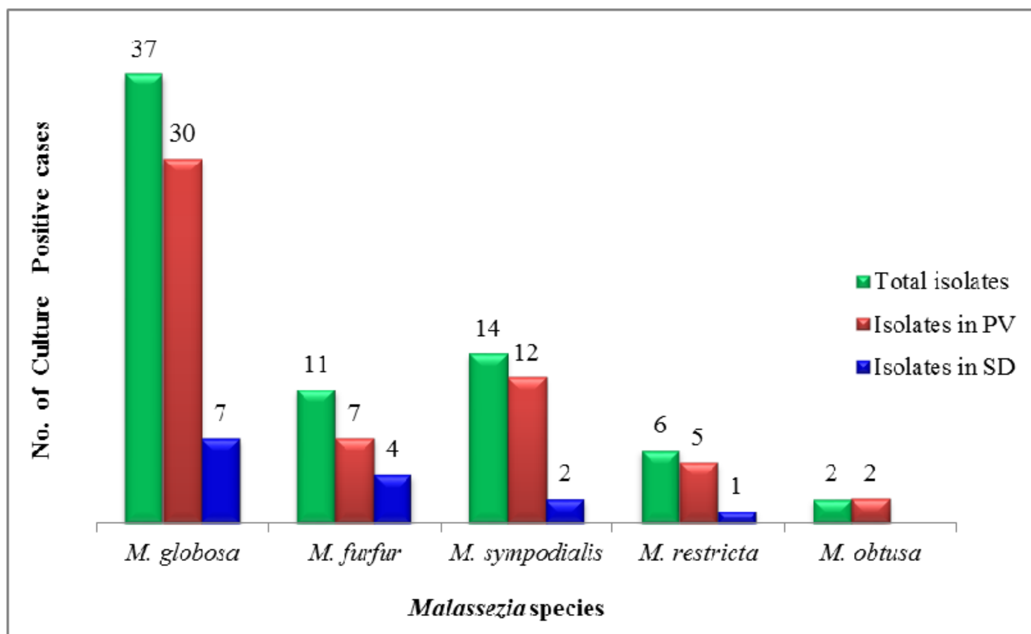


Table 28: The MIC ranges of antifungal agents tested against *Malassezia* species isolated from PV and SD lesions (n = 70)

Malassezia **species**	**FLU (µg/ml)**	**KETO (µg/ml)**	**ITRA (µg/ml)**	**VORI (µg/ml)**	**Amp B (µg/ml)**
M. globosa **(n = 37)**	0.06 – 8	0.06 – 4	0.03 - 4	0.03 - 8	0.03 - 2
M. sympodialis **(n = 14)**	0.06 – 4	0.06 – 0.25	0.03 - 1	0.03 – 0.5	0.06 - 1
M. furfur **(n = 11)**	0.06 – 1	0.03 - 2	0.03 – 0.125	0.03 - 1	0.03 - 4
M. restricta **(n = 6)**	0.06 – 1	0.06 - 2	0.03 – 4	0.06 - 4	0.03 - 4
M. obtusa **(n = 2)**	0.25 - 2	0. 5	0.125 – 1	0.06 – 2	0.06 – 0.5

FLU – fluconazole, KETO – ketoconazole, ITRA – itraconazole,

VORI – voriconazole, Amp B – amphotericin B.

A modification of the CLSI Document M27-A3 broth microdilution method for testing the antifungal susceptibility of yeasts was followed for the determination of MICs of the above antifungal agents for all the *Malassezia* species isolated. In this study, the MIC range of Malassezia species were within the following range: Fluconazole : 0.06 – 8µg/ml; Ketoconazole: 0.03 – 4µg/ml; Itraconazole : 0.03 – 4µg/ml; Voriconazole : 0.03 – 8µg/ml; Amphotericin B : 0.03 – 4µg/ml.

Table 29: MIC_{50} and MIC_{90} of the antifungal agents tested against *Malassezia* isolates from PV and SD lesions (n = 70)

Malassezia species (n = 70)	Fluconazole (64-0.06 μg/ml)		Ketoconazole (16-0.0313 μg/ml)		Itraconazole (16-0.0313 μg/ml)		Voriconazole (16-0.0313 μg/ml)		Amphotericin B (16 – 0.0313 μg/ml)	
	MIC_{50} μg/ml	MIC_{90} μg/ml	MIC_{50} μg/ml	MIC_{90} μg/ml	MIC_{50} μg/ml	MIC_{90} μg/ml	MIC_{50} μg/ml	MIC_{90} μg/ml	MIC_{50} μg/ml	MIC_{90} μg/ml
***M. globosa* (n = 37)**	2	8	4	4	2	4	2	8	0.06	2
***M. sympodialis* (n = 14)**	0.5	4	0.125	0.25	0.06	1	0.125	0.5	0.06	1
***M. furfur* (n = 11)**	0.25	1	0.125	2	0.125	0.125	0.5	1	0.25	4
***M. restricta* (n = 6)**	0.06	1	0.125	2	0.06	4	4	4	0.125	8
***M. obtusa* (n = 2)**	0.25	2	0.25	0.5	0.125	1	0.06	2	0.06	0.5

MIC_{50} - The minimal inhibitory concentration at which 50% of the isolates are susceptible to the antifungal agent tested.

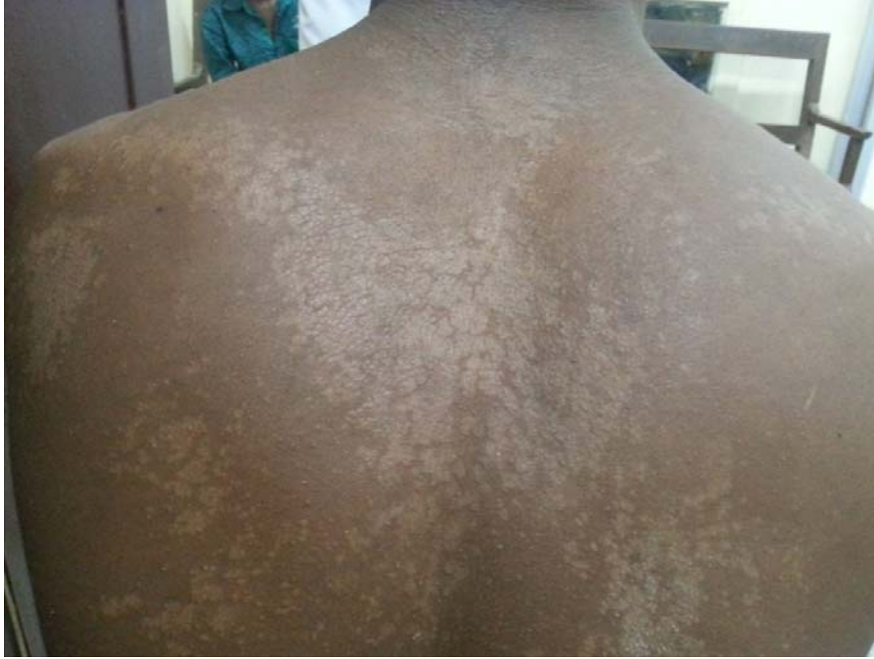
MIC_{90} - The minimal inhibitory concentration at which 90% of the isolates are susceptible to the antifungal agent tested.

MIC_{50} of the above antifungal agents for most of the *Malassezia* species were less than 1μg/ml, though a few strains of *M. globosa* and *M. restricta* had MIC values as high as 8 μg/mlfor fluconazole and voriconazole in the case of some *M. globosa* strains and 4μg/ml for itraconazole, voriconazole and amphotericin B in the case of some *M. restricta* strains.

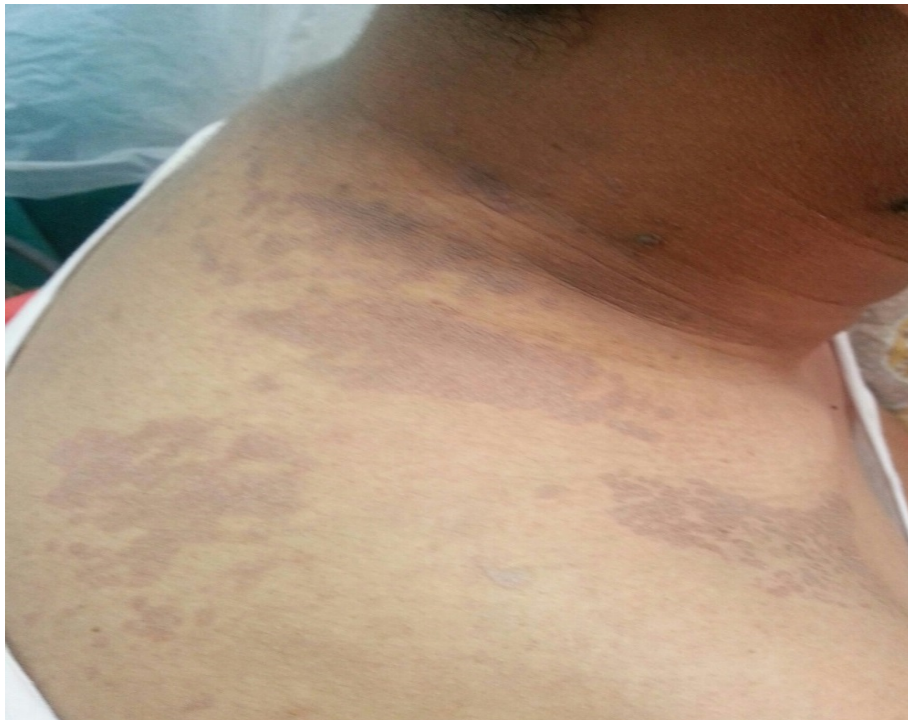
MIC_{50} and MIC_{90} values of amphotericin B were consistently low, indicating good sensitivity of all species to this drug.

COLOUR PLATES

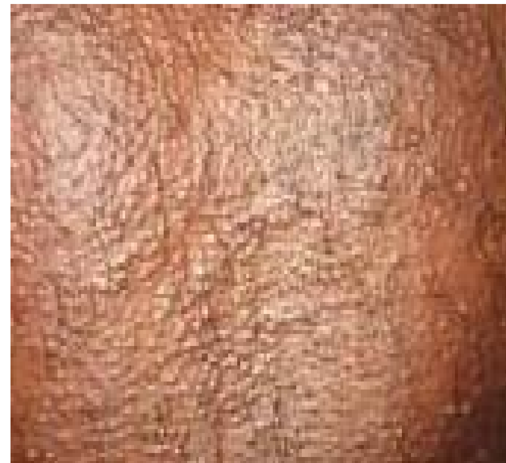
HYPOPIGMENTED PV ON THE BACK



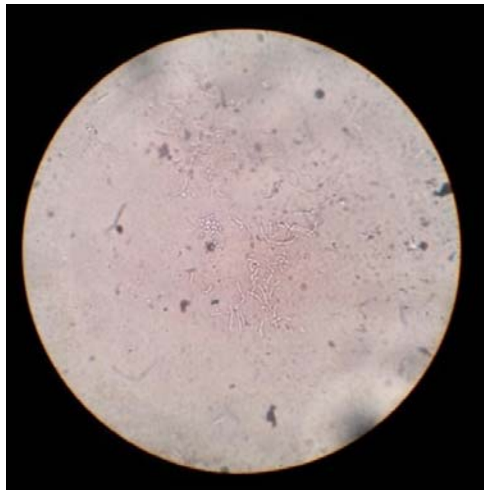
HYPERPIGMENTED PV – BACK, CHEST &NECK

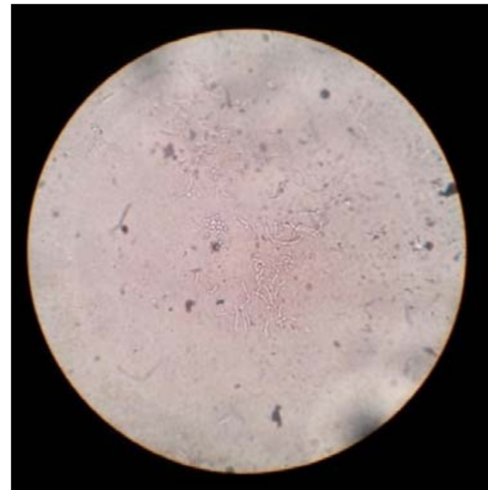


SEBORRHEIC DERMATITS OF SCALP AND BACK

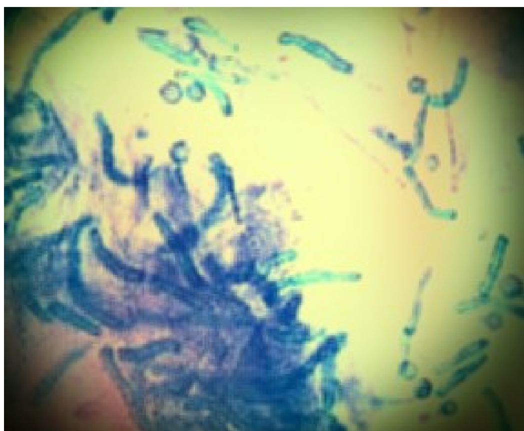


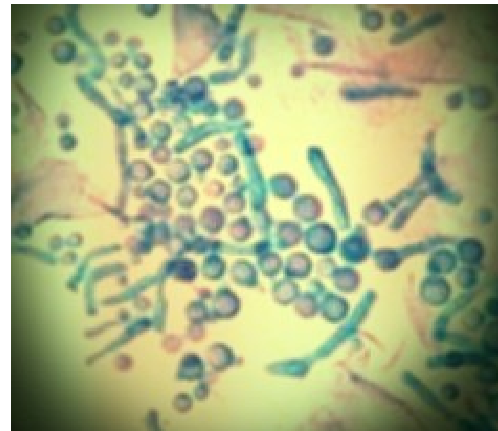
DIRECT MICROSCOPIC EXAMINATION KOH MOUNT OF SKIN SCRAPINGS SHOWING YEAST CELLS AND SHORT HYPHAE





KOH WITH PARKER BLUE INK MOUNT

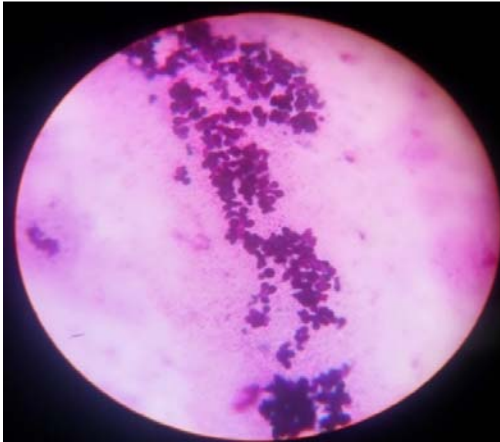




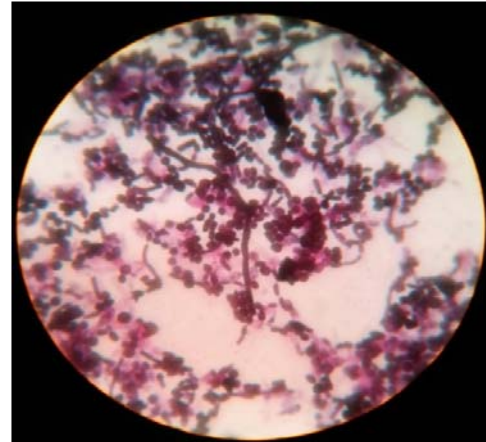
MACROSCOPIC APPEARANCE OF *MALASSEZIA* COLONIES ON SDA WITH OLIVE OIL OVERLAY

GRAM STAINING OF COLONIES GROWN ON SDA

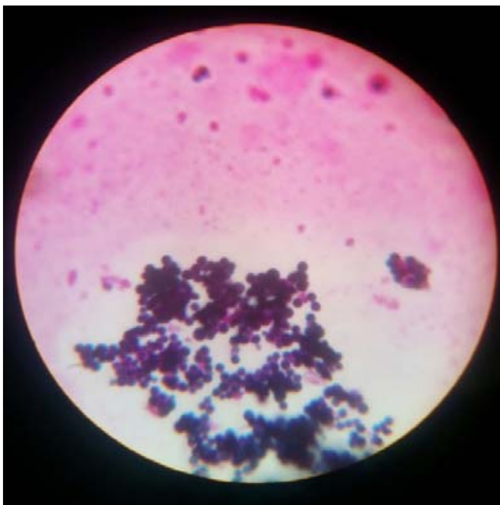
***M. furfur*- oval yeast cells**



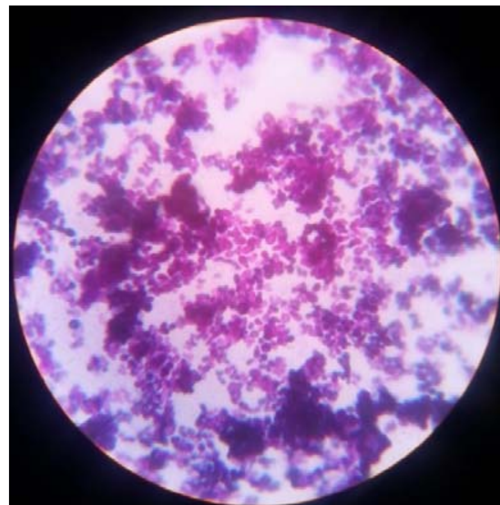
Yeast cells with hyphae



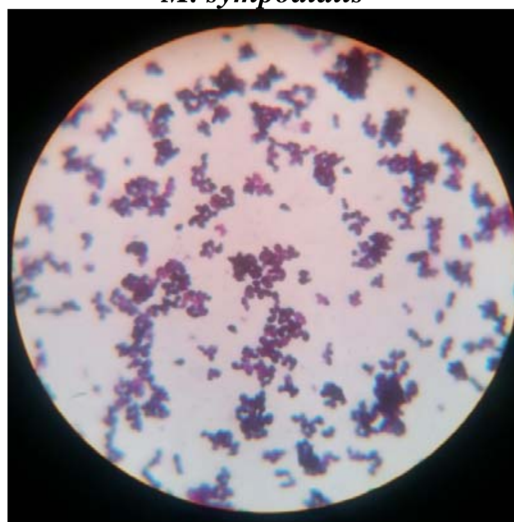
M. globosa



M. restricta

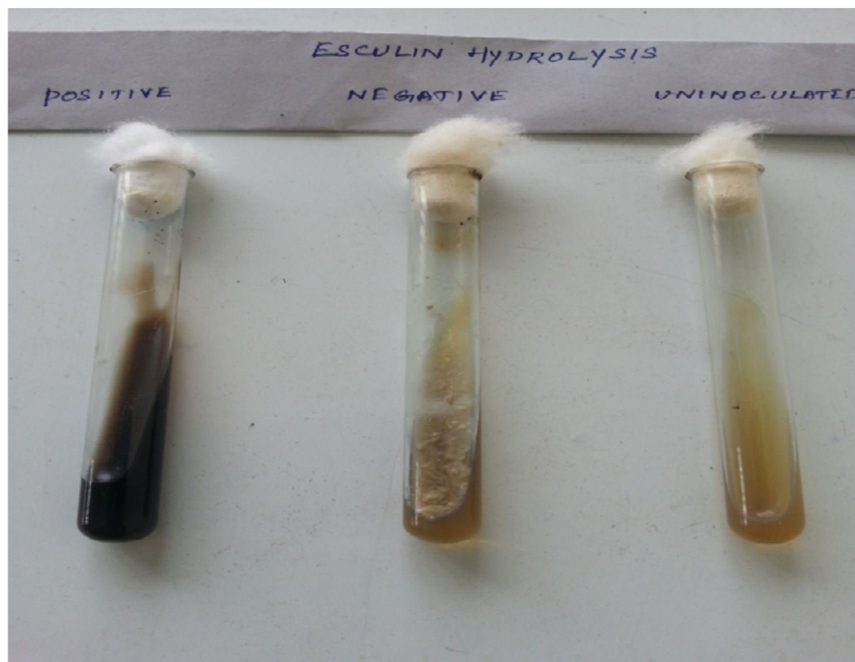


M. sympodialis



UREASE TEST

ESCULIN HYDROLYSIS TEST



CATALASE TEST

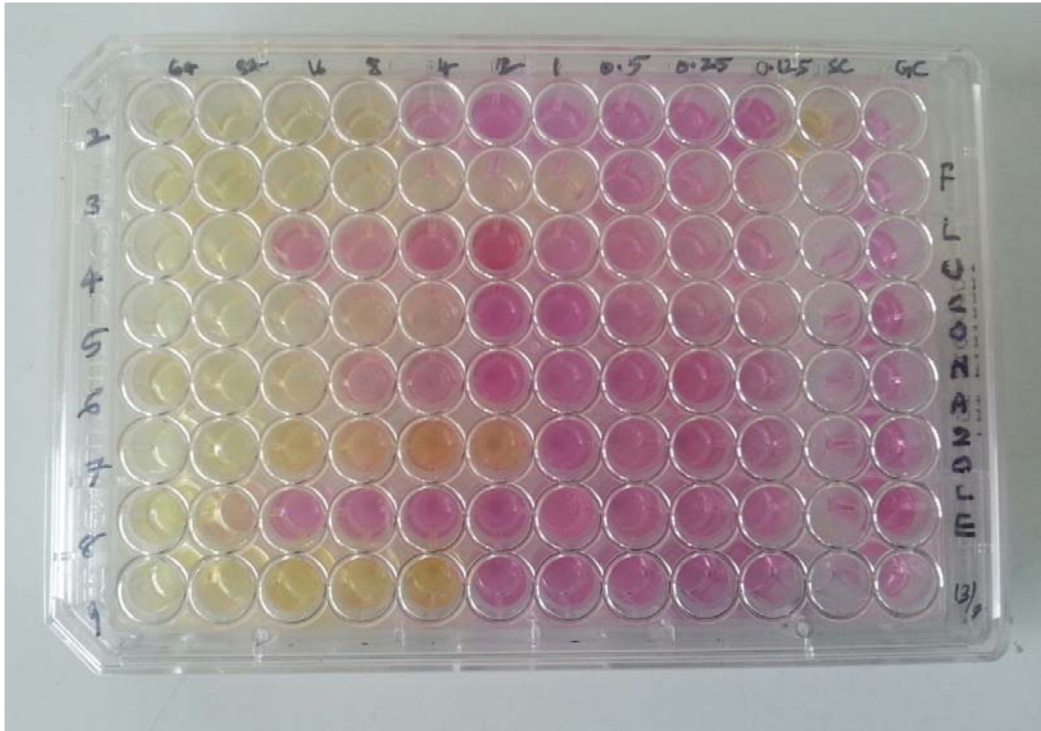
TWEEN ASSIMILATION TEST

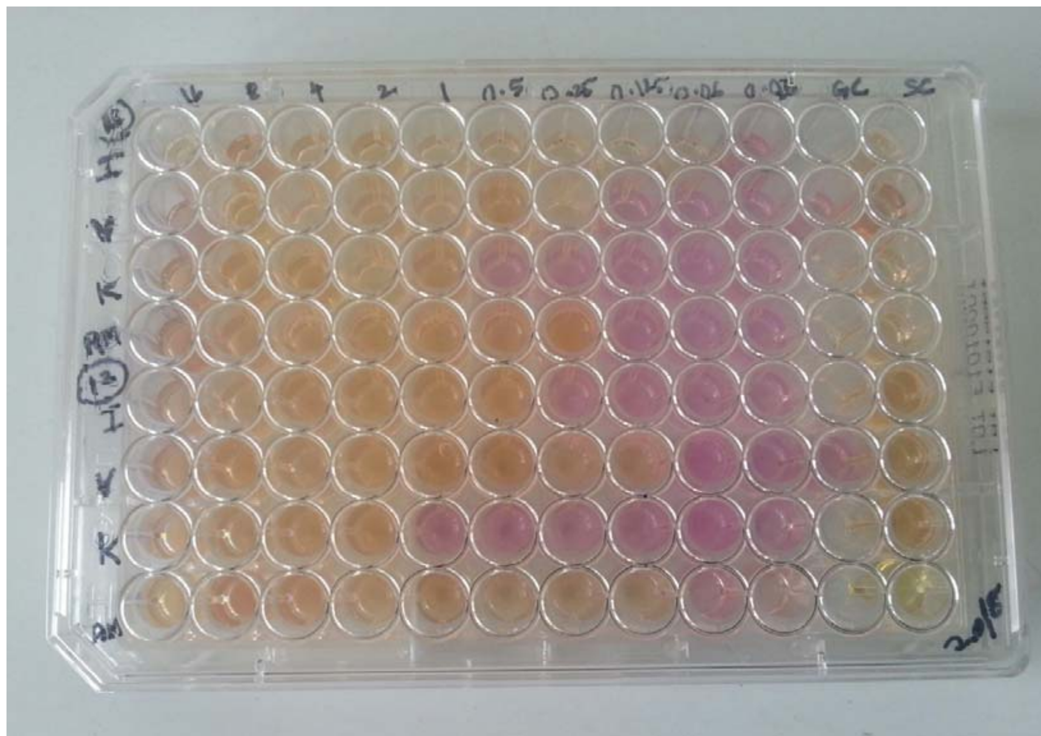
M. sympodialis

M. globosa

M. furfur

ANTIFUNGAL SUSCEPTIBILITY TESTING – MICROBROTH DILUTION METHOD





DISCUSSION

DISCUSSION

In this cross sectional study conducted for a period of one year from October 2013 to September 2014 in the Institute of Microbiology, Madras Medical College in association with the Department of Dermatology, Rajiv Gandhi Government General Hospital(RGGGH), Chennai, the epidemiological profiles of the two most common skin infections caused by *Malassezia* species namely, pityriasis versicolor and seborrheic dermatitis were analysed in 100 patients of whom 73 were clinically diagnosed to have pityriasis versicolor and 27 were clinically diagnosed to have seborrheic dermatitis **[Table 1].**

PITYRIASIS VERSICOLOR:

PV is a chronic, recurrent, superficial pigmentary dermatosis caused by *Malassezia* yeasts, affecting in general all ages ,except children and the elderly, and medical attention is sought only for cosmetic concerns.

In this study, the age group most commonly affected with PV was 20 - 29 years (42%) followed by 30 – 39 years (22%) **[Table 2].** This is in accordance with the study by Sanjeev Grover *et al* [96] in 2003, where 39.6% of those affected with PV belonged to 21 – 30 years age group and 30% belonged to 31 – 40 years age group. Siddartha Dutta *et al*[67] in 2002 and Gatha Rao *et al*[61] in the same year, also reported a 39.6% and 30% incidence repectively of PV in the 21 – 30 years age group. Studies by Terragni *et al*[51] in 1991, Michalowski *et al*[52] in 1965 and Cullen *et al* [53] in 1963 have also shown pityriasis versicolor to be common in

adolescents and young adults. This may be due to increased sebum production in the skin in these ages.

In the present study, males (72.6%) were more commonly affected with pityriasis versicolor than females (27.4%) **[Table 2]**. This is concordant with the study by Gatha Rao *et al*[61] in 2002, who observed 73.3% incidence in male patients. Gurumohan Singh *et al*[54] in 1996 and Deepak Kumar *et al*[62] in 2005 reported 75.3% and 55.4% incidence in males. Imwidthya *et al*[60] in 1988 have observed a higher incidence in females, which may be due to their increased beauty consciousness. The higher incidence in males in this study, may be attributed to increased outdoor activities of males for occupational purposes, putting them at a higher risk of sun exposure and humidity, which favours the growth of *Malassezia* yeasts. It might also be due to the proportionately higher male population attending RGGGH.

In this study, the major site of involvement of pityriasis versicolor was back (51%), followed by face and neck (29%) **[Table 3].** This is similar to the study by Kristany *et al*[83] in 2008 where 76.5 % lesions were in the back of the trunk, followed by 16% in chest. Gatha Rao *et al,*[61] reported 70% lesions in the back of trunk. Gupta *et al* in 2001 and Imwidthaya *et al*[60] in 1988 also observed PV lesions to be common on the back, followed by face. The higher involvement of the trunk, face and neck may be due to the presence numerous sebaceous glands in these sites and also due to the hot and humid climate prevalent in South India, resulting in excessive sweating in these sites.

In the present study, hypopigmented lesions (63.1%) were more common compared to the hyperpigmented lesions (36.9%)**[Table 4].** This correlates with the studies conducted by Imwidthya *et al,*[60] Gatha Rao*et al*[61] and Crespo Erchiga*et al,*[64] where hypopigmented lesions constituted 83%, 75% and 58% respectively of the PV lesions. Pityriacitrin and similar indole alkaloids produced by *M. furfur* and probably other *Malassezia* species induces apoptosis of human melanocytes and this is one likely explanation for the higher incidence of hypopigmented lesions observed in this study.[85]

In this study, 28.8 % of patients had pruritus **[Table 5]**. This is in accordance with the study conducted by GathaRao*et al*[61] in 2002, where 30% of the patients had pruritus.

In the present study, 32.9% of the patients had history of recurrence **[Table 7].** Kristany *et al*[83] observed a 26% recurrence in Indonesian population. On the contrary, Gatha Rao *et al*[61] observed recurrent PV in only 3% of the Indian patients. *Malassezia* species are commensals on healthy skin and tend to become pathogenic when host immunity is altered (as in chronic intake of steroids and other immunosuppressant drugs, HIV infection, tuberculosis and malignancy) and thus infections caused by *Malassezia* tend to be recurrent.

In the present study, a positive family history of lesions similar to pityriasis versicolor was observed in 27.4% of patients **[Table 6]**. Faergemann and Fredreichson[49] in 1979 observed a positive family history in 18% of the PV patients, Gatha Rao *et al*[61] observed 38% and Deepak Kumar *et al* [62] in 2005

observed 6% of PV patients with history of similar lesions in other family members. Though a positive family history has been noticed in more cases than chance would permit, whether it is genetically determined or due to increased exposure of family members to the causative fungal agent, is yet to be determined.[83]

In this study, majority (54.8%) of the patients with pityriasisversicolor presented with moderate amount of scales in the lesions. 28.8% had mild scales and 16.4% had severe degree of scaling in the lesions **[Table 8].** This is in accordance with the studies of Tschen[56] in 1984 and Assaf and Weil[59] in 1996 who observed that scales were present in all patients with pityriasis versicolor and also with the studies by Gatha Rao *et al*[61] in 2002 and Ghosh *et al*[95] in 2008, where 75% and 89% of patients with PV respectively, were found to have moderate to severe scales in the lesions.

In the present study, 38.4% of the patients had excessive sweating, 31.5% gave history of using bath oils, 17.8% had history of diabetes mellitus or other immunocompromised states like HIV infection, rheumatoid arthritis, post renal transplantion status, 9% were on steroids or other immunosuppressive drugs **[Table 16]**. Excessive sweating as a risk factor in the causation of PV was statistically insignificant (p value 0.39) in this study. Gatha Rao *et al*[61] observed immunocompromised status like tuberculosis, diabetes mellitus or malignancy in 18.3% of the patients. Baillon,[57]Reed,[58] Burke[66] and Faergemann[47] have found

the use of steroids, pregnanacy, poor general health, use of oral contraceptives and hyperhidrosis as predisposing factors for pityriasis versicolor.

The isolation rate of *Malassezia* species from clinically diagnosed cases of pityriasis versicolor was 76.7% in this study**[Table 9]**. Faergemann *et al*[49] in 1979 reported a 100% recovery rate, Sidhartha Dutta *et al,*[67] 59% in 2002, Kindo *et al,*[78] 68.75% in 2004 and Ebrahimzadeh *et al,*[50] 81% in 2009. The differences in the isolation rates in various studies may be due to the differences in sampling techniques and the use of different media for culture, like modified Dixon's medium, Leeming and Notman agar and SDA with olive oil.

In the present study, the duration of lesions in the majority (60.3%) of PV patients was between 1 to 6 months **[Table 11].** 86.4% of those who presented with this duration of lesions were positive by culture. This correlation between the duration of lesions and culture positivity is statistically significant (**p value 0.015**). According to Kristany *et al,*[83] 72% of the PV patients presented with duration of lesions in the 1 month – 1year range and 73% of them were culture positive.

In this study, the recovery rate of *Malassezia* species was more from the hyperpigmented type (92.6%) of PV, compared to the hypopigmented type (67.4%) **[Table 10].** This correlation was found to be statistically significant (**p value 0.013**). According to Kristany *et al,*[83] hyperpigmented lesions were found in 19% of the study population and all of them were culture positive. This is

explained by the fact that hyperpimented lesions have been found to contain more hyphae and spores than the hypopigmented ones.[36]

In the present study, the most common species isolated from culture positive cases of PV was *M. globosa* (53.6%) followed by *M. sympodialis* (21.4%) **[Table 12].** This is concordant with the studies by Siddhartha Dutta *et al*[67] in 2002, Tarazooie *et al*[63] in 2004, Crespo Erchiga *et al* [64] in 2000 and Thayikannu *et al*[68] in 2013 who observed the most common isolate to be *M.globosa*. But Gupta *et al*[65] in 2001 and Kindo *et al*[78] in 2004 reported *M.sympodialis* to be the most common isolate and Makimura *et al*[49] in 2000 reported *M. furfur* to be the most common isolate recovered from PV lesions.

In this study, *M. globosa* was the most common species isolated from both hypopigmented (67.7%) and hyperpigmented (36%) types of PV **[Table 14].** It was also the most common species isolated from PV lesions on the back (59.4%) and face (58.4%) **[Table 13].** But this correlation between site of PV lesion and *Malassezia* species isolated was statistically insignificant (p value 0.066). This is in concordance with the studies by Shah *et al*[98] in 2013 and Thayikannu *et al,*[68] who also reported *M. globosa* to be the most common isolate obtained from both hypopigmented and hyperpigmented types of PV. The higher esterase activity and lipase activity and hence the higher pathogenicity may be the likely cause for *M. globosa* being the most common isolated species.[98]

SEBORRHEIC DERMATITIS:

The etiological role of yeasts of the genus *Malassezia* has been established in seborrheic dermatitis, a superficial, flaky dermatoses affecting body sites rich in sebaceous glands.

In this study, people in the 40 – 49 years age group were commonly affected with SD (44%), followed by those in 30 – 39 years age group (33%) **[Table 17]**. The median age of occurrence of SD was 38 years. The median age of occurrence was 27.3 years in Iranian population according to Hedayati *et al*[69] in 2010, and 35 years according to Khosravi *et al*[77] in 2009. Various studies indicate that the colonization pattern of *Malassezia* species varies in different age groups[70,71]. Bergbrant*et al*[72] in 1992, also observed that the colony counts are generally lower in children and decreases again in elderly individuals. This condition may be related to the differences in sebum secretion in these age groups.

In the present study, 74% males were found to be affected with SD in contrast to 26% of females **[Table 17].** This is in accordance with the study by Khosravi *et al*[77] in 2009, where 60% of the affected individuals were males. Studies by Saghazadeh *et al*[74] in 2010 and Shivprakash *et al*[76] in 2014, observed SD to be common in females. The lesser number of female patients in this study may be due to the negligent attitude of women in the lower socioeconomic strata towards SD, as it is only a minor cosmetic ailment.

In this study, face (33.3%) and scalp (29.6%) were the common sites involved in SD **[Table 18].** This is in accordance with the studies by Hedayati *et al*[69] and Shivprakash *et al*[76] where scalp was the most common site involved. Seborrrheic dermatitis of the scalp (dandruff) being easily noticeable and widely publicized by the cosmetic industry, it is relevant that patients with this form of SD present more commonly and early to the dermatology clinic.

52% of those with clinically diagnosed SD were culture positive for *Malassezia* in the present study **[Table 20]**. In studies by Kindo *et al*[78] and Hedayati *et al,*[69] 68% and 77% respectively, of the specimens were culture positive. These differences may be due to the differences in the culture media used. Modified Dixon's agar was used in the above mentioned studies.

In this study, 66% of SD lesions of 3 months to 1 year duration were culture positive and 50% of lesions of more than 1 year duration were culture positive **[Table 24].** This correlation was not statistically significant (p value 0.39). This is in accordance with the study by Khosravi *et al*[83] in Iranian population, where 72% of patients with duration of lesions in the 1 month – 1year range were culture positive.

75% of scalp lesions and 60% of lesions on the chest and back were culture positive in the present study **[Table 21].**But the correlation was statistically insignificant (p value 0.36). This is more or less concordant with Hedayati *et al*[69] who have reported 78% of scalp lesions and 71% of the trunk lesions to be culture

positive. No statistical correlation has been noticed between the lesion site and culture result in this study also.

In the present study**,** *M. globosa* (50%) was the most frequently encountered species in SD cases, followed by *M. furfur* (28.6%) **[Table 22].** Shivprakash *et al*[76] reported that *M. globosa* and *M. restricta* were recovered commonly form SD lesions in North Indian population and *M. globosa, M. restricta* and *M. furfur* from South Indian population. According to Hedayati *et al,*[69]*M. globosa* and *M. furfur* were the most common isolates obtained from SD lesions in Iranian patients. Gaitanis *et al*[79] in 2006 similarly reported *M.globosa* to be the common among Greek SD patients. Kindo *et al*[78]reported *M.furfu r*to be common in SD patients in India. SD lesions in Swedish patients are more frequently found to be colonized with *M.obtusa*[82]. The differences in the isolates recovered may be attributed to the geographical differences and sampling and culture techniques used. *M. globosa,* by virtue of its possessing high lipase and esterase enzyme activities has been said to be the most common causative agent.

In the present study, *M. globosa* was the most common species isolated from the face (50%), scalp (50%) and chest (66.7%) **[Table 23].** *M. furfur* was the second most common species from these three sites. According to Hedayati*etal,*[69] *M.globosa* (55.8%) was the most common species isolated from scalp and face lesions SD and *M. furfur* (32.5%)from trunk lesions. This result also agrees with the study by Gupta *et al*[70]in which contact plate method was used to isolate *Malassezia* species from the various body sites of patients with SD. The

predominance of *M. furfur* in the tropics might be due to the presence of pityriacitrin, an indole alkaloid[84] which has the ability to protect the fungus against ultraviolet light, rendering *M. furfur* more resistant to sun exposure.

PV and SD lesions in patients with HIV and AIDS have been found to be extensive and refractory to standard therapy.[27] In this study, 11% of those with SD were diabetic, 7.4% were infected with HIV and 3.7% had history of steroid intake **[Table 25]**. In 2006, Rincon *et al*[86] found extensive SD in patients with HIV whose CD4 counts were below 500 cells/ cu.mm.

On analysing the results obtained in PV and SD patients in this study, both the conditions were found to be common in males **[Tables 2 & 17].** Both the conditions were common in young adults, PV being common in 20 – 29 years age group **[Table 2]** and also adolescents, and SD in the adults of 40 – 49 years age group **[Table 17]**. Most of the PV and SD cases presented within 3 months to 6 months of illness. 33% of the patients had recurrence in both the conditions **[Tables 7&19]**. The common site to be involved in PV was back and that in SD, face and scalp **[Tables 3&18].**

Culture positivity for *Malassezia* spp. was higher in PV (77%) compared to SD (52%) **[Table 26]**. In both the conditions, *M. globosa* was the most common species isolated. The second most common agent was *M. sympodialis* in PV and *M. furfur* in SD **[Table 27].** A significant number of patients with diabetes mellitus, history of steroid intake, HIV infection and other immunocompromised

states like rheumatoid arthritis, post-renal transplant state were encountered in both the conditions **[Tables 16 & 25].**

MIC OF THE ISOLATES:

The MIC ranges obtained in the present study are comparable to those of the studies by Margarita *et al,*[88] Tiwari *et al,*[90] Gupta *et al,*[91] Schmidt *et al,* [92] Hammer *et al,* [93] and *Marcon et al.*[94] The comparisons are tabulated below:

Malassezia species	Study	FLU (µg/ml)	KETO (µg/ml)	ITRA (µg/ml)	VORI (µg/ml)	AMP B (µg/ml)
M. globosa	Present study	0.06 – 8	0.06 – 4	0.03 - 4	0.03 - 8	0.03 - 2
	Others[88, 91 - 94]	0.125 - 16	0.008 – 0.125	0.016 – 8	0.03 - 8	0.06 - 1
M. sympodialis	Present study	0.06 – 4	0.06 – 0.25	0.03 - 1	0.03 – 0.5	0.06 - 1
	Others[88, 91 - 94]	0.03 – 4	0.06 – 1	0.06 – 0.5	0.03 -0.5	0.03 - 2
M. furfur	Present study	0.06 – 1	0.03 - 2	0.03 – 0.125	0.03 - 1	0.03 - 4
	Others[88, 91 - 94]	0.25 - 64	0.03 – 0.125	0.03 – 0.5	0.03 – 0.25	0.5 - 8
M. restricta	Present study	0.06 – 1	0.06 - 2	0.03 – 4	0.06 - 4	0.03 - 4
	Others[88, 91 - 94]	0.5 - 4	0.016 – 4	0.016 – 2	0.06 - 8	0.06 - 2
M. obtusa	Present study	0.25 - 2	0. 5	0.125 – 1	0.06 – 2	0.06 – 0.5
	Others[88, 91 - 94]	0.25 - 8	0.06 - 4	0.03 – 0.5	0.03 - 2	0.03 - 8

FLU – fluconazole, KETO – ketoconazole, ITRA – itraconazole,VORI – voriconazole, Amp B – Amphotericin B.

In the present study, the lowest MIC_{50} value for *M.globosa*was observed with amphotericin B (0.06µg/ml), for *M. sympodialis* with itraconazole and amphotericin B (0.06µg/ml), for *M. furfur* with ketoconazole and itraconazole(0.125µg/ml), for *M. restricta* with fluconazole and itraconazole(0.06µg/ml), and for *M. obtusa* with voriconazole and amphotericin B (0.06µg/ml).

Low MIC ranges were noted with ketoconazole and itraconazole, indicating high sensitivity of *Malassezia* species to these azoles. This is in concordance with the studies by Gupta *et al,* [91] Schmidt *et al*[92] and Hammer *et al,* [93] where MIC ranges of ketoconazole were between 0.03 – 2 µg/ml.

The main purpose of MIC determination is the detection of resistance. As the effect of testing variables on MICs for *Malassezia* yeasts and their reproducibility has not been evaluated as for other yeasts, a reliable comparison of MIC data is rendered difficult.

SUMMARY

SUMMARY

This cross sectional study done for a period of one year at a tertiary care hospital in Chennai, included a total of 100 patients who were clinically diagnosed with either pityriasis versicolor or seborrheic dermatitis. The epidemiological profile and antifungal susceptibility pattern of the *Malassezia* species isolated from the lesions in study population were analysed and the results are summarized below:

- Of the total 100 patiens enrolled in the study, 73 were clinically diagnosed to have pityriasis versicolor and the remaining 27, seborrheic dermatitis. The study population consisted of patients belonging to all age groups and both sexes.

- Pityriasis versicolor commonly occurred in the 20 – 29 years age group (42.5%) and SD in the 40 -49 years age group (44.5%).The median age of occurrence was found to be 24 years for PV and 38 years for SD.

- Males were affected more commonly than females in both PV (72.6% males) and SD (74% males).

- In both sexes, the 20 – 29 years age group was commonly affected in the case of PV(32.9% of males and 9.6% of females) and the 40 – 49 years age group in the case of SD (29.6% males and 14.9% of females belonged to this age group).

- Majority of the pityriasis versicolor patients presented with lesions on the back (50.7%), followed by face and neck (24.7%). Face (33.33%) and scalp (29.63%) were the common sites involved in SD, followed by chest and back (18.52%).

- Pruritus was present in 28.8% of the cases of PV.

- 27.4% of those with PV had a family history of similar lesions.

- 32.9% of the patients with PV and 33% of the patients with SD had recurrent lesions. SD involving scalp was found to occur recurrently compared to lesions on the other sites.

- 63.1% of the patients had hypopigmented type (achromic PV) of pityriasis versicolor and 36.9% had hyperpigmented (chromic) type.

- Majority (54.8%) of the patients with pityriasis versicolor presented with moderate amount of scaling in the lesions. Severe degree of scaling was commonly noticed in the hyperpigmented type of PV.

- The overall isolation rate of *Malassezia* species was 70% of the total study population of 100.

- The isolation rate of *Malassezia* species was higher from PV lesions (76.7% of the 73 PV cases), while it was only 52% from the SD lesions (27 SD cases).

- Among the PV cases, 92.6% of the hyperpigmented lesions were culture positive compared to 67% in the hypopigmented type. This result was statistically significant (p 0.013).

- In SD, 75% of scalp lesions and 60% of lesions on the chest and back were culture positive for *Malassezia* species.

- The duration of lesions in majority of patients with pityriasis versicolor was between 1 to 6 months (60.3%). 86.4% of cases with this duration of lesions were culture positive for *Malassezia*. 66% of SD lesions of 3 months to 1 year were culture positive. This correlation between the duration of lesions and culture positivity was statistically significant (p value 0.015) in the case of PV lesions.

- *M. globosa* was the most common species isolated (53.6%), followed by *M. sympodialis* (21.4%), in PV. *M. globosa* was the most frequently encountered species (50%) in SD cases, followed by *M. furfur* (28.6%).

- *M. globosa* was the most common species isolated form back (59.4%) and face (58.4%) lesions of PV and *M. sympodialis* was the predominant species isolated from chest lesions (44.5%). In SD, *M. globosa* was the most common species isolated from the face (50%), scalp (50%) and chest (66.7%). *M. furfur* was the second most common species form these three sites.

- *M. globosa* was the most common species isolated from both the hypopigmented (67.7%) and hyperpigmented (36%) types of PV.

- *M. globosa* was the most common species isolated from both new (39.3%) and recurrent cases (14.2%) of PV and SD.

- Risk factors like excessive sweating, use of bath oils, immunocompromised status were present in 38.3%, 31.5% and 12.3% of the PV cases respectively. In the case of SD, 11% of the cases were diabetics and 7.4% of them were HIV positive.

- Antifungal susceptibility by broth microdilution method using Christensen's urea broth, was performed for the 70 *Malassezia* isolates using fluconazole, ketoconazole, voriconazole, itraconazole and amphotericin B.

- MIC range of *Malassezia* species were 0.06 – 8 µg/ml for fluconazole, 0.03 – 4 µg/ml for ketoconazole, itraconazole and amphotericin B, and 0.03 – 8 µg/ml for voriconazole.

- Low MIC ranges and MIC_{50} values were noted with amphotericin B, ketoconazole and itraconazole for most of the *Malassezia* species, excepting a few strains of *M. globosa* and *M. restricta.*

CONCLUSION

CONCLUSION

Yeasts of the genus *Malassezia* are part of commensal flora of human and animal skin, which under the influence of various predisposing factors, are capable of causing a spectrum of conditions from superficial dermatologic lesions causing only cosmetic disfigurement to serious, life threatening systemic infections and fungemia. This study was aimed at analyzing the epidemiological attributes and other risk factors involved in the clinical patterns of the common superficial infections namely, pityriasis versicolor and seborrheic dermatitis caused by *Malassezia* species in this part of India.

Adolescents and young adults of both sexes were the ones commonly affected with the superficial skin infections analyzed and hence they assume cosmetic significance. *Malassezia globosa* was the most common species isolated from both hypopigmented and hyperpigmented pityriasis versicolor and also from seborrheic dermatitis. In the case of PV, the isolation rate of *Malassezia* species was significantly higher from the hypepigmented type, and also higher in patients who presented with PV lesions of 1 to 6 months duration.

The in vitro antifungal susceptibility testing results were comparable with those of previous studies. However, for *Malassezia* species, standard testing conditions and clinical breakpoints have not yet been established and it remains unclear whether the in vitro activity of the antifungal agents tested is predictive of clinical outcome. Hence, routine antifungal susceptibility testing along with analysis of clinical outcome should be performed in future studies.

Confirmation of a clinical diagnosis of *Malassezia* infections by direct microscopic examination of the clinical material collected and culture techniques is important, as different species are involved in the etiological role in different geographical locations and also due to interspecies differences in susceptibility patterns to antifungal agents. As culture forms the basis for species level identification through biochemical tests and choosing of an appropriate antifungal agent through determination of MIC, the information provided by culture will be very valuable in paving way for a tailored clinical approach, thus reducing chronicity, recurrences and systemic complications associated with these infections and also for a better understanding of the epidemiology of *Malassezia* species.

APPENDICES

APPENDIX - 1

LIST OF ABBREVIATIONS USED

AhR	-	Aryl hydrocarbon Receptor (AhR)
AIDS	-	Acquired immunodeficiency syndrome
ATCC	-	American type culture collection
CFU	-	Colony forming units
CFW	-	Calcoflour White stain
CLSI	-	Clinical Laboratory Standards Institute
DMSO	-	Dimethyl Sulfoxide
ELISA	-	Enzyme Linked Immunosorbent Assay
FFA	-	Free fatty acids
HIV	-	Human Immunodeficiency Virus
HPE	-	Histopathological Examination
KOH	-	Potassium hydroxide
MIC	-	Minimum Inhibitory Concenteration
PAS	-	Periodic Acid Schiff
PFGE	-	Pulsed Field Gel Electrophoresis
PUVA	-	Psoralen Ultraviolet A
PV	-	PityriasisVersicolor
RFLP	-	Restriction Fragment Length Polymorphism
RPMI	-	RoseWell Park Memorial Institute
ROS	-	Reactive Oxygen Species
SDA	-	Sabouraud Dextrose Agar
SD	-	Seborrheic Dermatitis
UV	-	Ultraviolet

APPENDIX II

MEDIA, STAINS AND REAGENTS

1. 10% KOH with Parker Quink's blue black ink

Potassium hydroxide	l0g
Glycerol	l0ml
Distilled water	80ml

10 g of KOH crystals are dissolved in distilled water by stirring slowly and glycerol is added. Parker Quink's blue black ink is added to the prepared solution in equal volume before use.

2. Gram stain

Methyl violet	l0g Methyl violet in l00ml absolute alcohol in 1litre of distilled water (primary stain)
Gram's Iodine	l0g Iodine in 20g KI (mordant)
Acetone	Decolourising agent
Carbolfuchsin 1%	Secondary stain (counter stain)

3. Catalase

3% hydrogen peroxide

4. Christensen's Urease test medium

Peptone	1g
Sodium chloride	5g
Dipotassium hydrogen phosphate	2g
Phenol red	6ml
Distilled water	1 litre
10% sterile solution of glucose	l0ml
Sterile 20% urea solution	l00ml

Sterilize the glucose and urea solutions by filtration. Prepare the basal medium without glucose and urea, adjust to pH 6.8-6.9 and sterilize by autoclaving in a flask at 121°C for 30min. Cool to about 50°C, add the glucose & urea.

5. Sabouraud dextrose agar

Dextrose	40g
Peptone	l0g
Chloramphenicol	500 mg
Cycloheximide	500 mg
Agar	20g
Distilled water	l000ml
p H = 5.4	

65 g of dehydrated medium (HiMedia) should be reconstituted in 1 litre of distilled water. The pH is adjusted to 5.4. Cycloheximide is dissolved in acetone and chloramphenicol is dissolved in 95% alcohol and added to the medium. Sterilize by autoclaving at 121ºC for 20 min.

6.Esculin Agar:

Casein enzyme hydrolysate	13 g
Sodium chloride	5 g
Yeast extract	5 g
Beef heart infusion	2 g
Esculin	1 g
Ferric citrate	0.5 g
Agar	15 g
Distilled water	1000 ml
Final pH	7.3 ± 0.2

41.5 g of dehydrated medium (HiMedia) should be reconstituted in 1 litre distilled water. Dissolve by boiling and adjust pH to 7.3. Dispense in 3 ml quantities in tubes and autoclave at 121˚ C for 20 min. Cool the tubes in a slant position.

7. Tween Compounds:

Tween compounds are water – soluble, nonionic surfactants, which are polymers of ethylene oxide linked to sorbitan and a lipophilic group.

Tween 20 - polyoxyethylene (20) sorbitanmonolaureate

Tween 40 - polyoxyethylene (20) sorbitanmonopalmitate

Tween 60 - polyoxyethylene (20) sorbitanmonostearate

Tween 80 - polyoxyethylene (20) sorbitanmonooleate

Sterilised at 160˚C for 1 hour in hot air oven.

ANNEXURES

ANNEXURE 1 - MASTER CHART

Sl. No.	Name	Age	sex	OP/IP No	Lesion	Duration	Site of lesion	Occupation	PV type	Any IC Status	Pruritis	scales	excessive sweating	recurrence	Family history	Bath oils	growth	catalase	Esculin	Gth at 42	Tween assimiln.	MIC FLC	MIC KTC	MIC ITC	MIC VRC	MIC AMPH
1	Sasikumar	19	M	34568	PV	3 months	face & neck	student	hypopigmented		N	moderate	Y	N	Y	N	M. globosa	Pos	Neg	Neg	nil	0.06	0.5	0.06	0.25	2
2	Venkatesan	39	M	23674	PV	2 months	chest	driver	hypopigmented		N	moderate	N	N	N	N	M. restricta	neg	Neg	Neg	nil	0.06	0.06	0.03	4	0.06
3	Janarthanan	18	M	29759	PV	2 months	back	student	hypopigmented		N	moderate	Y	N	N	N	M. furfur	Pos	Neg	pos	20,40,60,80	0.25	0.03	0.125	1	0.06
4	Kumar	49	M	131447	PV	2 months	back & chest	painter	hyperpigmented	PT	Y	severe	Y	Y	N	N	M. restricta	neg	Neg	Neg	nil	0.25	0.06	0.03	0.06	0.03
5	Ismail	32	M	129118	PV	1 month	face	Cooley	hyperpigmented		Y	moderate	N	Y	Y	Y	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
6	Nirmala	29	F	20302	PV	4 months	face & neck	housewife	hypopigmented		N	mild	N	N	Y	N	M. globosa	Pos	Neg	Neg	nil	2	4	1	0.5	4
7	David Prasanna	22	M	27163	PV	8 months	back & chest	student	hypopigmented		N	moderate	Y	Y	N	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
8	Prabhu	42	M	36627	SD	4 weeks	neck	vendor	NA		NA	NA	NA	N	N	y	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	Sathyaraj	25	M	39309	PV	6 months	back	plumber	hypopigmented		Y	moderate	N	N	N	Y	M. globosa	Pos	Neg	Neg	nil	0.06	0.5	0.03	0.03	0.06
10	Prakash	21	M	26412	PV	6 weeks	back	student	hypopigmented		N	moderate	Y	N	N	N	M. globosa	Pos	Neg	Neg	nil	0.5	0.5	1	0.5	0.12
11	Dinesh Kumar	14	M	20403	PV	5 months	back & chest	student	hyperpigmented	IDDM	N	moderate	N	N	Y	N	M. sympodialis	Pos	pos	pos	40,60,80	0.25	0.06	0.06	0.06	0.06
12	Vignesh	20	M	42998	PV	1 year	arms & chest	student	hypopigmented		N	mild	Y	N	Y	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
13	Arasu	28	M	43562	PV	9 months	arms, back & chest	driver	hyperpigmented		Y	severe	Y	Y	N	N	M. restricta	neg	Neg	Neg	nil	0.06	1	2	4	0.5
14	Parameswari	13	F	38471	PV	2 months	face & neck	student	hypopigmented		Y	mild	N	N	N	Y	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
15	Soorya	19	M	45209	PV	1 month	back & chest	student	hypopigmented		N	moderate	Y	N	N	N	M. globosa	Pos	Neg	Neg	nil	0.5	0.5	0.06	0.03	0.25
16	Mani	25	M	46140	SD	3 months	arms & chest	business	NA		NA	NA	NA	N	N	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
17	Indumathi	22	F	22342	PV	5 months	neck	student	hyperpigmented		N	moderate	Y	N	Y	N	M. sympodialis	Pos	pos	pos	40,60,80	0.5	0.25	0.125	0.125	0.25
18	Susaiyappan	40	M	55570	PV	3 weeks	back	farmer	hypopigmented	RA	N	mild	Y	Y	N	N	M. globosa	Pos	Neg	Neg	nil	2	2	1	8	2
19	Ganesan	34	M	47862	SD	4 months	back	tailor	NA		NA	NA	NA	Y	N	N	M. globosa	Pos	Neg	Neg	nil	2	4	0.06	0.06	0.5
20	Rahul	20	M	39567	SD	10 months	scalp	student	NA		NA	NA	NA	Y	Y	N	M. globosa	Pos	Neg	Neg	nil	2	1	1	0.05	2
21	Padma	25	F	43218	PV	5 months	face & neck	student	hypopigmented		Y	mild	N	Y	N	N	M. globosa	Pos	Neg	Neg	nil	2	1	0.125	0.125	0.25
22	Selvam	22	M	41757	PV	9 months	back	student	hypopigmented		N	moderate	N	N	Y	N	M. globosa	Pos	Neg	Neg	nil	0.5	0.5	0.5	1	0.25
23	Priyadarshini	18	F	49790	PV	2 months	back & neck	student	hypopigmented		N	moderate	Y	N	Y	N	M. furfur	Pos	Neg	pos	20,40,60,80	0.06	0.06	0.06	0.125	0.25
24	Bharathan	21	M	12528	PV	1 month	arms & chest	student	hypopigmented		N	mild	N	N	N	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
25	Balaji	28	M	38407	PV	5 months	back	shopkeeper	hyperpigmented		N	severe	N	N	N	N	M. globosa	Pos	Neg	Neg	nil	0.12	0.5	0.06	8	0.5
26	Anand	22	M	149822	PV	3 months	back	student	hypopigmented		N	mild	N	N	N	N	M. restricta	neg	Neg	Neg	nil	1	2	4	4	4
27	Muthulakshmi	35	F	46074	PV	1 month	arms	housewife	hyperpigmented		Y	mild	Y	N	Y	N	M. obtusa	Pos	pos	Neg	nil	0.25	0.5	1	0.06	0.06
28	Narasimhan	46	M	47960	PV	2 months	back	teacher	hypopigmented	RA	Y	moderate	Y	N	Y	Y	M. globosa	Pos	Neg	Neg	nil	2	4	1	1	2
29	Krsihnaveni	18	F	48260	PV	5 months	face	housewife	hyperpigmented		N	moderate	Y	N	N	N	M. sympodialis	Pos	pos	pos	40,60,80	0.06	0.06	0.03	0.03	0.06
30	Devi	38	F	11574	PV	3 weeks	neck	housewife	hypopigmented		N	moderate	Y	N	N	Y	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
31	Rani	41	F	55159	PV	6 months	face & neck	housewife	hyperpigmented	DM	N	severe	N	Y	N	N	M. globosa	Pos	Neg	Neg	nil	0.5	0.5	1	1	0.03
32	Subramani	38	M	55244	PV	6 months	back	driver	hyperpigmented	PT	N	mild	Y	Y	N	Y	M. sympodialis	Pos	pos	pos	40,60,80	0.125	0.25	0.125	0.125	0.25
33	Murugan	37	M	57044	PV	4 months	back	plumber	hypopigmented		Y	severe	N	N	Y	N	M. globosa	Pos	Neg	Neg	nil	2	4	1	1	0.06
34	Rajan	40	M	56498	PV	1 month	back	farmer	hypopigmented	RA on steroids	N	moderate	Y	N	N	N	M. globosa	Pos	Neg	Neg	nil	0.5	1	1	0.5	0.5
35	Naveen Kumar	23	M	58177	PV	2 months	chest	student	hypopigmented		Y	mild	N	N	N	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
36	Balaji	15	M	57991	PV	5 months	chest	student	hyperpigmented		Y	mild	N	Y	N	Y	M. sympodialis	Pos	pos	pos	40,60,80	0.5	0.125	0.03	0.06	0.125
37	Saravanan	30	M	25065	PV	4 months	back & chest	Cooley	hyperpigmented		N	moderate	N	Y	Y	N	M. furfur	Pos	Neg	pos	20,40,60,80	0.5	0.125	0.03	0.5	0.03
38	Mathaiyan	38	M	25308	SD	3 weeks	nape of neck, face	truck driver	NA		NA	NA	NA	N	N	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
39	Anandhan	20	M	59036	PV	1 year	back	student	hypopigmented		N	severe	Y	Y	N	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
40	Yadhin	18	M	35611	PV	10 months	chest	student	hyperpigmented		N	moderate	N	Y	Y	N	M. obtusa	Pos	pos	Neg	nil	2	0.5	0.125	2	0.5
41	Surya	15	M	29226	PV	3 months	back & chest	student	hypopigmented		Y	moderate	N	Y	N	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
42	Shankar	35	M	59527	PV	2 months	arms & chest	electrician	hyperpigmented		N	severe	Y	N	Y	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
43	Ramesh	24	M	34185	PV	4 months	arms, back & chest	Cooley	hyperpigmented	Post-op GI surgery	N	severe	N	N	Y	Y	M. globosa	Pos	Neg	Neg	nil	2	4	4	2	0.25
44	Prasad	21	M	86710	PV	2 months	back	student	hypopigmented		N	mild	N	N	N	Y	M. globosa	Pos	Neg	Neg	nil	0.5	0.5	2	1	1
45	Chitrasan	20	M	42444	PV	5 weeks	back & chest	student	hypopigmented		N	moderate	N	N	N	N	M. sympodialis	Pos	pos	pos	40,60,80	0.06	0.125	0.5	0.25	0.06

46	Amutha	43	F	37801	PV	4 months	face	housewife	hypopigmented		N	mild	N	Y	N	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
47	Vijayaraghavan	44	M	47301	PV	3 months	back	teacher	hyperpigmented	post renal transplant	Y	moderate	Y	N	N	Y	M. sympodialis	Pos	pos	pos	40,60,80	0.5	0.125	0.125	0.06	0.25
48	Suresh Kumar	29	M	86461	PV	2 months	back	painter	hyperpigmented	inflam. Polyarthritis	N	severe	N	Y	N	Y	M. globosa	Pos	Neg	Neg	nil	2	0.5	0.5	0.25	0.5
49	Syed Pranutheen	69	M	90995	PV	1 month	chest & neck	retired	hypopigmented	COPD on steroids	Y	moderate	N	Y	N	N	M. sympodialis	Pos	pos	pos	40,60,80	0.06	0.06	0.06	0.5	0.06
50	Ramalingam	20	M	91509	PV	I month	chest	student	hypopigmented		N	mild	Y	Y	N	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
51	Lazar	49	M	91530	PV	2months	back	driver	hypopigmented	DM	Y	moderate	N	N	N	Y	M. globosa	Pos	Neg	Neg	nil	0.12	0.5	1	0.5	1
52	Ajith	13	M	91746	PV	6 months	chest	student	hyperpigmented		N	mild	Y	N	Y	N	M. globosa	Pos	Neg	Neg	nil	2	4	4	8	0.25
53	Yadhav	20	M	92306	PV	2 years	back, face, chest	student	hypopigmented		N	moderate	N	N	N	Y	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
54	Ramakrishnan	23	M	56210	PV	1 year	back & chest	student	hyperpigmented		N	moderate	N	N	N	N	M. furfur	Pos	Neg	pos	20,40,60,80	0.25	0.25	0.125	0.25	0.5
55	Rajesh	29	M	93405	PV	4 months	face, neck & back	vendor	hyperpigmented		N	severe	N	Y	Y	N	M. furfur	Pos	Neg	pos	20,40,60,80	0.03	0.125	0.03	0.03	4
56	Sumathi	36	F	80226	PV	1 month	chest	cook	hypopigmented		N	moderate	N	N	N	Y	M. sympodialis	Pos	pos	pos	40,60,80	0.5	0.25	0.25	0.125	0.125
57	Shamsudeen	33	M	79854	PV	1 month	back	teacher	hypopigmented		N	severe	Y	N	N	Y	M. globosa	Pos	Neg	Neg	nil	2	4	0.06	1	0.125
58	Kanniyamma	32	F	34521	PV	5 months	chest	housewife	hyperpigmented		N	moderate	Y	Y	N	N	M. globosa	Pos	Neg	Neg	nil	1	1	1	1	0.06
59	vijay Kumar	26	M	98241	PV	6 months	back & chest	business	hyperpigmented		Y	moderate	N	Y	N	N	M. sympodialis	Pos	pos	pos	40,60,80	0.25	0.06	0.03	0.06	0.25
60	Baskaran	48	M	93667	PV	5 weeks	chest & back	vendor	hypopigmented		Y	moderate	N	N	N	Y	M. sympodialis	Pos	pos	pos	40,60,80	0.5	0.25	0.125	0.125	0.06
61	Rajan	17	M	104867	PV	7 weeks	back & chest	student	hypopigmented		N	mild	N	N	Y	N	M. globosa	Pos	Neg	Neg	nil	2	1	0.06	2	0.25
62	Satham Hussain	24	M	104826	PV	7 months	back & chest	student	hypopigmented		N	mild	N	N	N	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
63	Sheik Mubaraq	20	M	103256	PV	10 months	face & neck	student	hypopigmented		N	moderate	Y	N	N	Y	M. globosa	Pos	Neg	Neg	nil	8	4	4	2	0.06
64	Monisha	17	F	105172	PV	3 months	face	student	hypopigmented		N	moderate	N	N	N	Y	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
65	Krishnan	65	M	10985	PV	2 months	chest & back	farmer	hypopigmented	HT & DM	N	moderate	N	N	N	N	M. globosa	Pos	Neg	Neg	nil	2	1	1	0.5	0.06
66	Janaki	17	F	57323	PV	2 weeks	face & neck	student	hypopigmented		N	moderate	Y	N	Y	Y	M. sympodialis	Pos	pos	pos	40,60,80	0.06	0.25	0,5	0.25	0.25
67	Decemberi	24	F	57324	PV	4 weeks	face & neck	student	hypopigmented		N	mild	N	Y	N	N	M. globosa	Pos	Neg	Neg	nil	4	4	2	2	0.06
68	Vijayakumar	34	M	92965	PV	2 months	back	driver	hyperpigmented		N	moderate	N	N	N	N	M. globosa	Pos	Neg	Neg	nil	2	4	4	4	0.25
69	Munusamy	48	M	87365	PV	5 months	back	cooley	hypopigmented		N	mild	N	Y	N	N	M. globosa	Pos	Neg	Neg	nil	2	1	1	2	0.12
70	Jeyaseeli	24	F	113183	PV	1 month	face	student	hypopigmented		Y	mild	N	Y	N	Y	M. globosa	Pos	Neg	Neg	nil	0.5	1	0.06	2	0.06
71	Ravi	34	F	108512	PV	2 months	face & neck	housewife	hypopigmented		Y	moderate	Y	N	N	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
72	Murali	36	M	115182	SD	6 months	scalp	office staff	NA		NA	NA	NA	Y	N	y	M. globosa	Pos	Neg	Neg	nil	4	4	0.06	1	0.25
73	Balaguru	23	M	115462	PV	1 month	neck & chest	student	hypopigmented		Y	moderate	N	N	Y	N	M. restricta	neg	Neg	Neg	nil	0.125	0.5	0.06	0.06	0.125
74	Sheela	31	F	114775	SD	2 years	neck & back	housewife	NA		NA	NA	NA	N	N	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
75	Muthukumaran	38	M	78605	SD	6 months	scalp,nasolabial area	watchman	NA		NA	NA	NA	Y	N	N	M. furfur	Pos	Neg	pos	20,40,60,80	0.25	0.5	0.125	0.125	0.25
76	Priya	24	F	128791	SD	1 year	scalp,ear lobes & neck	student	NA		NA	NA	NA	Y	Y	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
77	Devaki	46	F	123265	SD	10 months	scalp	housewife	NA	DM	NA	NA	NA	Y	N	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
78	Nithyarajan	46	M	106341	SD	2 years	face	shopkeeper	NA		NA	NA	NA	N	N	N	M. furfur	Pos	Neg	pos	20,40,60,80	0.5	0.125	0.125	0.06	0.06
79	Padmalakshmi	27	F	99876	PV	1 year	back & neck	nurse	hyperpigmented		N	moderate	N	Y	N	Y	M. furfur	Pos	Neg	pos	20,40,60,80	0.125	0.125	0.06	0.5	0.25
80	Gunasekaran	45	M	119800	PV	6 months	back	drama artist	hyperpigmented		N	severe	N	N	N	N	M. globosa	Pos	Neg	Neg	nil	2	1	2	0.125	0.03
81	Senthil	37	M	104831	SD	6 months	scalp & neck	driver	NA		NA	NA	NA	Y	N	N	M. sympodialis	Pos	pos	pos	40,60,80	1	0.06	0.06	0.125	1
82	Ramesh Kumar	34	M	93625	SD	1 year	neck & back	electrician	NA		NA	NA	NA	N	N	N	M. sympodialis	Pos	pos	pos	40,60,80	4	0.125	1	0.5	0.125
83	Lakshmanan	45	M	110832	SD	1 year	face	cook	NA	HT/DM	NA	NA	NA	N	N	Y	M. restricta	neg	Neg	Neg	nil	0.06	0.125	0.5	4	0.5
84	Michael	28	M	128901	SD	2 months	face & neck	porter	NA		NA	NA	NA	N	N	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
85	Charumathi	43	F	126834	SD	6 months	face	housewife	NA		NA	NA	NA	N	N	N	M. globosa	Pos	Neg	Neg	nil	0.06	1	2	1	2
86	Nancy	30	F	97531	SD	4 months	face	housewife	NA		NA	NA	NA	N	N	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
87	Balasundaram	55	M	104562	SD	8 months	scalp & neck	security	NA	DM	NA	NA	NA	Y	N	N	M. furfur	Pos	Neg	pos	20,40,60,80	0.25	2	0.125	0.25	0.06
88	Sathyapriya	43	F	103487	SD	2 months	face & scalp	housewife	NA		NA	NA	NA	N	Y	N	M. globosa	Pos	Neg	Neg	nil	2	4	0.12	2	1

89	Neelakandan	44	M	127895	SD	3 months	back & chest	tailor	NA	RVD	NA	NA	NA	N	N	N	M. furfur	Pos	Neg	pos	20,40,60,80	0.5	0.125	0.125	0.5	1
90	Cerin	36	M	120765	SD	2 months	face & back	sportsman	NA		NA	NA	NA	N	N	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
91	Mohan Raj	35	M	105298	PV	1 month	arms & chest	driver	hypopigmented		N	moderate	Y	N	N	Y	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
92	Kumaresan	45	M	99854	SD	3 months	back & chest	security	NA		NA	NA	NA	Y	N	N	M. globosa	Pos	Neg	Neg	nil	1	1	2	1	1
93	Jerome	40	M	87845	SD	3 months	face & neck	driver	NA	steroids	NA	NA	NA	N	N	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
94	Nishanth	23	M	123075	SD	4 months	back	student	NA		NA	NA	NA	N	N	Y	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
95	Rafiq	43	M	128673	SD	6 months	scalp & back	musician	NA		NA	NA	NA	N	N	N	M. globosa	Pos	Neg	Neg	nil	2	4	4	2	0.5
96	Raji	41	F	137643	SD	1 year	back	actor	NA		NA	NA	NA	N	N	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
97	Nambiar	46	M	129712	SD	4 months	face	vendor	NA	RVD	NA	NA	NA	N	N	N	nil	NA	NA	NA	NA	NA	NA	NA	NA	NA
98	Unnamalai	29	M	131321	PV	8 months	back	electrician	hyperpigmented		N	moderate	N	N	N	N	M. globosa	Pos	Neg	Neg	nil	2	4	0.12	0.125	0.125
99	sambavi	24	F	139146	PV	6 months	back	housewife	hyperpigmented		N	moderate	N	N	N	Y	M. furfur	Pos	Neg	pos	20,40,60,80	1	0.5	0.125	0.5	0.5
100	Mallika	32	F	148765	PV	1 year	back and face	cook	hypopigmented		Y	mild	N	N	N	N	M. globosa	Pos	Neg	Neg	nil	2	4	0.12	0.5	1

ANNEXURE –2

PROFORMA

- Name : OP NO:
- Age: Ward:
- Sex:
- Occupation:
- Address:

- Presenting complaints

- Duration of illness

- History of

  increased sweating

  use of bath oils

  prolonged usage of corticosteroids or antibiotics

  Diabetes mellitus

  Cushing's syndrome

  Acquired Immunodeficiency Syndrome

  Immunosuppressive therapy

  Transplantation or malignancy

- Previous history of similar illnes

- Previous Medical History

- Prior antifungal therapy

- Personal history

- Family history of similar illness

Clinical Examination:

- Site of lesion
- Type of lesion

Hypopigmented or hyperpigmented

Scaling

Pruritus

Clinical Diagnosis

Microbiological investigations:

- Direct examination: 10% KOH mount with Parker Quink's ink
- Culture : Fungal growth on

SDA (containing chloramphenicol &actidione) with sterile olive oil overlay

SDA (containing chloramphenicol &actidione) without olive oil overlay

- Macroscopic appearance of colony
- Gram staining
- Urease test
- Catalase test
- Lipid dependance
- Tween assimilation test
- Esculin hydrolysis test
- Growth at 41˚ C
- Antifungal susceptibility pattern of the isolate

ANNEXURE –3

CONSENT FORM

STUDY TITLE: Isolation of Malasseziaspecies from patients clinically diagnosed with pityriasisversicolor and seborrheic dermatitis and determination of antifungal susceptibility of the isolates

I……………………………………………, hereby give consent to participate in the study conducted by Dr. Jersey Gayathiri M., Post graduate at Institute of Microbiology, Madras Medical College, Chennai and to use my personal clinical data and the result of investigations for the purpose of analysis and to study the nature of the disease, I also give consent to give my clinical Specimen (skin or scalp scrapings) for further investigations. I also learn that there is no additional risk in this study. I also give my consent for my investigator to publish the data in any forum or journal.

Signature/ Thumb impression
of the patient/ relative

Place
Date

Patient Name & Address:

Signature of the investigator:

MASTER CHART

INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE, CHENNAI-3

EC Reg No.ECR/270/Inst./TN/2013
Telephone No : 044 25305301
Fax : 044 25363970

CERTIFICATE OF APPROVAL

To

Dr. Jersey Gayathiri M.,
Post Graduate in MD Microbiology,
Institute of Microbiology,
Madras Medical College, Chennai-3.

Dear **Dr. Jersey Gayathiri M.,**

The Institutional Ethics Committee of Madras Medical College, reviewed and discussed your application for approval of the proposal entitled ***"Isolation Of Malassezia Species From Patients Clinically Diagnosed With Pityriasis Versicolor And Seborrheic Dermatitis And Determination Of Antifungal Susceptibility Of The Isolates"*** No.13122013

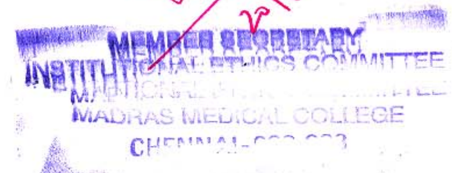
The following members of Ethics Committee were present in the meeting held on 11.12.2013 conducted at Madras Medical College, Chennai-3.

1. Dr. G. Sivakumar, MS FICS FAIS -- Chairperson
2. Prof. B. Kalaiselvi, MD -- Member Secretary
   Vice Principal, MMC, Ch-3
3. Prof. Ramadevi, -- Member
   Director i/c, Instt. of Biochemistry, Chennai.
4. Prof. P. Karkuzhali, MD for Dr. V. Ramamoorthy -- Member
   Prof. Instt. of Pathology, MMC, Ch-3
5. Thiru. S. Govindasamy, BABL -- Lawyer
6. Tmt. Arnold Saulina, MA MSW -- Social Scientist

We approve the proposal to be conducted in its presented form.

Sd/Chairman & Other Members

The Institutional Ethics Committee expects to be informed about the progress of the study, and SAE occurring in the course of the study, any changes in the protocol and patients information / informed consent and asks to be provided a copy of the final report.



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