IDENTIFICATION OF *MALASSEZIA* SPECIES IN PITYRIASIS VERSICOLOR

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CERTIFICATE

Certified that the dissertation entitled **"IDENTIFICATION OF *MALASSEZIA* SPECIES IN PITYRIASIS VERSICOLOR"** is a bonafide work done by **Dr. N. RATHNAPRIYA,** Postgraduate, Institute of Microbiology, Madras Medical College, Chennai, under my guidance and supervision in partial fulfillment of the regulation of the Tamil Nadu Dr. M.G.R Medical University for the award of **M.D. Degree, Branch – IV (Microbiology)** during the academic period of May 2005 to March 2008.

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DECLARATION

I, **Dr. N. RATHNAPRIYA,** solemnly declare that this dissertation titled **"IDENTIFICATION OF *MALASSEZIA* SPECIES IN PITYRIASIS VERSICOLOR"** is a bonafide record of work done by me in the Institute of Microbiology, Madras Medical College and Department of Dermatology, Government General Hospital, Chennai under the guidance of **Prof. Dr. S. GEETHALAKSHMI, M.D., Ph.D.,** Institute of Midcrobiology, Madras Medical College, Chennai.

This dissertation is submitted to the Tamilnadu Dr. M.G.R. Medical University Chennai, in partial fulfillment of the University regulations for the award of degree of M.D., Branch IV (Microbiology) examination to be held in March 2008.

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CONTENTS

INTRODUCTION

The skin is the body's largest and thinnest organ. Approximately, 15 percent of all patients who seek medical attention have either some skin disease or skin lesion, many of which are infectious[5].

Fungi are ubiquitous in the environment. Fungal infections of the skin are a health concern worldwide. Clinical fungal infections are generally divided into four types namely, cutaneous, subcutaneous systemic and opportunistic mycosis[57].

Superficial mycosis are by far the most common. Superficial fungal infections are defined as infections in which a pathogen is restricted to the stratum corneum, with little or no tissue reaction. One of the superficial fungal infections of the skin is Pityriasis versicolor[72].

Tinea versicolor, also known as Pityriasis versicolor, is one of the most common pigmentary disorders worldwide[76]. In the United States, the name Tinea versicolor is commonly used, whereas Pityriasis versicolor is the common name in Europe. Perhaps, European colleagues are correct, since the name "Tinea" suggests that the infection is caused by a dermatophyte, which, of course, is untrue[21].

The causative organism of Pityriasis versicolor is the lipophilic yeasts, of the genus *Malassezia.* Clinically, patients with Pityriasis versicolor have scaly irregularly shaped macules and papules with brawny scales which may be hypopigmented or hyperpigmented, as

implied by the name versicolor, they may appear yellowish to brown, pale yellow or dark brown, occasionally reddish or pinkish in color[76].

The genus *Malassezia* was previously called *Pityrosporum* and the species, *Pityrosporum ovale* and *Pityrosporum orbiculare* were identified by micromorphological studies[76,47].

Although, *Malassezia* yeasts are a part of the normal microbial flora, under certain conditions they can cause superficial skin infection. The study of the clinical role of *Malassezia* species has been surrounded by controversy because of their fastidious growth requirement and relative difficulty in isolation, cultivation and identification[75].

Till date there are 10 species of *Malassezia.* Among these, *M.globosa, M.sympodialis*, *M.furfur* are the most common species from the patients with Pityriasis versicolor, *M.restricta, M.sloofiae*, *M.obtusa* are the other lipophilic *spp.* and *M.pachydermatis* is the only non-lipophilic species. Three new species, *M.dermatis, M.nana* and *M.japonica* have been described using ribosomal RNA sequences and not with a cultivation identification system[76,60].

The yeasts of the genus *Malassezia* have been associated with a number of diseases affecting the human skin such as Pityriasis versicolor, *Malassezia* (Pityrosporum) folliculitis, seborrheic dermatitis (dandruff), atopic dermatitis, psoriasis, and less commonly with other dermatologic disorders such as confluent and reticulated papillomatosis,

onychomycosis, otitis externa, obstructive dacrocystitis, pneumonia, peritonitis, and transient acantholytic dermatosis[35].

Malassezia spp. also cause disseminated infections in infants and young children and even adults, who are receiving parentral nutrition through indwelling catheters[49,54].

The fungal agent, belonging to the genus *Malassezia,* which historically caused only superficial infections, is now an agent of disseminated infections in patients receiving parentral therapy[18].

In view of the wide spectrum of diseases caused by *Malassezia spp.* it was, therefore, felt necessary to study the most common *Malassezia spp.* associated with patients having Pityriasis versicolor and analyse the risk factors leading to the infection.

REVIEW OF LITERATURE

Historical Considerations

Eichstedt, first recognised the disease, Pityriasis versicolor to be a fungal disease, in 1846. (pityron means scale forming in Greek, versicolor means variation in colour)[83].

Robin, further described the fungus as spherical budding cells grouped in clusters, associated with hyphae, in scales of Pityriasis versicolor in 1853. He considered it to be a dermatophyte and named it *Microsporum furfur*[27].

In 1873, Rivolta described double contoured budding cells in a case of Psoriasis, and considered it to belong to Cryptococcus[42].

Malassez, in 1874, observed budding “spores” of various shapes, which occurred in abnormal skin conditions[83].

Baillon, created the generic name *Malassezia* and named the agent of Pityriasis versicolor as *Malassezia furfur* in his text, Traite de Botanique in 1889[83].

Subsequently in 1913, Castellani and Chalmers for the first time isolated lipophilic oval-budding yeasts from normal skin and seborrheic dermatitis and coined the name, *Pityrosporum ovale* for the species[57,10].

Bizzozero, in 1884, observed the morphological diversity in the epidermal scales, and named the organisms with spherical cells, *Saccharomyces sphaericus* and those with oval cells, *S.ovalis*[83].

Sabouraud in 1904, assigned *Pityrosporum malassezii* as the species responsible for seborrheic dermatitis[72]. In 1927, Acton and Panja created a second *Malassezia* species, *M.ovalis,* for the scalp organism[1].

In 1925, Weidman isolated a yeast from a severe exfoliative dermatitis of an Indian rhinoceros *(Rhinoceros unicornis),* which he cultured without the addition of lipids and named it *Pityrosporum pachydermatis* later, it was described as *P.canis,* by Gustafson in 1955[57].

In 1935, C.W. Dodge described *M.pachydermatis* and after four years in 1939, Rhoda Benham discovered the lipophilic nature of the genus *Malassezia*[57].

In 1951, Gordon isolated and authenticated a round, double contoured budding yeast that produced spherical to oval buds in scales of Pityriasis versicolor as well as on normal skin. He named the organium *P.orbiculare,* based on the micromorphology and preserved it to be distinct organism from *P.ovale*[83,31].

In 1964, Dixon agar was used for the isolation of these species, which was subsequently modified to obtain better results[42].

Since early 1980s, *Malassezia* species have also been considered as agents responsible for causing opportunistic systemic infections, particularly in premature neonates[42].

The genus *Malassezia* created by Baillon predates Sabouraud's Pityrosporum, and is recognised as the correct name in taxonomic publications[71].

All the species were accommodated in the genus *Malassezia* by Yarrow and Ahearn in 1984[87].

Simmons and Guého, in 1990 recognised *M.sympodialis* as an independent species[78].

The designation *Malassezia*, was used previously to describe mycelial phase of the organism, whereas yeast phase was divided into two distinct species based on microscopic morphology. *Pityrosporum orbiculare* was considered to inhabit trunk and was regarded as an etiological agent of Pityriasis versicolor. *Pityrosporum ovale* was usually limited to scalp and face and was associated with seborrheic dermatitis. Midgley maintained the two species, *Malassezia furfur (P. orbiculare)* and *M.ovalis,* which was further divided into three forms, in 1989[58,51].

Cunningham and colleagues, in 1990, defined serovars A, B and C on the basis of distinct cell surface antigens. Microscopically, serovars A and B had round, whereas serovar C had oval blastospores. Historically, serovars A and B were described as *P.orbiculare* and

serovar C as *P.ovale.* It had also been shown that serovar A predominated over trunk, serovar B over trunk and scalp while serovar C was more prevalent on scalp of normal individuals. Cunningham's serovars A, B and C are now identified with genus *Malassezia* as *M.sympodialis*, *M.globosa* and *M.restricta,* respectively[17].

Guého *et al,* in 1996 described four new species, *M.restricta, M. globosa, M.sloofiae* and *M.obtusa* on the basis of their morphologic, physiologic and genetic characteristics. These four new species, along with the previously described species namely *M.furfur*, *M.pachydermatis* and *M.sympodialis* brought the number of *Malassezia* species upto seven. Therefore, totally seven species were characterised by Guého *et al*[33].

In 2004, three new species, *M.dermatis, M.nana, M.japonica,* have been described using ribosomal RNA sequences and not with a cultivation identification system[76].

Taxonomy

The genus *Malassezia* has been placed in the phylum Basidiomycota in the family Crytococcaceae. Although, no teleomorph has been described for any of the *Malassezia spp.*, their affinities have been indicated by the possession of characteristics that are found in other Basidiomycetous yeasts. These features include the structure of the cellwall, which is composed of several layers and such properties as the ability to breakdown urea and the positive reaction to staining with Diazonium Blue B[57].

Genetic studies, which include the estimation of the guanine / cytosine content of DNA and analysis of ribosomal RNA sequences, have confirmed the taxonomic position of the genus[34].

The genus *Malassezia* has several species that differ from each other in cellular characteristics, morphology, guanine / cytosine content of DNA in serotypes, RNA / DNA sequences, requirement of long-chain fatty acids from C_{12} to C_{24} series, catalase activity and temperature requirements[33].

All species are lipid dependent, except *Malassezia pachydermatis,* which is mainly associated with animals. Six lipid-dependent species namely, *M.globosa, M.sympodialis, M.furfur, M.restricta, M.obtusa* and *M.sloofiae,* were recovered from normal as well as diseased skin of humans[33].

Seven species of *Malassezia* can be identified by comparing the morphological and physiological characteristics of isolates corresponding to the species[27].

Three new species, *M.dermatis, M.nana* and *M.japonica,* have been described using ribosomal RNA sequences and not with a cultivation identification system[76].

Nomenclature of *Malassezia* species[34]

New Nomenclature	Old Nomenclature
M.furfur (Robin) Baillon 1889	*Microsporum furfur* Robin 1853 *Pityrosporum ovale* Castellani and Chalmers 1913
M.pachydermatis (Weidman) Dodge 1935	*Pityrosporum pachydermatis* Weidman 1925 *Pityrosporum canis* Gustafson 1955
M.sympodialis Simmons and Guého 1990	*Pityrosporum ovale* form 3 *Malassezia furfur* serovar A *Malassezia ovalis* form 3
M.globosa Midgley, Guého and Guillot 1996	*Pityrosporum orbiculare* Gordon 1951 *Malassezia furfur* serovar B
M.obtusa Midgley, Guillot and Guého 1996	*Pityrosporum ovale* form 2 *Malassezia ovalis* form 2
M.restricta Guého, Guillot and Midgley 1996	*Malassezia furfur* serovar C
M.sloofiae Guillot, Midgley and Guého 1996	*Pityrosporum ovale* form 1 *Malassezia ovalis* form 1
M.dermatis 2004	None
M.nana 2004	None
M.japonica 2004	None

Despite its two morphological forms, it is not regarded as a dimorphic fungus, in true sense, as both types of morphological structures are simultaneously found on the skin, irrespective of temperature variations[17].

The distinct taxa matched very well with serological and morphological differences previously documented by Cunningham *et al*, and Midgley, respectively[58].

DISEASES ASSOCIATED WITH *MALASSEZIA* SPECIES

Pityriasis versicolor[27,61]

This is the most common skin disease caused by *Malassezia* species. This is a chronic, mild, recurrent superficial fungal infection of the stratum corneum, characterised by patchy discolouration of the skin ranging from hypopigmentation to hyperpigmentation with various shades. The lesions may be hypopigmented, hyperpigmented, leukodermal, erythematous or dark brown with dust like or furfuraceous scales[3,62].

Pityriasis versicolor is an opportunistic infection of the skin. Transformation of the yeast cells to their mycelial form is unique to Pityriasis versicolor. Under appropriate conditions, *Malassezia* fungus converts from the saprophytic yeast to the predominantly parasitic mycelial form, associated with clinical disease[4,46]. *M.globosa* is suggested as the aetiological agent of Pityriasis versicolor[4,60,66].

Most of the patients of Pityriasis versicolor are healthy individuals, who seek medical advice on cosmetic grounds. The lesions occur mostly in the upper trunk, neck, back and upper aspects of arm. They are usually asymptomatic, but sometimes they may be associated with pruritus. The disease is related to various host and environmental factors, which are discussed later[3,4].

Seborrheic dermatitis[35]

Seborrheic dermatitis is characterised by whitish, dry, loose flakes on scalp. Mild, non-inflammatory form of seborrheic dermatitis is called Dandruff. Seborrhoea is a combination of Latin word “sebum” meaning grease and Greek word “rhoea” for flow[80].

Patients with immunocompromised states including Acquired Immuno Deficiency Syndrome (AIDS)[56], suffer from seborrheic dermatitis[21]. Sites most commonly involved are the scalp, nasolabial folds, ears, eyebrows and chest[39,71].

M.globosa and *M.restricta* have been shown to be most closely associated with seborrheic dermatitis[35].

Atopic dermatitis[35]

This is a chronic inflammatory disorder marked by intense pruritis and characteristic eczematous lesions with erythema, fine scaling and thickening of epidermis[24,62].

Genetic factors are known to play an important role in the development of this disorder. If both parents are carriers of the disease, the risk of children is as high as 70 percent.

Lesions are localized to scalp, face and neck. Most commonly, *M.sympodialis,* followed by *M.furfur* and *M.dermatis* were isolated from skin of patients with atopic dermatitis[35].

Immunoglobulin E (IgE) antibodies against *M.furfur* demonstrated in 40 - 65 percent of patients with atopic dermatitis[53].

Malassezia folliculitis[35]

The folliculitis caused by *Malassezia* species consists of discrete pruritic follicular pustules mainly localized to anterior portion of chest and upper back and to a lesser extent on shoulder and arms.

Malassezia folliculitis is common in patients with immunosuppressive conditions like transplant recipients, Diabetes mellitus, Acquired Immuno Deficiency Syndrome (AIDS) and in those with history of prolonged usage of broad-spectrum antibiotics.

M.furfur has been detected in follicular contents in patients with folliculitis.

Psoriasis[35]

Psoriasis is a skin disease characterized by hyperproliferation and hyperkeratinisation of the epidermis.

Psoriasis is known to occur in genetically predisposed individuals and the lesions occur on scalp, forehead and trunk.

M.globosa was the most common species isolated from patients with Psoriasis.

Systemic infections

Apart from skin diseases, the other major type of infection caused by *M.furfur* is central venous catheter-related fungemia[32]. This infection has most often been described in neonates, especially those receiving lipid infusions[27,11]. Cases in immunocompromised patients, both children and adults have also been reported[18].

M.pachydermatis also has been reported to cause fungemia in neonates. Although *M.pachydermatis* does not require exogenuous lipids for growth, fatty acids stimulate its growth[54]. There are case reports of pneumonia and peritonitis caused by *M.furfur*[52].

An outbreak of *M.pachydermatis,* in an intensive care nursery unit, associated with colonisation of health care worker's pet dogs has been reported[27].

Other diseases

M.furfur has been demonstrated in cases of confluent and reticulate papillomatosis[35].

Malassezia yeasts have been isolated from some cases of onychomycosis[35,85].

A pathogenic role of *M.sympodialis* in neonatal cephalic pustulosis, a skin disorder also known as Neonatal acne has been suggested[27,53].

PITYRIASIS VERSICOLOR

Definition[72,53]

Pityriasis versicolor is neither contagious nor due to poor hygiene. The infection results from a change to the mycelial state of lipophilic yeasts of the genus *Malassezia*, which colonise the stratum corneum and are typical skin flora[38].

Pityriasis versicolor is a chronic infection of the skin caused by *Malassezia* yeasts, characterised by multiple discrete scaly macules or patches, discolored or depigmented areas, with skip regions of normal skin in between mainly on the upper trunk, with or without pruritus. The disease often has a relapsing nature and needs to be treated frequently[76].

Synonyms[83,72]

Tinea versicolor, Dermatomycosis furfuracea, Tinea flavea, Liver spots, Chromophytosis, Achromia parasitica.

Epidemiology[83]

The geographic distribution of *Malassezia spp* is worldwide. Infact, it is part of the normal flora of human skin, predominantly *M.sympodialis. Malassezia* species are lipophilic unipolar yeasts. The natural habitat for *Malassezia spp.* is the skin of warm-blooded animals. Although, Pityriasis versicolor occurs most frequently in tropical climates

with high ambient temperatures and high humidity, it also a common disorder in temperate climates[15,38,65].

No racial or gender difference has been established. People of any age may develop the disease. Interestingly, *Malassezia* has an oil requirement for growth, accounting for the increased incidence in adolescence and preference for sebum-rich areas of the skin[6,44].

Pityriasis versicolor is known to be more extensive in Acquired Immunodeficiency Syndrome patients, although it does not differ clinically from the disease pattern seen in HIV negative patients[22].

Aetiology[57,53]

Pityriasis versicolor occurs when the *Malassezia* yeasts transforms to the mycelial form, which is unique for the disease.

Pathogenesis[76]

Malassezia fungus grow readily on skin surface rich in sebum, which consists of sterol ester, squalene, triglycerides and free fatty acids.

The causative fungus interferes with melanin production, leading to hypopigmentation or hyperpigmentation of the skin.

1. Hypopigmentation may be due to lipoperoxidation process, produced by *Malassezia*[83].

2. Dicarboxylic acid produced by *Malassezia* fungi, such as azelaic acid, competitively inhibit Dihydroxy phenylalanine (DOPA) - Tyrosinase reaction and therefore produce a direct cytotoxic effect on hyperactive melanocytes, inducing hypopgimentation. Due to this cytotoxic effect, repigmentation can take months or years to happen[63].

3. Scales produced in Pityriasis versicolor prevent tanning from occurring[83].

4. The size and distribution of melanosomes within melanocytes influence the type of lesion. Hypopigmentation occurs when the melanosomes are abnormally small and in hyperpigmentation, the melanosomes are large[76].

5. Ultrastructural studies have shown pronounced melanocyte damage, varying from altered melanosomes and mitochondria, to actual degeneration[76,8].

6. Flurochromes, especially pityrialactone, are linked with golden yellow fluorescence in Pityriasis versicolor due to *M.furfur*[76]. Indole pigments formed only by *M.furfur,* have been recently found to be potent ultraviolet light filters and interfere with normal tanning[35,63].

7. Hyperpigmentation may be due to increased thickness of the keratin layer. Hyperpigmentation may be due to the stimulating

effect of inflammatory cell infiltrate on melanocytes, resulting in the production of more pigment[83,2].

8. *Malassezia* secretes a potent ultraviolet light protectant, Pityriacitrin that confers resistance to ultraviolet radiation[35].

Due to these reasons, hypopigmentation or hyperpigmentation, may require months to return to normal, even after treatment.

Immunology

Immunopathogical responses in Pityriasis versicolor are difficult to interpret. Antibodies against *Malassezia furfur* have been found to be in similar titres in healthy controls and patients with Pityriasis versicolor[23].

Depressed cellular immunity considered to be a possible factor in the development of Pityriasis versicolor. Cellular immunity is not weakened, however, Pityriasis versicolor was reported in 33 percent of renal transplant recipients, perhaps reflecting a localized immunological deficit or host susceptibility factors[82].

Predisposing / Risk factors

Pityriasis versicolor occurs when the yeast transforms to its mycelial form due to one or more predisposing factors. These factors can be classified as endogenous or exogenous. Cause may be multifactorial[36].

EXOGENOUS FACTORS[26]

Exogenous factors consists of the environmental factors like temperature, humidity and also clothing, cosmetics, bathoils used by the individual.

1. Heat and Moisture[84,7,53,38,65]

Increased temperature and high humidity provides warm, humid environment, favouring the growth of this fungus in tropical countries, especially during summer months.

2. Occlusion of skin[84]

Occlusion of skin by either clothing or cosmetics results in increased carbon dioxide concentration, an altered microflora and altered pH range, thus promoting this infection.

3. Bath oils[73]

Applying bath oils or skin lubricants has been suggested to favour the growth of *Malassezia.*

ENDOGENOUS FACTORS[26,53]

Endogenous factors consists of host factors like age, gender, heredity, increased sweating and associated diseases.

1. Age[53]

Pityriasis versicolor is more common in adolescents and young adults due to increased activity of sebaceous glands and also because of the hormonal changes occurring during that age[35].

Newborns show an increased rate of colonisation of *Malassezia spp.* (upto 20 percent), due to the influence of maternal hormones and acquisition of the organism from the Neonatal intensive care unit environment[53].

In middle aged and elderly persons, the colonisation rates are less, because of decrease in skin lipids[21,19].

2. Gender[84]

Studies have shown variable male to female ratios, but they appear to be nearly equal.

3. Heredity[83,26,53]

A positive family history of approximately 17 percent was noted, while conjugal cases were less commonly reported. Hence, hereditary factors seem to play a role.

4. Site[76,53,29]

Lesions occur mostly in the sebum rich areas like upper trunk, upper back, neck, and upper aspects of arms. Face is commonly involved in infants and children[45].

5. Increased sweating[9,26,53,83]

Many studies have shown that individuals with increased sweating (hyperhidrosis), are more prone to have Pityriasis versicolor.

6. Systemic corticosteroids[26,53,83,70]

Systemic steriods will suppress the normal skin flora and allow the growth of opportunistic pathogen like *Malassezia* fungus, leading to Pityriasis versicolor.

7. Prolonged antibiotic usage[27,76,53]

Prolonged broad spectrum antibiotic usage suppresses the normal flora and increases the risk of the opportunistic infection, due to *Malassezia.*

8. Immunocompromised states[56,41,26]

Immunosuppressed patients like transplant recepients and patients with HIV infection, show an increased incidence of Pityriasis versicolor.

9. Pregnancy, Oral contraceptive pills usage[57]

Increase in hormonal levels during pregnancy and oral contraceptive pill usage, stimulates the activity of sebaceous glands, leading to the development of Pityriasis versicolor.

10. Other associated dieases[83,40,53]

Pityriasis versicolor is most often seen in patients with Diabetes mellitus, Cushing's dieases, renal failure and malignancy.

LABORATORY DIAGNOSIS OF PITYRIASIS VERSICOLOR

Wood's lamp examination[72]

Wood's lamp examination was first discovered by Margaret and Deveze in 1925, while investigating a case of hair infection by dermatophytes.

Wood's glass consists of barium silicate containing about 9 percent nickel oxide, which transmits light rays of wavelength above 365nm. Ultravoilet light source like mercury vapour lamp, when used along with Wood's glass is called Wood's lamp.

On Wood's lamp examination, Pityriasis versicolor lesions produce golden yellow fluorescence. Only *M.furfur* produce the indole compounds that fluoresce under Wood's light. Positive Wood's light examination response is seen only in one third of Pityriasis versicolor cases, which limits the usefulness of this test[36].

Specimen collection

The skin of the affected area is thoroughly cleansed with 70 per cent alcohol, to remove surface contaminants. Then, the skin scrapings are collected from the active edges of the lesions[57].

METHODS TO OBTAIN SPECIMEN

1. Scrub Technique[57]

This technique is ideal for collecting specimen for culture and direct examination. Skin scrapings are collected using flame sterilized or disposable blunt 15 no. scalpel blade.

2. Scotch Tape Method[13]

This is an effective and reliable method for direct microscopic examination, especially in children. A piece of Scotch tape is applied on the skin lesion and pressed firmly to recover the scales. The tape is removed and then placed over 1-2 drops of 10-20 per cent potassium hydroxide solution on the centre of a glass slide and examined under light microscope.

3. Contact Plate Method[57,50]

In this method, the agar surface of the culture plate is pressed against the area of skin to be sampled.

Direct Microscopic Examination

Stains Used

Staining of the skin scrapings helps to identify the yeast cells and hyphae easily, rapidly and helps to avoid any confusion with artifacts.

1. 10-20 percent KOH solution with or without Dimethyl sulphoxide (DMSO) wet mount.

   The aqueous potassium hydroxide solution digests keratinised epithelial cells and helps to identify the fungal elements easily. DMSO also acts as a clearing agent[76].

2. Parker Quink's Stain.

   This stain is a mixture of equal volumes of KOH and Parker Quink's permanent blue / black fountain pen ink. Parker Quink's ink is an extremely useful stain were the fungi stains light blue against the clear background of digested keratinocytes[57,14].

3. Methylene blue[26,83] and Albert's stain[35] are used as an alternative method to stain the yeast and hyphae[64].

4. Calcofluor White stain[76,67]

   Calcofluor White is a fluorescent textile whitener, which stains the cellulose and chitin of the fungal cell wall. This is a simple, rapid, and highly reliable stain to identify fungal elements, under fluorescence microscopy.

Direct Microscopic Feature

Under direct microscopic examination, using 10 percent aqueous KOH or Parker Quink's ink stains, *Malassezia* species appear as clusters of round to oval yeast cells, 2 to 7 μm in size with occasional

budding and short, curved septate hyphae 2-5 μm wide and upto 25 μm long. This characteristic appearance is unique for Pityriasis versicolor and is described as “Banana and Grapes” or “Spaghetti and Meat Balls” appearance[57,16].

Histopathological examination of the biopsy specimen obtained from the skin lesion, show hyphae and yeast cells in the stratum corneum when stained with Haematoxylin and Eosin stain, Periodic acid-Schiff stain or Gormori’s methenamine silver stain[83,53,43].

Culture Characteristics

Growth requirement[57,61]

Malassezia is a lipophilic fungus, therefore lipids are incorporated into culture media, which include Olive oil, glycerol monostearate and Tweens.

With the exception of *M.pachydermatis,* rest of the *Malassezia spp.* have an absolute requirement of medium or long chain fatty acids in the culture medium. Optimum temperature for incubation is between 32°C and 35°C.

Culture media

- Sabouraud Dextrose Agar with antibiotics and cycloheximide, overlaid with Olive oil, which was frequently used in the past[57].

- Modified Dixon's Agar provides substantial growth and differences between species in colonial morphology are more pronounced in this media[57].

- Leeming and Notman's medium has whole fat cow's milk, as lipid source. Enhanced recovery of *Malassezia spp.* with this media has been reported[36,51].

- GYP-S agar is glucose-yeast extract-peptone agar with Olive oil, Tween 80 and glycerol monostearate can also be used but this medium is not as good as Modified Dixon's agar[42].

COLONY MORPHOLOGY ON MODIFIED DIXON'S AGAR[57]

Colony morphology of the various *Malassezia spp.* on Modified Dixon's agar are as follows.

M.furfur

Cream coloured, smooth, convex or umbonate colonies

Texture is soft and easier to emulsify

On Gram's stain, yeast cells are elongated, spherical or oval cells upto 6µm in diameter with broad-based budding.

Guanine / cytosine content of DNA is 66.4

M.sympodialis

Cream to buff coloured, flat or slightly convex colonies, with a smooth, shiny surface.

On Gram's stain, yeast cells are small, oval 2.5 - 5µm in diameter with sympodial budding.

Guanine / cytosine content of DNA is 62.2

M.globosa

Cream to buff coloured, rough colonies with a deeply folded surface.

Texture is very brittle and difficult to emulsify.

On Gram's stain, yeast cells are spherical, 6-8µm in diameter with narrow-based budding.

Guanine / cytosine content of DNA is 53.5

M.sloofiae

Cream to buff coloured colonies with finely folded surface.

On Gram's stain, yeast cells are small and cylindrical, 1.5-3.5µm

in diameter with broad-based budding.

Guanine / cytosine content of DNA is 68.7

M.restricta

Cream coloured, dull, smooth colonies with rough edges.

Texture is hard, brittle and difficult to emulsify.

On Gram's stain, yeast cells are oval 2-4 μm in diameter with narrow-based budding.

Guanine / cytosine content of DNA is 54.5

M. obtusa

Cream coloured, flat colonies with smooth surface

Texture is sticky and difficult to emulsify.

On Gram's stain, yeast cells are cylindrical 1.5-4 μm, in diameter with broad based budding.

Guanine / cytosine content of DNA is 61.4

M.pachydermatis

This is the only lipid independent species.

Cream coloured, smooth, typically convex colonies with matt surface.

Texture is brittle and difficult to emulsify.

On Gram's stain, yeast cells are small, cylindrical,

2.5-4 µm in diameter with broad-based budding.

Guanine / cytosine content of DNA is 55.6

PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS

Malassezia spp. can be identified by the various physiological and biochemical tests. They are as follows.

Urease Test[57]

All the *Malassezia spp.* are urease positive. Christensen's urease medium, modified to grow these lipophilic yeasts, by the addition of 0.5 percent Tween 40 and 0.1 percent Tween 80 is used.

Test for Lipid Dependence[34]

All the *Malassezia* isolates after isolation using the lipid containing media like Modified Dixon's agar are subcultured onto media without any lipid supplement like SDA without Olive oil overlay.

Only lipid-independent species is *Malassezia pachydermatis,* which grows on SDA without Oliveoil overlay. All other *Malassezia spp.* are lipid dependent, hence they do not grow on media without lipid supplement.

Catalase Test[34]

Catalase test is done using 3 percent hydrogen peroxide. *M.restricta* is the only catalase negative species, rest of the *Malassezia* species are positive for catalase reaction.

Growth at 41°C[34]

M.furfur, M.sympodialis, M.pachydermatis and *M.sloofiae* grow at 41°C. *M.globosa, M.obtusa* and *M.restricta* produce little or no growth at 41°C.

Esculin Splitting[84]

Splitting of esculin is revealed by darkening of the esculin agar medium. *M.sympodialis* and *M.obtusa* give positive results and *M.pachydermatis* gives variable results. Other species do not split esculin.

Cremophor Test[42]

1-10 percent Cremophor (castor oil) promotes the growth of most of the *Malassezia* isolates. This test is not specific for any particular *Malassezia* species.

Tween Assimilation Test[34]

This test detects the ability of the *Malassezia* isolates, to utilise Tweens as the sole source of lipid. For each isolate, the ability to utilise

different Tweens like Tween 20, 40, 60 and 80 is tested by incorporating them in Sabouraud Dextrose Agar with cycloheximide and antibiotics.

COMPOSITION OF TWEENS[34]

Tween 20	-	Polyoxyethene sorbitan monolaureate
Tween 40	-	Polyoxyethene sorbitan monopalmitate
Tween 60	-	Polyoxyethene sorbitan monostearate
Tween 80	-	Polyoxyethene sorbitan monooleate

Sugar assimilation and fermentation tests are not applicable for *Malassezia spp.* Because of their lipid dependence, conventional carbon assimilation test cannot be employed, as for the identification of other yeast genera[57,30].

Anti Fungal Susceptibility Testing[57]

In vitro antifungal susceptibility testing, according to NCCLS, M27-A_2 is not applicable for the Genus *Malassezia,* due to the Olive oil requirement for growth.

Immunodiagnosis[42]

Solid phase ELISA was developed for determination of antibody titres specific for the *Malassezia* species (serovars A, B and C).

Fluorescent microscopy helps to identify fungal elements easily.

Molecular Methods[35,57]

Molecular methods like Restriction Fragment Length Polymorphism (RFLP), Polymerase Chain Reaction (PCR) fingerprinting and Multilocus Enzyme Electrophoresis (MLEE) are also used to identify *Malassezia* isolates, especially for epidemiological studies[28].

Pulsed field gel electrophoresis (PFGE) and Randomly Amplified Polymorphic DNA (RAPD) are useful to identify medically important *Malassezia* species.

TREATMENT[36]

Topical Antifungal Agents[37,25]

1-2% Selenium sulphide shampoo

20-25% Sodium thiosulphate cream

10% Sulphur ointment

1-2% Imidazole derivatives like

2% Ketoconazole with Zinc pyrithione shampoo

1% Bifonazole shampoo

Whitfield's ointment

Topical tar based preparations

Hydroxypyridones-ciclopirox olamine, piroctone olamine shampoo

Allylamines-1% Terbinafine[74]

Systemic Antifungal Agents[68]

Ketoconazole 400 mg once a month[68].

Fluconazole 400 mg for 3 days or as Pulse therapy 150 mg weekly for 2-3 months.

Itraconozole 200 mg daily for 7 days

Intermittent application of Propylene glycol 50 percent in water twice daily for 2 weeks in case of relapse.

AIMS AND OBJECTIVES

1. To identify the yeasts isolated from clinically diagnosed patients with Pityriasis versicolor.

2. To evaluate Modified Dixon's Agar for isolation of the *Malassezia* species.

3. To compare Modified Dixon's Agar and Sabouraud Dextrose Agar with Olive oil overlay for isolation of *Malassezia spp.*

4. To speciate the *Malassezia* isolates using Tween Assimilation Tests.

5. To analyse the risk factors which promote *Malassezia* fungus infection.

MATERIALS AND METHODS

This cross sectional study was done in the Institute of Microbiology, Madras Medical College and the Mycology section of the Department of Dermatology, Government General Hospital, Chennai from July 2005 to June 2006.

The study group included both male and female outpatients of all age groups, attending the mycology section of the Department of Dermatology, with complaints of hypopigmented or hyperpigmented macular lesions. The skin scrapings were collected from 112 clinically diagnosed patients with Pityriasis versicolor. The study was proceeded after obtaining Ethical Committee clearance.

SPECIMEN COLLECTION[57]

Skin scrapings were collected after thoroughly cleansing the skin with 70 percent alcohol, to remove the skin surface contaminants.

The skin scrapings were collected by scraping the active edge of the lesion, using flame sterilized blunt 15 no. scalpel blade and processed as per standard mycological procedures.

DIRECT MICROSCOPIC EXAMINATION

Potassium Hydroxide Wet Mount[57]

Skin scrapings were subjected to 10 percent Potassium Hydroxide (KOH) wet mount, to detect the presence of yeast cells and hyphal elements. (Appendix I)

A drop of 10 percent KOH was placed on the skin scrapings on a clean glass slide of size 2 inches length and 1 inch breadth and a coverslip was placed over it. The wet mount was examined under the microscope after 15 to 20 minutes. The characteristic “spaghetti and meat balls” or “banana and grapes” appearance was identified. This appearance is unique for Pityriasis versicolor.

Parker Quink’s Stain[57,14]

Skin scrapings were also subjected to wet mount preparation using Parker Quink’s stain and examined for yeast cells and hyphal elements. Parker Quink’s stain is a mixture of 10 percent KOH and equal volume of Parker Quink’s permanent blue / black fountain pen ink. *Malassezia spp.* takes up the Parker Quink’s stain and appear light blue against the digested keratinocytes.

CULTURE[57,48]

Skin scrapings were inoculated simultaneously on two different culture media namely Modified Dixon’s Agar slants and Sabouraud Dextrose Agar slants overlaid with Olive oil and incubated at 32°C for one week and the slants were examined daily for a week.

COLONY MORPHOLOGY

Modified Dixon's Agar (Appendix II)

On Modified Dixon's agar, cream to buff coloured colonies with smooth, convex surface was observed. Modified Dixon's agar plates were inoculated to isolate *Malassezia* yeast in pure culture.

Sabouraud Dextrose Agar (Appendix III)

On SDA with cycloheximide and antibiotics, cream coloured colonies with smooth, flat or slightly convex surface was observed.

GRAM'S STAIN[57]

Gram's stain was done for the colonies and found small, oval to spherical Gram positive budding yeast cells, 3-6µm in diameter.

CULTURE CHARACTERISTICS OF *MALASSEZIA SPP.* ON MODIFIED DIXON'S AGAR

M. furfur

Cream coloured, smooth, convex colonies showing 3-6µm diameter spherical or oval yeast cells with broad based budding.

M. sympodialis

Cream to buff coloured, flat or slightly convex colonies with a smooth, shiny surface showing 2.5-5µm oval yeast cells with sympodial budding.

M. globosa

Cream to buff coloured with deeply folded surface showing 6-8µm spherical yeast cells with narrow based budding.

M. sloofiae

Cream to buff coloured with finely folded surface, showing 1.5-3.5µm small cylindrical yeast cells with broad based budding.

M. restricta

Cream coloured, dull, smooth, colonies with rough edges, showing 2-4µm oval yeast cells with narrow based budding.

M. obtusa

Cream coloured flat colonies with smooth surface showing 1.5-4µm cylindrical yeast cells with broad based budding.

M. pachydermatis

Cream coloured smooth convex colonies showing 2.5-4µm cylindrical yeast cells with broad based budding.

PHYSIOLOGICAL AND BIOCHEMICAL TESTS[34]

Malassezia species were identified by the following physiological and biochemical tests.

1. Test for Lipid Dependence
2. Urease Test

3. Catalase Test

4. Growth at 41°C

5. Tween Assimilation Test

These tests were done for speciation of *Malassezia* isolates after identification by Gram's stain.

1. Test for Lipid Dependence

Primary inoculation of the skin scrapings from patients with Pityriasis versicolor directly onto lipid containing medium like Modified Dixon's agar and sterile Olive oil overlaid SDA, allowed the growth of the lipid dependent *Malassezia spp.* All isolates were further subcultured onto Sabouraud Dextrose agar with cycloheximide and antibiotics without Olive oil overlay and incubated at 32°C, for one week.

Only non-lipid dependent species, *M.pachydermatis* grew on SDA without Olive oil overlay, other *Malassezia* species grew only on lipid containing media.

2. Urease Test

This test detects the ability of the yeast cells to produce urease enzyme, which splits urea into ammonia and carbon dioxide. All cultures were inoculated on Christensen's urease medium, modified by the addition of 0.5 percent Tween 40 and 0.1 percent Tween 80. All *Malassezia spp.* gave positive urease test. (Appendix IV)

3. Catalase Test

This test detects the presence of catalase enzyme in the *Malassezia* isolates.

Half of the test tube was filled with 3 percent hydrogen peroxide and a small amount of the culture to be tested was picked from Modified Dixon's agar with a sterile wooden stick and inserted into hydrogen peroxide solution. The production of gas bubbles indicated a positive reaction. Among the seven *Malassezia* species, all were catalase positive except *M.restricta*.

4. Growth at 41°C

All the cultures were inoculated on two plates of Modified Dixon's Agar. One plate was incubated at 41°C and the other plate at 32°C. *M.furfur*, *M.pachydermatis*, *M.sympodialis* and *M.sloofiae* grew at 41°C within 2-3 days but *M.globosa, M.obtusa* and *M.restricta* did not grow at 41°C.

5. Tween Assimilation Test

This is the confirmatory test for speciation of *Malassezia* isolates. This test was done to detect the ability of *Malassezia spp*. to utilise Tweens as the sole source of lipid. For each isolate, the ability to utilize Tweens was tested by the following procedure.

Procedure

To 16 ml of sterile Sabouraud Dextrose agar containing 0.05 percent chloramphenicol and 0.5 percent cycloheximide, 2ml of a suspension of the yeasts to be identified were added to the medium at 50°C. The suspension was obtained by inoculating 5ml of the sterile distilled water, with a loopful of fresh culture. Several similar appearing colonies of the fresh culture were emulsified in sterile distilled water and the turbidity was adjusted to 10^5 cells / ml, using McFarland's 0.5 standard as corresponding to 10^8 / 30 yeast cells / ml[59,49].

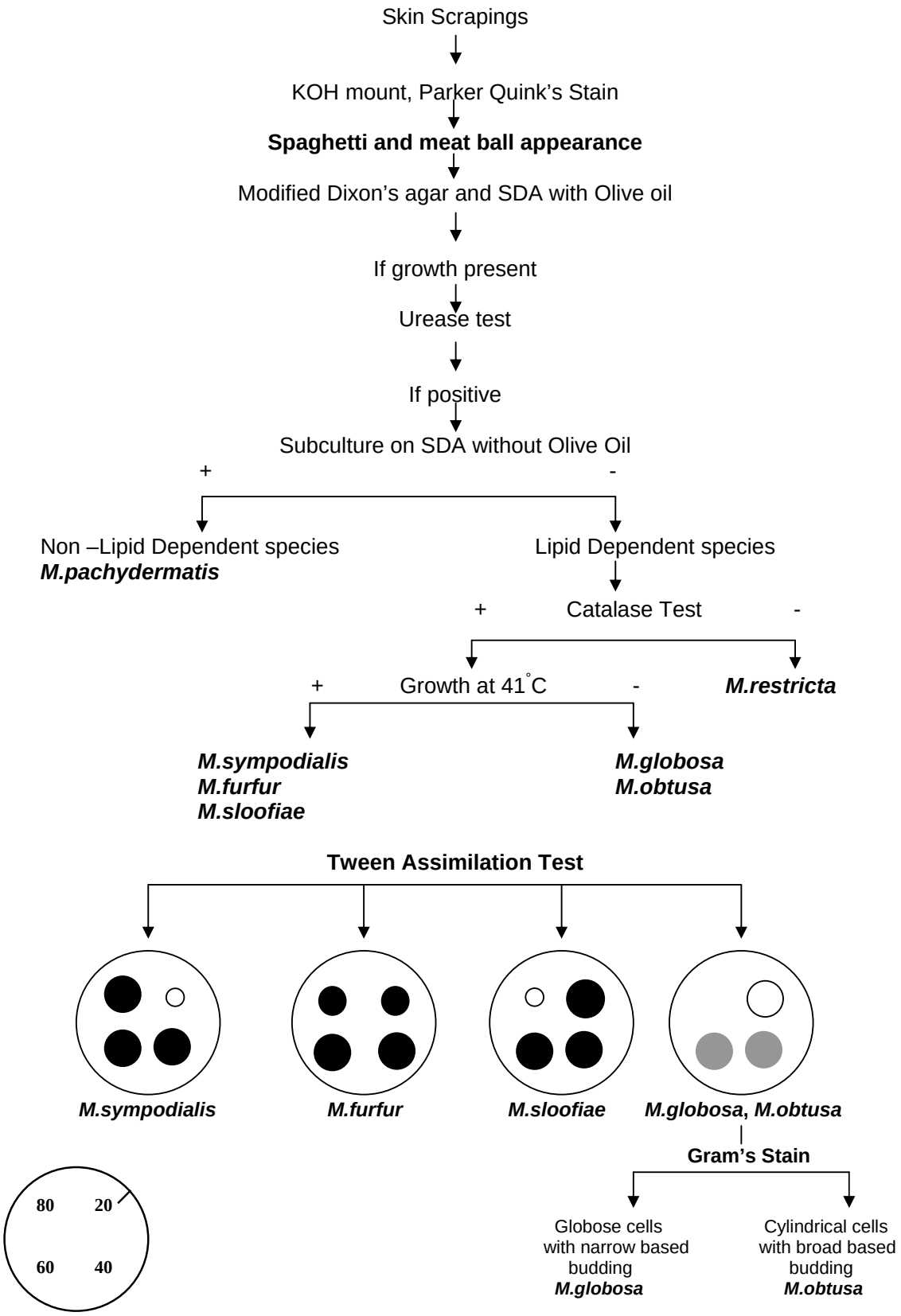
The seeded agar was then vigorously mixed and poured into a 9cm diameter Petri dish. Once the medium had solidified, four holes were made by means of a 2mm diameter punch and filled with 5µl of Tween 20, 40, 60, and 80 respectively (Otto Kemi, Hi media). All the Tweens were water soluble and formed a concentration gradient around each well. The plates were systematically incubated for one week at 32°C. Specific Tween assimilation pattern developed within two to three days of the incubation period.

Interpretation

Utilisation of Tweens was assessed by the zone of assimilation and degree of growth of the lipophilic yeast, around the individual wells containing the Tweens 20, 40, 60 and 80 respectively.

The hydrolysis of Tweens produced precipitation of the corresponding insoluble fatty acids, in combination with or without growth, which is characteristic for each *Malassezia* species.

Flow Chart Showing Identification Scheme of *Malassezia* Isolates



PHYSIOLOGICAL AND BIOCHEMICAL TESTS[34]

Species	Catalase test	Urease test	Growth at 41°C	Tween				Lipid Dependence	Gram's Stain
				20	40	60	80		Type of budding
M. furfur	+	+	+	+	+	+	+	+	Broad based
M. sympodialis	+	+	+	-	+	+	+	+	Sympodial
M. globosa	+	+	-	-	-	-	-	+	Narrow based
M. restricta	-	+	-	-	-	-	-	+	Narrow based
M. obtusa	+	+	-	-	-	-	-	+	Broad based
M. sloofiae	+	+	+	+	+	+	-	+	Broad based
M. pachydermatis	+	+	+	+	+	+	+	-	Broad based

RESULTS

Table1: Prevalence of Pityriasis versicolor in Government General Hospital, Chennai

Total outpatients attending Dermatology Department	Total outpatients attending mycology section (Dermatology)	Total outpatients with Pityriasis versicolor
76492	19501 (25.49%)	3237 (16.6%)

Pityriasis versicolor accounted for 16.6 percent patients with dermatomycoses in Government General Hospital, Chennai.

Table 2: Age distribution in the study population n=112

S.No.	Age group	No. of cases	Percentage
1	11-20	30	26.87
2	21-30	34	30.35
3	31-40	18	16.07
4	41-50	16	14.28
5	51-60	10	8.93
6	61-70	4	3.57

Majority of the study population belonged to the age range 21-30 years.

Table 3: Gender distribution in the study population n=112

S.No.	Age group	Male	%	Female	%
1	11-20	18	16.0	12	10.7
2	21-30	24	21.4	9	8.0
3	31-40	11	9.8	7	6.3
4	41-50	10	8.9	5	4.5
5	51-60	7	6.3	3	2.7
6	61-70	4	3.6	2	1.8
	Total	74	66%	38	34%

Male patients out numbered female patients.

Table 4: Seasonal distribution in patients with Pityriasis versicolor n=112

S.No.	Months	No. of Cases	Percentage
1	July-Aug'06	31	27.77
2	Sept-Oct'06	25	22.32
3	Nov-Dec'06	10	8.92
4	Jan-Feb'07	12	10.71
5	Mar-April'07	14	12.50
6	May-June'07	20	17.85

Maximum number of cases occurred during July to August.

Table 5: Type of lesion in patients with Pityriasis versicolor n=112

S.No.	Type of lesion	No. of cases	Percentage
1	Hypopigmented	101	90.2
2	Hyperpigmented	11	9.8

Majority of patients had hypopigmented lesions.

Table 6: Duration of complaints in patients with Pityriasis versicolor n=112

S.No.	Duration	No. of cases	Percentage
1	< 2 months	56	50.0
2	< 4 months	25	22.3
3	< 6 months	20	17.9
4	> 6 months	11	9.8

Majority of patients presented within 2 months of onset of the lesion.

Table 7: Distribution of lesion in various sites in patients with Pityriasis versicolor

n=112

S. No.	Site	No. of cases	Percentage
1	Chest	24	21.42
2	Back	21	18.75
3	Shoulder	11	9.82
4	Neck	8	7.14
5	Face	6	5.36
6	Abdomen	5	4.46
7	Upper limb	3	2.68
8	Chest & Back	12	10.71
9	Chest & Shoulder	10	8.94
10	Neck & Back	4	3.57
11	Face & Neck	3	2.68
12	Chest & Abdomen	2	1.79
13	Chest & Upper limb	2	1.79
14	Back & Shoulder	1	0.89

Chest and back regions were the most commonly affected sites.

Lower limb, hip palms, soles, and gluteal regions were not involved.

Table 8: Direct Microscopic Examination

n=112

S.No	Wet mount	Potassium Hydroxide	Parker Quink's Ink
1	Only yeast cells	8 (7.14%)	8 (7.14%)
2	Yeast cells and hyphae (spaghetti and meat ball appearance)	104 (92.86%)	104 (92.86%)

Both mounts found to be equally sensitive.

Table 9: Isolation rate of *Malassezia* fungus in patients with Pityriasis versicolor

n=104

S.No.	Direct Microscopy	Culture on Modified Dixon's agar	
		Positive	Negative
1	104 Cases	66 (63.46%)	38 (36.54%)

Out of 104 Pityriasis versicolor patients, only 66(63.46%) were culture positive for *Malassezia* yeast.

Table 10: Comparison of Modified Dixon's Agar with SDA with Olive oil overlay for isolation of *Malassezia* yeast

n=104

S. No.	Culture media	No. of culture positive	Percentage
1.	Modified Dixon's Agar	66	63.46
2.	SDA with Olive oil overlay	58	55.77

Modified Dixon's Agar had 7.69% higher isolation rates than SDA with Olive oil overlay.

Table 11: Isolation rate of *Malassezia* species in patients with Pityriasis versicolor

n=104

S. No.	Species	Number	Percentage
1	*M. globosa*	37	56.06
2	*M. sympodialis*	24	36.36
3	*M. pachydermatis*	2	3.03
4	*M. globosa* + *M.sympodialis*	3	4.55

M. globosa was the predominant species isolated in Pityriasis versicolor cases.

Table 12: Analysis of predisposing factors in patients with Pityriasis versicolor

n=112

S. No.	Risk factor	No. of cases	Percentage
1	Increased sweating	96	85.71
2	Known Diabetic	7	6.25
3	Systemic steroid usage	4	3.57
4	Bath oil application	6	5.36
5	Other risk factors	Nil	-

Increased sweating was identified to be the predominant risk factor.

Table 13 : Association of risk factor with culture results for Malassezia yeasts in Modified Dixon's Agar in patients with Pityriasis versicolor

Risk factor		Culture on Modified Dixon's Agar						Chi-square test	P value
		Positive		Negative		Total			
		No.	%	No.	%	No.	%		
Increased sweating	Yes	66	68.75	30	31.25	96	100	χ^2=15.1	P=0.0001
	No	-	-	8	100	8	100		
Known diabetic	Yes	6	85.71	1	14.28	7	100	χ^2=1.67	P=0.21
	No	60	61.86	37	38.14	97	100		
Systemic steroids usage	Yes	4	100	-	-	4	100	χ^2=2.4	P=0.12
	No	62	62	38	38	100	100		
Bath oil application	Yes	6	100	-	-	6	100	χ^2=3.7	P=0.06
	No	60	61.22	38	38.78	98	100		

Increased sweating was found to be the statistically significant risk for Pityriasis versicolor with P value of 0.0001.

Table 14: Association of family history in patients with Pityriasis versicolor

S. No.	Relationship	No. of cases	Percentage
3	Brothers/Sisters	2	9.82
1	Parents	11	8.03
2	Siblings	9	1.78
4	Total	22	19.63

No conjugal relationship was not seen in this study.

Table 15: Association of past history in patients with Pityriasis versicolor

n=112

S. No.	Past history	No of cases	Percentage
1	Similar illness	11	9.824
2	Topical treatment with antifungal preparations	8	7.14

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ABBREVIATIONS

AIDS	-	Acquired Immunodeficiency Syndrome
DBB	-	Diazonium Blue B
DMSO	-	Dimethyl sulphoxide
DOPA	-	Dihydroxy phenylalanine
DNA	-	Deoxyribonucleic acid
ELISA	-	Enzyme Linked Immunosorbent Assay
G+C	-	Guanine+cytosine ratio
HIV	-	Human Immunodeficiency Virus
KOH	-	Potassium Hydroxide
MLEE	-	Multilocus Enzyme Electrophoresis
NCCLS	-	National Committee for Clinical Laboratory Standards
PCR	-	Polymerase Chain Reaction
PFGE	-	Pulsed Field Gel Electrophoresis
RAPD	-	Randomly Amplified Polymorphic DNA
RFLP	-	Restriction Fragment Length Polymorphism
RNA	-	Ribonucleic Acid
SDA	-	Sabouraud Dextrose Agar

PROFORMA

NAME: OUT PATIENT NO:

AGE: ADDRESS:

SEX: OCCUPATION

PRESENTING COMPLAINTS

DURATION OF ILLNESS:

PREVIOUS HISTORY OF SIMILAR ILLNESS OR TREATMENT

H/O INCREASED SWEATING

- SYSTEMIC STEROIDS USAGE
- PROLONGED ANTIBIOTIC USAGE
- DIABETES MELLITUS
- CUSHING'S DISEASE
- MALIGNANCY
- ACQUIRED IMMUNODEFICIENCY SYNDROME
- TRANSPLANTATION

PERSONAL HISTORY APPLICATION OF BATH OIL

FAMILY HISTORY OF SIMILAR ILLNESS

CLINICAL EXAMINATION

- SITE OF LESION
- TYPE OF LESION-HYPOPIGMENTATION / HYPERPIGMENTATION
- SCALY LESION
- ANY PRURITUS

CLINICAL DIAGNOSIS

LABORATORY DIAGNOSIS

SPECIMEN: SKIN SCRAPINGS

DIRECT MICROSCROPIC EXAMINATION: 10% KOH MOUNT

PARKER QUINK'S INK MOUNT

CULTURE:

SDA WITH CYCLOHEXIMIDE AND ANTIBIOTICS OVERLAID WITH STERILE OLIVE OIL

SDA WITH CYCLOHEXIMIDE AND ANTIBIOTICS WITHOUT OLIVE OIL

MODIFIED DIXON'S AGAR

MACROSCOPIC COLONY MORPHOLOGY

MICROSCOPIC GRAM'S STAIN

PHYSIOLOGICAL AND BIOCHEMICAL TESTS

TEST FOR LIPID DEPENDENCE

CATALASE TEST – 3% HYDROGEN PEROXIDE

GROWTH AT 41°C

MODIFIED CHRISTENSEN'S UREASE TEST

TWEEN ASSIMILATION TEST

FINAL DIAGNOSIS

APPENDIX

I. POTASSIUM HYDROXIDE SOLUTION

Potassium hydroxide	-	10 gm
Glycerol	-	10 ml
Distilled water	-	80 ml

To a solution of 10% potassium hydroxide, 10 ml of glycerol was added to prevent drying. The ingredients were mixed and stored at room temperature.

II. MODIFIED DIXON'S AGAR

Composition

Malt extract	-	3.6 gm
Dessicated oxbile	-	2 gm
Peptone	-	0.6 gm
Tween 40	-	1 ml
Glycerol	-	0.2 ml
Olive oil	-	0.2 ml
Agar	-	1.2 gm/L
Chloramphenicol	-	5 mg
Cycloheximide	-	50 mg
Distilled water	-	100 ml

Final pH was adjusted to 6.0.

III. SABOURAUD DEXTROSE AGAR

Composition

Peptone	-	1 gm
Dextrose	-	4 gm
Agar	-	2 gm

Chloramphenicol - 5 mg

Cycloheximide - 50 mg

Distilled water - 100 ml

Final pH was adjusted to 5.6.

Olive oil was sterilized at 160°C for 1 hr in hot air oven and overlaid on SDA after inoculation of the culture media.

Media Preparation

Modified Dixon's Agar and Sabouraud Dextrose Agar were prepared as follows.

All the ingredients were dissolved by boiling in a water bath. Cycloheximide was dissolved in 10 ml of acetone and added to the boiling medium. Similarly, chloramphenicol was dissolved in 10 ml of 95% alcohol and added to the boiling medium. The medium was removed from heating, mixed well and then dispensed in tubes and autoclaved at 121°C for 15 minutes and the final pH was adjusted as needed. The tubes were cooled in slanted position and later the slants were stored in the refrigerator.

IV MODIFIED CHRISTENSEN'S UREA AGAR

Composition

Urea Agar base - 2.9 gm

Agar - 1.5 gm

Tween 40 - 0.5 ml

Tween 80	-	0.1 ml
Distilled water	-	100 ml

Christensen’s urea agar base was dissolved in 10 ml of distilled water and sterilized by filtration. Agar was suspended in water, heated with gentle mixing to boiling and autoclaved at 121°C for 15 minutes. The sterilised urea agar base was added to be cooling agar at approximately 50°C and dispensed in sterile tubes and cooled in slanted position. Phenol red was used as the indicator for this medium.