1. INTRODUCTION

The term "mycology" is derived from Greek word "mykes" meaning mushroom. The fungal disorders caused by the pathogenic fungi includes 80 different genera and are emerging as a significant cause of the infections in the world developing as an important clinical condition that deserves public health attention. To a certain extent, mycology is a disregarded field in medical research limiting the availability of documentated data on the overall prevalence of fungal infections in the world. Superficial fungal infection involving the keratinized layers of skin, hair and nails affects millions of individuals worldwide, as these infections tend not to be self-limiting, thus contributing to further spread in the absence of treatment resulting in the great alteration of the cosmetic appearance.

1.1 AETIOLOGICAL AGENTS

Dermatophytes are a uniquely pathogenic group of fungi that cause most common fungal infections globally and contained within three anamorphic (asexual or imperfect) genera, *Trichophyton*, *Epidermophyton* and *Microsporum* of class Hyphomycetes of the Deuteromycota (Fungi Imperfecti). At present there are 42 species of dermatophytes recognized namely 23 species of *Trichophyton*, 18 species of *Microsporum* and 2 species of *Epidermophyton*. Those dermatophytes capable of reproducing sexually producing ascomata with asci and ascospores are classified in the teleomorphic genus *Arthroderma*, family Arthrodermataceae of the Onygenales, phylum Ascomycota. The significant characteristics of some clinically important dermatophytes are tabulated in table 1.1

1.2 ECOLOGY

Based on their ecology and host preference, dermatophytes have been grouped into geophilic, zoophilic and anthropophilic species.

a) Geophilic dermatophytes: These dermatophytes are chiefly associated with keratinous materials have been dissociated from living animals and are in the process of decomposition. These species cause human and animal infection. Geophilic species are thought to have been ancestral to the pathogenic dermatophytes, preadapted to cutaneous pathogenesis by their ability to decompose keratin and their consequent case association with animals living in hair
and feather-lined nests in contact with soil. Ex: *M. gypseum, M. fulvum, T. ajelloi, T. vanbreuseghemii*.

**b) Zoophilic dermatophytes:** These dermatophytes have gradually evolved from soil to parasitize animals. These fungi are primarily animal parasites. Human infections are acquired either by direct contact with an infected animal or indirectly by contact with fomites. Ex: *M. canis, M. gallinae, T. mentagrophytes var mentagrophyte, T. verrucosum, T. equinum*.

**c) Anthrophilic dermatophytes:** These dermatophytes have evolved from zoophilic species. Humans are the normal hosts for this species and transmission occur directly or indirectly. Ex: *E. floccosum, M. audouinii, T. mentagrophytes var interdigitale, T. rubrum, T. schoenleinii, T. tonsurans, T. violaceum*.

### 1.3 PREDISPOSING FACTORS

The supreme factors that prime tinea infections belongs to the diverse categories comprising environmental, socioeconomical, occupational, metabolic-endocrine disorders, immunosuppression and drug therapy. As the dermatophytes flourish gloriously at temperatures of 25-28ºC, the infection of human skin is corroborated by warm and humid conditions, prosperous bulk tourism, augmenting migration, unstable socioeconomic status, overcrowding, intensified urbanization acts as the most approving social factors for tinea infections worldwide which are also not restrained to age, sex, race and miserable hygiene. Many working practices may also complicate these infections specifically when conditions favor disease transmission. Inspite of the socioeconomical and cultural variables, the dermatophytosis can also be a part of every human's individuality based on their immune condition, genetically related or metabolic disorders and drug therapy. Since, the disposition of dermatophytosis and its aetiological agents fluctuates with its geographical regions and various factors at an unparalleled pace, the management of these infections would be a conclusive challenge to mankind.

### 1.4 HOST-PATHOGEN INTERACTION AND PATHOGENESIS

#### 1.4.1 Anatomy of the host

The dermatophyte infection commences with the deposition of infected scales, hyphae or arthropores on the skin of the hosts (Fig 1.1). The factors that play a crucial role apart from the fungal inoculums are trauma and increased hydration of skin and maceration. After the host skin has been inoculated under
suitable condition, the infection progresses through many stages such as period of incubation, period of enlargement, refractory period and stage of involution.

Fig 1.1 Mode of transmission, Route of entry and Pathogenicity of dermatophytes

**Skin**

During the incubation period of 1-3 week, the dermatophyte grows in stratum corneum with minimal clinical signs of infection. In consequence of certain inhibitory factor in serum, the fungus restricts itself in the stratum corneum after entering the skin through minor trauma and spreads from the site of inoculation in a radial fashion producing clinical symptoms of an expanding ring with a central clearing (Fig 1.2). Subsequent to establishment of infection in stratum corneum, two factors are very important in determining the size and the duration of lesion namely, the rate of growth of organism and the epidermal turnover rate in which the former must be equal or exceed the later or the organism will be shed quickly. After a definite period, the fungus ceases to grow after the clinical lesion has reached a certain size as these are the stages of refractory period and of involution.
Hair

From the adjacent stratum corneum, the fungus intrudes the hair follicle at the mid-follicular level descending within the intrapilary portion of hair until they reach the border of the keratogenous zone (Fig 1.3). The terminal tuft of hyphae in this location is termed Adamson’s fringe. The spores are either formed within the hair shaft (endothrix infection) or on the surface of the hair shaft (ectothrix infection) which rely on the infecting species. Spore formation within the hair shaft results in a significant weakening of hair shaft with subsequent fracture of the shaft at the scalp line and the remnants of hair within the follicle is called “black-dot. In favus, hair invasion occurs without spore formation and with less hyphae culminating normal length of hair growth. The kerion type infection of results from diffusion of metabolites or toxins from fungus and an immunologic response to dermatophyte antigen.

*T. tonsurans* and *T. violaceum* produce endothrix infection. Ectothrix infection of hair may occur with either small spore producing species like *M. canis* and *M. audounii* or with large spore producing species like *T. verrucosum* and *T. mentagrophytes.*
Nails

The dermatophyte invasion commences from the hyponychium and distal nail bed of stratum corneum, progresses proximally in the nail bed and invade the ventral surface of nail plate. Subungual hyperkeratosis results from a hyper proliferative reaction of the nail bed in response to the infection.

![Fig 1.4 Anatomy of nail](image)

The two possible routes of infection are:

- a. Penetration of the fungus through the eponychium and the hyphae advances toward the nail bed in the comparatively soft keratin of ventral layer.

- b. Hyphae entering the lateral nail grooves, follow the lamellar structure of nail plate transversely and reach the ventral layer (Fig 1.4).

The fungi grow for preference in the softer keratin where they are adjacent to the living tissue of nail bed with the end result is a progressively dystrophic nail unit.

1.5 ENZYMATIC MACHINERY

The enzymatic machinery comprising of secreted hydrolytic enzymes with different substrate specificities such as proteases, lipases, elastases, collagenases, phosphatases and esterases, is one of the most important factors during these infectious process allowing the hydrolysis of structural components of the epidermal tissue and the invasive character of these pathogens. These enzymes act on the substrates, producing peptides that are hydrolyzed to amino acids, which are used by the fungus as a carbon, nitrogen and sulphur. The metabolization of some amino acids promotes the alkalinization of the host's microenvironment, making it suitable to the action of keratinases with optimal activity in alkaline pH (Fig 1.5), which
allows the maintenance of the infection and makes the complete installation, development and permanence of the dermatophyte in the host tissue possible.

1.6 VIRULENCE AND PATHOGENICITY

Proteolytic and keratinolytic activities of dermatophytes have been a subject of interest for several years to understand the pathogenicity of infection.

1.6.1 Proteases

The proteases of dermatophytes can be divided into endoproteases (or endopeptidases) and exoproteases (or exopeptidases). The exoproteases cleave peptide bonds only at the N- or the C- terminus of polypeptide chains (Fig 1.6).

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**Fig 1.5** Schematic representation of dermatophyte pathogenesis

**Fig 1.6** The keratin degradation pathway by the dermatophytes
Based on the nature of the functional group at the active site and the type of enzymatic mechanisms, there are eight catalytic types of peptidases: asparagine, aspartic, cysteine, glutamic, metallo, serine, threonine and unknown. It is now known that dermatophyte secreted endoproteases are member of two large protein families, the subtilisins (serine proteases) and the fungalysins (metalloproteases).

1.7 CLINICAL MANIFESTATIONS

The clinical features of dermatophyte infections results from a combination of keratin destruction and an inflammatory host response. The wide variation in infection depends upon the species and probably the strain of the infecting fungus concerned, size of the inoculum, site of the body infected and immune status of the host (Table 1.2).

1.8 DIAGNOSIS, TREATMENT AND PREVENTION

Conventional approaches

Direct microscopic examination is usually performed using clearing reagents (KOH, NaOH) alone but its sensitivity may be greatly enhanced by the use of stains or fluorochromes like PAS, chlorazol black E, Congo red, calcofluor white or Chicago sky blue stain. Specific culture media may be used to overcome the growth of rapidly growing contaminating moulds which may hamper the recovery of dermatophytes. Identification at the species level which may be useful to initiate an appropriate treatment or for setting prophylactic measures, relies on macroscopic and microscopic morphology. Additionally, in case of atypical isolates, some biochemical or physiological tests may be performed such as the search for urease activity or the in vitro hair perforation test.

Molecular approaches

The morphological similarity, variability, and polymorphism of dermatophytes have meant that species identification for dermatophytes is time consuming and requires a significant degree of knowledge and technological expertise. Molecular biology-based techniques have solved problems concerning the morphology-based identification of dermatophytes and have improved our knowledge on the epidemiology of dermatophytosis. In the early 1980s, nucleic acid-based methods were used to try to resolve questions about the evolutionary relationships between dermatophytes. Other molecular methods that are not suitable for fundamental studies, have been recently developed to facilitate the
delineation of species and strains. Diagnostic and epidemiological applications are envisaged. DNA base composition and DNA–DNA hybridization, Mitochondrial DNA restriction fragment length polymorphism, Nuclear ribosomal RNA genes sequencing (large ribosomal subunit (25S rRNA) sequences, a technique and small ribosomal subunit (18S rRNA), Chitin synthase 1 gene sequencing, Internal transcribed spacer region ribosomal DNA sequencing.

Treatment

After the clinical and microbiological investigation, the choice of treatment was determined by the site and extent of lesions, the fungal species involved, and the efficacy, safety profile, and pharmacokinetics of the available antifungal agents. The first-line therapy was based on the use of topical agents namely imidazole antifungal. When such therapy is ineffective, oral therapy with antifungal agents such as terbinafine, itraconazole, ketoconazole, and fluconazole was instituted. The combined therapy with topical and oral antifungal with anti-inflammatory agents has been employed in an attempt to increase the cure rate.

Prevention

Prevention control of dermatophytic infection depends on area of the body involved, the causal agent and source of infection. To avoid large outbreaks among school children, good hygiene should be maintained and avoidance of sharing headgear, combs and hairbrushes should be stressed to students. Tinea corporis and tinea cruris caused by anthropophilic dermatophytes should be prevented by washing, cloth disinfecting, bedding used by patient before being used by other people. When zoophilic species infection is detected, infected animal reservoir should be sought out and treated. The spread of tinea pedis infection may be controlled by good foot hygiene, using antifungal powders, avoiding excessive moisture and wearing ventilated foot wear. The prevention from zoophilic dermatophytes can be done using rodent control and the vaccines which are available for cattle and other animal groups (Palacio et al., 2000).

The research work presented in this thesis is comprised of the various aspects of dermatophytosis, with potential light into the virulence mechanisms and prevention strategy.
1.9 GENERAL STRATEGY OF DERMATOPHYTOSIS

Dermatophytosis is a common infectious disease caused by dermatophytes. Although common, the meticulous size of the dermatophytosis resists measurement, the World Health Organization (WHO) estimated that superficial fungal infections affect roughly 20-25% of the world population. Surpassing the importance, tinea infections not only fluctuate with their epidemiology and ecology but also rely on a shrewd range of environmental and civilized factors. The ultimate factors that beforehand tinea infections suit to the diverse categories including both the natural and anthropogenic associated activities. Strengthened urbanization acts as the most approving social factors for tinea infections worldwide while working practices may also complicate these infections specifically when conditions favor disease transmission. The dermatophytosis can also be a part of every human's individuality based on their immune condition, genetically related or metabolic disorders and drug therapy. As the temperament of dermatophytosis and its aetiological agents fluctuate with its geographical regions and various factors at an unparalleled pace, the management of these infections would be a decisive challenge to mankind in the years to come. Although the general health of patient is not altered, the cosmetic appearance is greatly altered (Hainer, 2003). The estimated lifetime risk of acquiring a dermatophyte infection is between 10-20% encountered in India because this being a tropical country (Thappa, 2002).

The pathogenesis of dermatophytosis is usually multifaceted, whereas the clinical presentation of this infection is inconsistent depending upon the site of infection, the management of this condition requires an early measurement and rapid treatment. In the international scenario, the pathophysiology of tinea infections is quite complex, but their physical appearance are largely a consequence of host and pathogen related factors (Lakshmipathy and Kannabiran, 2010). The infection begins as a sequential progression of adhesion, penetration, invasion and penetration of arthrospores of dermatophytes. These mechanisms were evidenced in the studies of Baldo et al., 2011; Tainwala and Sharma, 2011; Samdani, 2005). The fungal agents of dermatophytosis has found to be diverse as shown by different studies. Most of the studies reported from apex to base of India have shown the predominance of *T. rubrum* (Sarada and Kumari, 2015; Vignesh et al., 2015; Lakshmanan et al., 2015; Sharma et al., 2015, Singh et al., 2015; Najotra et al., 2015. but a very few studies had reported *T. mentagrophytes* (Bhatia and
Sharma, 2014, Agarwal et al., 2014), T. violaceum (Mane et al., 2013), T. verrucosum (Ghosh et al., 2014), T. tonsurans (Sha et al., 2013) as the exceptional cases. The available literature on the studies of the aetiological agents on dermatophytes were prevalent every nook and corner of India, there are no studies on dermatophytosis pertaining to this part of Southern India, i.e. Salem.

In India, the laboratory diagnosis of dermatophytosis is extrapolated from the data available from the global countries, which may or may not be appropriate for Indian patients as it may be economical. Hence, rapid diagnosis by direct microscopy at cost effective rate is required which can significantly improve the clinical outcome and reduce the infection related morbidities. Despite direct microscopy, culture readily becomes the gold standard for the diagnosis of dermatophytes. Hence, an early detection of lesions, prompt diagnosis of dermatophytosis are important for successful therapeutical regimen. Thus, the standard diagnosis of dermatophytosis comprises direct microscopy along with culture. For many years, conventional laboratory methods based on the detection of phenotypic characteristics, such as microscopy and in vitro culture, have played an essential role in dermatophyte identification. Worldwide, the current developments and applications of nucleic acid amplification technology have provided the opportunity to enhance the quality and speed of dermatophyte diagnosis (Liu et al., 2000). But in Indian circumstances, a large amount of the patients are not aware of the disease or under self-treatment excluding the option for a clinician. If possible, some patients can only afford for diagnosis by direct microscopy but not for culture.

The most noteworthy aspects in the laboratory diagnosis of dermatomycosis has lead to the augmentation of knowledge on all the factors that participate in pathogenesis, such as: proteases, secretory enzymes, adhesion possibilities and ability to modulate defense mechanisms of the host (Vermout et al., 2008; Stojanov et al., 2011). These fungi secrete multiple serine and metallo endoproteases (subtilisins and fungalysins, respectively) formerly called keratinases. Elucidation of the primary stimuli and intracellular pathways that regulate proteases secretion, as is the understanding of dermatophytic adhesion process This will undoubtedly expedite the uncovering of the mechanisms underlying the infection process and finally leading to the design of new therapeutic approaches. The knowledge about the range of potentially keratinolytic proteases produced by dermatophytes is persistently on the rise, the rational basis for the development of therapeutic and
prophylactic strategies have been emerged (Vermout et al., 2008). While intercontinental scrutiny to study proteases was broadening, the Indian situation remains sparse to study the proteolytic activities of anthropophilic dermatophytes. Consequently, such an attempt has been made in this thesis to screen the proteolytic activities of dermatophytes isolated from Salem zonal clinics, Tamil Nadu.

A variety of protein or keratin substrates were used for screening of proteases namely, casein, gelatin, soy protein, skim milk protein, chicken feathers, animal hair, hooves, human skin, nails and hair. The application of chicken feathers as a keratin source has broadened the scope on the understanding of the proteolytic activity of dermatophytosis. Therefore, a better understanding of the protease screening is very much essential especially in determining the virulence and pathogenesis of dermatophytosis. In Southern India, as there are only two studies evaluating the protease production in dermatophytes (Venkatesan et al., 2010; Gokulshankar et al., 2011), since there are increasing report as the proteolysis and the genes associated with it worldwide, one such study has been made in this thesis.

The molecular biology based techniques holds great promise in assessing the dermatophytes, given that sequencing is unbiased, allowing for accurate identification of dermatophyte species. The 18S rDNA can be used to identify the dermatophyte species using universal primer. Hence defining fungal DNA requires sequencing at a significant higher cost making PCR not favorable for rapid diagnosis. Due to this limitation, in India, most of the studies to date were primarily based on the conventional methods. Most new techniques for identifying dermatophyte strains are based on the amplification of the 18S rRNA gene. This highly conserved gene is present in the genome of all eukaryotes, but it contains hyper variable regions that can be used for identifying specific fungal species. The sequence of the organisms provides new information on the relationship between genome size, genetic complexity and ecological versatility in fungi. Hence such an approach has been made in this thesis with regard to the partial sequencing of the protease producing strains by using 18S rDNA gene sequencing.

In dermatophytes, metallo and serine protease coding genes (MEP1-5 and SUB1-7) remain the most important contributing factor to virulence and
pathogenesis. In abroad, there are several reports pertaining this attribute of these proteases in dermatophytes namely, the isolation of SUB and MEP genes (Descamps et al., 2002), recombination and expression in yeast (Monod et al., 2002, Jousson et al., 2004) and animal models (Vermout et al., 2004), gene silencing (Vermout et al., 2007), adherence studies (Baldo et al., 2008), differential host selection studies (Preutt et al., 2010), and various mutant studies (Zhang et al., 2013). But in Indian scenario, as there is only one reports regarding the dermatophyte gene sequencing of secreted subtilases (Latka et al., 2015), an endeavor has made in this thesis to sequence the protease encoding genes of dermatophytes isolated from the clinical setting of Salem zone, Tamil Nadu. The investigation of protein profiles, the recognition of some orthologous secreted proteases and the likeness of their amino acid sequences adumbrated that each species secretes a unique crew of homologous proteins (Giddey et al., 2007). The characteristics of excretory or secretory proteins circulating throughout the body of an organism (localized to or released from the cell surface) make these molecules extremely attractive targets for novel vaccines and therapeutics, which are currently the focus of major drug discovery research programs (Ranganathan and Garg, 2009).

In this intention, bioinformatics act as the backbone computational tools and databases that substantiate genomic and related research, which extensively circumscribes the study of DNA structure or function, gene expression and protein production, structure and function (Rajamurugan et al., 2012). Consequently, the best method for predicting the structure of a protein depends on whether it has sequence homology to a protein of known structure. If there is such a similarity, relatively accurate models can be built using the known structure as a template. In the absence of such similarity, models can be built using de novo prediction methods, which do not rely on a template structure. In many cases, hybrid template-based/de novo methods may be most appropriate: portions of a given target may be modeled based on a template, while it may only be possible to model long variable loops or extra domains or extensions not contained in the template using de novo methods. In other hands, the widespread use of combinatorial chemistry and virtual screening (VS) is used to ascertain novel protease inhibitors. High-speed computational methods can now enrich the fraction of suitable lead candidates in a chemical database, thereby creating the potential to greatly
enhance productivity and dramatically reduce drug development costs. With an ever-increasing number of drug discovery projects having access to high-resolution crystal structures of their targets, high performance ligand-receptor docking is the clear computational strategy of choice to augment and accelerate structure-based drug design.

In this thesis to study certain aspects of dermatophytosis which are of substantial significance in knowing this infection and which have now not been adequately studied has been dealt. This study comprises determination of aetiological agents and clinico-mycological profile of tinea infections in and around Salem hospitals, Tamil Nadu, India. The efficacy of Potassium hydroxide (KOH) mount in relation to the results obtained by culture for the rapid diagnosis of this condition has also been evaluated. The vital aspect of this study is focusing on the endoproteases responsible for virulence through preliminary screening of dermatophytes for protease enzymes on media containing different protein substrate has been done. The genotypic confirmation of the strain exhibiting highest proteolytic activity has also been revealed by 18S rDNA sequencing. Another crucial aspect of this thesis is the evaluation of multiplex PCR for the determination of metalloproteinase (MEP1-5) and subtilisin (SUB1-7) genes in strains with maximum proteolytic activity followed by sequence analysis. The Prediction of protein structure of the metalloproteinase (MEP1-5) and subtilisin (SUB1-7) genes through investigation of signal peptides, catalytic triads and protein structures by 2D and 3D prediction tools has been assessed to evaluate the functionality of subtilisin protease. The novel facet of this thesis is the recognition of new-fangled lead compounds as potential targets for the identified metallo- or subtilisin protease. Finally, the identification of novel protease inhibitors against metallo- and serine-proteases through virtual screening of drug molecules has been addressed.

The results obtained in this study will help in prioritizing the resources, thus enabling the correct therapeutic and preventive management of this conditions, which will have significant clinical consequences on the strain, producing chronic infection making the treatment of these lesions difficult, thereby decreasing the quality of patient life.