2. REVIEW OF LITERATURE

Globally, dermatophytosis is one of the most common causes of infection affecting greater than 20-25% of the world’s total population (Pires et al., 2014). Due to the defect involving poor virulence models and genetic tools, the progression of this condition and effective therapeutics has been stunted, presenting new challenges for the delivery of a safe and effective antifungal therapy. However, several recent advances have been established that minimize these technical difficulties of working with the characterization of these fungi, resulting in a lack of sequenced dermatophyte genomes and their pathogenicity (Achterman and White, 2012). Regardless of these advances, an important number of fungi remain as serious threats to the health of susceptible patients and the outcomes are far from optimal, requiring a better knowledge of their pathogenic mechanisms and the development of new therapeutic strategies. In this review the various aspects pertaining to the study of dermatophytosis has been given pointing out the lacuna in the literature which has been addressed in this thesis.

The ancient Ayurvedic literature of India (800 BC to 100 AD), describes a skin condition called “Dadru”, which resembles present day ringworm clinically, but nothing has been said about causative agent (Dey et al., 1973). The term “Tinea” was first coined by Cassius Felix in about 400 AD., in his summary of Medicine including skin disease. Aulus Cornelius Celsus in his work entitled “De Re-Medicina” first reported the case of dermatophytic infection in the scalp (Rippon, 1988). The systemic study of fungi in seventeenth century began after the invention of microscope by Anton Van Leuwenhock and the man who deserves the esteem of being called the Founder of Science of Mycology was Pier “Antonia Micheli”, the Italian Botanist who in 1729 published “Nova Planetarium Genera” in which his research on fungi were included (Allexopoulous, 1972).

During the mid 19th century, the discovery of the fungal etiology of favus focused around three European physicians; Robert Remak, Johann L Schonlein and David Gruby (Weitzman and Summerbell, 1995). Due to the creeping nature of the lesions in circular dimensions, the Greeks named the disease Herpes while the Romans termed the lesions as tinea meaning any small insect larva (Rippon, 1974).
2.1 HISTORICAL REVIEW

In 1835, Robert Remak observed peculiar microscopic structures of patient suffering from tinea favosa while the fungal cause of tinea favosa was described by Professor J. L. Schonlein in Zurich (Emmons et al., 1977). In 1841 - 1844, Gruby depicted the clinical entities caused by dermatophytes and illustrated that fungi could be cultured and transmitted to other humans also (Chander, 1995). He also recognized ectothrix and endothrix hair invasion and gave the name *M. audouinii* to the agent of ringworm of childhood. In 1892, Raymond Sabouraud classified the dermatophytes into four genera, *Achorion, Epidermophyton, Microsporum* and *Trichophyton* and developed medium for culturing fungi and it is named in his honor as Sabouraud Glucose agar (Weitzman and Summerbell, 1995).

In 1925, Robert W Wood invented Wood’s lamp that was used for the detection of fungal infection of hair. In 1934, Chester Emmons modified the taxonomic scheme of Sabouraud and other scientists and established the current classification of dermatophytes on the basis of spore morphology and accessory structures. He eliminated the genus *Achorion* and emphasized on rest of the three genera (Chander, 1995). In 1952, Van Breuseghem described hair bait technique (Collier et al., 1998). In 1958, Gentles published a report on the oral administration of griseofulvin (Chander, 2009). In 1969, Taplin and his coworkers developed dermatophyte test medium (DTM) to isolate and distinguish dermatophyte from fungal or bacterial contaminants from cutaneous lesions (Chung and Bennet, 1992). In 1980’s azole derivatives were discovered (Chander, 2009).

2.2 EPIDEMIOLOGICAL SURVEY

Dermatophytosis has been afflicted humanity for centuries, as it is reported from different parts of the world. Since 1978, there have been reports of these dermatophytes restricted half way across the world because of the rapid transit, and the increasing mobility of people (Khaled et al., 2015). Several reports were found to be evident that no race in any geographical location was spared from dermatophytosis as these infections were studied in Turkey (Kantarcioğlu et al., 2015), Brazil (Pires et al., 2014), Saudi Arabia (Khaled et al., 2015; Hanafy et al., 2012), Poland (Budak et al., 2013; Kobylak et al., 2015), Bosnia and Herzegovina (Prohic et al., 2015), Portugal (Fernandes et al., 2013), China (Zhan et al., 2013; Yin et al., 2013), Nigeria (Samuel et al., 2013; Aleruchi et al., 2012; Ndako et al., 2015).
Pakistan (Shujat et al., 2014; Hanif et al., 2012; Naseri et al., 2014; Bakhshi et al., 2015), Abidjan (Fulgence et al., 2013), Italy (Mapelli et al., 2013), Greece (Tsoumani et al., 2011), Korea (Lee et al., 2012), Egypt (Azab et al., 2012), USA (Tirado-Gonzalez et al., 2012; Abdel-Rahman et al., 2010), UK (Ameen, 2010), Arad (Mihali et al., 2012), Kuwait (Yehia et al., 2010) and Spain (Boz-Gonzalez, 2012).

Even the nook and corner of India has not been escaped from these infections and these were evident from the studies in Northern India - Jammu and Kashmir (Najotra et al., 2015), Manipur (Singh et al., 2015), Himachal Pradesh (Bhatia and Sharma, 2014), Delhi (Sharma et al., 2012), Lucknow (Sahel and Mishra, et al., 2011), Moradabad (Kumar et al., 2012), Southern India - Mangalore (Surendran et al., 2014), Bellary (Sumathi et al., 2013; Sonth et al., 2013), Mysore (Jha and Murthy, 2013; Hanumanthappa et al., 2012), Andhra Pradesh (Doddamani et al., 2013), Vijayawada (Sarada and Kumari, 2015), Visakhapatnam (Maruthi et al., 2012), Hyderabad (Madhavi et al., 2011), Nellore (Santosh et al., 2015), Chennai (Lakshmanan et al., 2015), Pondicherry (Ramakrishnaiah et al., 2014), Kanchipuram (Vignesh et al., 2015), Namakkal (Shahitha et al., 2013), Tiruchirapalli (Balakumar et al., 2012). Studies from Eastern India - West Bengal (Samaddar et al., 2015; Ghosh et al., 2014), Kanpur (Omar et al., 2013), Tanda (Bhagra et al., 2014), Western India - Jaipur (Sharma et al., 2015; Sharma et al., 2015; Sarika et al., 2014; Vyas et al., 2013; Sharma et al., 2012), Gujarat (Gelotar et al., 2013; Hitendra et al., 2012), Rajasthan (Chand and Naruka, 2013), and Central India - Maharashtra (Mane et al., 2013; Yadav et al., 2013; Bose et al., 2013), Gwalior (Pandey and Pandey, et al., 2013). All these studies mainly focused on the clinico-mycological and epidemiology of the dermatophytic infections thus highlighting the prevalence of these infections throughout the country.

2.3 CLINICAL PATTERN, PREDISPOSING FACTOR AND AETIOLOGICAL AGENTS OF DERMATOPHYTOSIS

2.3.1 Clinical pattern

The clinical facet of dermatophytosis was extremely fluctuating, resulting from a combination of keratin destruction and an inflammatory host response. Consequential factors leading to the different clinical forms were the strain and species of the infecting fungus, size of the inoculum, the site of the body infected and the keratinisation at that site, as well as the immune status of the host.
(Degreef, 2008). The prevalence of dermatophytes in any community depends upon various factors, the genetic and racial constitution, the social and hygienic standards, customs and occupations, the nutritional status and age structure of the community, climatic factors, immunosupression and perhaps some degree of inherited susceptibility.

The prevalence of tinea capitis infections were reported in many studies worldwide. East-Innis et al., 2006 surveyed the aetiological agents of Tinea capitis in the Jamaican population while Khaled et al., 2007 described an unusual variant of tinea capitis in Tunis. The frequency, etiology, clinical forms and management of tinea capitis in Spain was assessed by Boz-Gonzalez, 2012 and Perez-Gonzalez et al., 2009. The clinical features of Tinea capitis favosa, a chronic inflammatory dermatophyte infection of the scalp was reported in Turkey by Ilkit, 2010. In China, the three familial cases of Tinea Capitis and Tinea Corporis was described by Yin et al., 2013. Mapelli et al., 2013 determined the aetiological agents of pediatric Tinea capitis in Milan, Italy and analyzed epidemiologic changes in relationship with changes in population. Fulgence et al., 2013 performed a cross sectional study among school children in southern Ivory Coast which was designed to assess the overall prevalence of tinea capitis and described its associated aetiological and epidemiological determinants. An unusual case of kerion celsi was described by Fernandes et al., 2013 in Portugal. Moto, et al., 2015 determined the prevalence Tinea capitis in children from selected schools from an urban slum in Nairobi city of Kenya. Asadi et al., 2009 and Kazemi et al., 2007 conducted a study to determine the epidemiologic condition of patients suspected with onychomycosis and tinea pedis in Iran. Viegas et al., 2013 designed a study to statistically describe the data obtained as results of analysis conducted during a four year period on the frequency of Tinea pedis and onychomycosis and their etiologic agents.

In India, the clinical facet of dermatophytosis varies on its geography and socioeconomical conditions. A case of “black dot” favosa variety of Tinea capitis was reported by Ghadgepatil, et al., 2015. Mane et al., 2013, assessed T. violaceum as a major cause of Tinea capitis in tertiary care hospitals The prevalence of onychomycosis was also reported in various parts of India namely, West Bengal (Samaddar et al., 2015), Gujarat (Gelotar, 2013), Rajasthan (Chand and Naruka, 2013), Shimla (Gupta et al., 2007), Maharastra (Veer et al., 2007). The incidence of Tinea corporis and Tinea cruris was reported in New Delhi by
Sonthalia et al., 2015. Although there are many studies reporting the cross-sectional studies of dermatophytosis in Tamil Nadu, the clinical profile of the dermatophytosis patients will help in the explaining the reasons for the mycological pattern of infection. Hence, in this study this aspect has been dealt with different clinical features and the clinical pattern of tinea infections.

2.3.2 Predisposing factor of dermatophytosis

As the dermatophytes flourish gloriously at temperatures of 25-28°C, the infection of human skin is attributed to warm and humid conditions, prosperous bulk tourism, augmenting migration (Havlikova, 2008), unstable socioeconomic status (Pires et al., 2014), overcrowding (Kumar, 2014), intensified urbanization (Achterman and White, 2012) and many working practices may also complicate these infections specifically sport centres and gymnastics (Mahmoudabadi and Rahnemaei, 2012) when conditions favor disease transmission. Sportsmen actively engaged in international sports activities, marathon runners, soldiers (Cohen et al., 2004), farmers (Mahmoudabadi and Izadi, 2011; Spiewak and Szostak, 2000, Spiewak, 1998), wrestlers (Zahra et al., 2012; Habibipour et al., 2012), miners. 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. The mediocre of metabolic and endocrine disorders or diseases which have a high fortune of these tinea infections comprise intensifying age, immunosupression and immunosuppressive drugs, including corticosteroids, vincristine, cyclophosphamide and azathioprine, the presence of diabetes mellitus, family history, malnutrition, rheumatoid arthritis, chronic liver and renal disorders, peripheral vascular disease, Leukemia, lymphoma, Cushing syndrome and disorders related to the skin such as hyperhidrosis, psoriasis, onychogriposis and nail trauma (Kuvandik et al., 2007).

Since, the disposition 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 conclusive challenge to mankind in the years to come and this study will implicate the understanding of the most predisposing causes for dermatomycoses.
2.3.3 Spectrum of moulds causing Dermatophytosis

2.3.3.1 Mycological agents of dermatophytosis worldwide

Most of the studies in Africa has reported *T. violaceum* as the predominant isolate (Ellabib *et al.*, 2001; Woldeamanuel *et al.*, 2006; Azab *et al.*, 2012 and Fulgence *et al.*, 2013) while few studies described *T. schoenleinii* (Khaled *et al.*, 2007), *M. ferrugineum* (Ndako *et al.*, 2012) and *T. soudanese* (Fulgence *et al.*, 2013) as the major aetiological agents. The studies in Nigeria reported *M. audouinii* (Hassan and Moses, 2010) and *T. mentagrophytes* (Aleruchi *et al.*, 2012) as the predominant aetiological agent. In America, *T. rubrum* (Monteiro *et al.*, 2005 and Flores *et al.*, 2009) was found to be prevalent while *T. raubitschekii* was first reported in the study of Lacaz *et al.*, 1999. *M. audouinii* was also reported by East-Innis in the Jamaican population. In the midst of Asian countries, several reports from Iran has documented the isolates of *M. ferrugineum* (Mahmoudabadi, 2006), *E. floccossum* (Dehghan, 2007 and Bassiri-Jahromi and Khaksari, 2009), *T. mentagrophytes* (Ansar *et al.*, 2011), *T. verrucosum* (Nejad, 2007 and Naseri *et al.*, 2013) as the causative agents. *T. mentagrophytes* was isolated by Yehia *et al.*, 2010 in Kuwait. *T. interdigitale* was the predominant isolate in Pakistan (Hanif *et al.*, 2012 and Shujat *et al.*, 2014). In China, *T. rubrum* was the predominant isolate (Zhan *et al.*, 2013, Yu and Jianhus, 2013 and Cheng *et al.*, 2014). *T. rubrum* was found to be the predominant isolate in most of the European countries (Valdigem *et al.*, 2006, Seebacher *et al.*, 2008 and Budak *et al.*, 2013). The other isolates found to be prevalent were *M. canis* (Tsoumani *et al.*, 2013 and Prohic *et al.*, 2015), *M. audouinii* (Fernandes *et al.*, 2013), *T. verrucossum* (Spiewak and Szostak, 2000), *T. eboreum* (Brasch and Graser, 2005), *T. violaceum* (Khaled *et al.*, 2015) and *E. floccossum* (Pakshir and Hashmi, 2006).

2.3.3.2 Mycological agents of dermatophytosis in India

Studies from India have reported, *T. rubrum* was the most prevalent isolate which was evidenced from the studies in Lucknow (Saahai and Mishra, 2011), Maharashtra (Bose *et al.*, 2013), Madhya Pradesh (Pandey and Pandey, 2013), Mumbai (Yadav *et al.*, 2013), Mumbai (Mane *et al.*, 2013), Delhi (Sharma *et al.*, 2012), Jammu and Kashmir (Najotra *et al.*, 2015), Haryana (Bhatia and Sharma, 2014), Shimla (Bhagra *et al.*, 2014), Imphal (Singh *et al.*, 2015), Gujarat (Patel *et al.*, 2010), Jaipur (Sharma *et al.*, 2012), Rajasthan (Sharma *et al.*, 2015), Rajasthan (Chand and Naruka, 2013), Trichirapalli (Balakumar *et al.*, 2012), Ahmedabad
(Hitendra et al., 2012), Mysore (Hanumanthappa et al., 2012), Hyderabad (Madhavi et al., 2011), Mangalore (Kumar et al., 2012 and Surendran et al., 2014), Bellary (Sonth et al., 2013 and Sumathi et al., 2013), Chennai (Lakshmanan et al., 2015), Kanchipuram (Vignesh et al., 2015), Gulbarga (Doddamani et al., 2013), Pondicherry (Ramakrishnaiah et al., 2014), Vijayawada (Sarada and Kumari, 2015). The exceptions were found as the isolates T. mentagrophytes in Lucknow, Haryana and Rajasthan (Sahai and Mishra, 2011, Bhatia and Sharma, 2014, Agarwal et al., 2014) T. violaceum in Mumbai (Mane et al., 2013), T. verrucosum in Kolkata (Ghosh et al., 2014), T. tonsurans in Mysore (Jha and Murthy, 2013).

Even if there were different studies emphasizing the dermatophytes in different regions of the country, the pattern of the organism was found to be vary depending upon diverse factors. Hence, in this thesis an attempt has been made to study the prevailing clinical pattern, aetiological agents and predisposing (risk) factors of dermatophytosis pertaining to this part of the study area.

2.4 LABORATORY DIAGNOSIS OF DERMATOPHYTOSIS

Laboratory investigations are very essential in conforming the diagnosis of dermatophytosis. Several modalities of diagnosis includes direct microscopic examination, culture, histology and the modern method of diagnosis which includes polymerase chain reaction (PCR). Direct microscopy provides an early and reasonably reliable method of diagnosing fungal infections. The hyphae stand out as highly refractile septate or aseptate threads. Several modifications of the basic KOH or NaOH preparation have been made with the incorporation of glycerin, DMSO, Parker Superchrome Blue-black ink, Sodium sulphide, eosin etc for the betterment of direct microscopic examination (Kurade et al., 2006). Some fungal stains such as calcofluor white stain, acridine orange, PAS, GMS also aided in the diagnosis of dermatophytosis (Chander, 2009).

2.5 THE EFFICIENCY OF POTASSIUM HYDROXIDE (KOH) MOUNTS IN RELATION TO THE CULTURE FOR RAPID DIAGNOSIS OF DERMATOPHYTOSIS

It was necessary to diagnose the infection with some laboratory evidences before treating them with antifungal drugs, whose duration of treatment is long and may have some serious side effects. Markus et al., 2001 found that application of a drop of 10% KOH in vivo for less than 1 minute before imaging allowed for easier
visualization of the hyphae. Pansiti et al., 2006 compared the efficacy of two different KOH-based staining methods and found a slightly higher sensitivity for KOH-chlorazole and a higher specificity for KOH-acridine orange suggesting the use of both techniques in order to improve detection of fungal infection, in onychomycoses. Levitt et al., 2010 determined the sensitivity and specificity of KOH smear and fungal culture for diagnosing of Tinea pedis concluding that KOH smear and fungal culture were complementary diagnostic tests for Tinea pedis, with the former being the more sensitive test of the two, and the latter being more specific. Hassab-El-Naby et al., 2011 designed a study to compare KOH or DMSO preparation with PAS of nail clippings in the diagnosis of onychomycosis and observed that KOH mount has a sensitivity of 75% versus 57.5% for PAS staining revealing KOH test as a first line preliminary tool in diagnosis of onychomycosis. Abdo et al., 2011 compared the standard KOH mount versus mycological culture in the diagnosis of tinea capitis with the aim of identification and isolation of the casual agent. Direct microscopy with KOH mount was found to be positive in 85.7% while mycological culture showed positive results in 60% of patients suggesting that KOH mount has higher sensitivity compared to that of mycological culture. Thus concluding, KOH mount was more sensitive than the mycological culture as it was easy to perform, rapid and gave significantly higher rates of positivity compared to the mycological culture. Bonifaz et al, 2013 compared the percentage of positivity and the degree of correlation of KOH, cultures and calcoflour white for the diagnosis of onychomycosis and found that KOH was positive in 66.67% of the cases, cultures in 33.33% and calcofluor white in 57.58%. Sarika et al., 2013 evaluated the sensitivity of present diagnostic procedures along with the rate of incidence of dermatophytic infections and aetiological agents with associated symptoms. 84% of the KOH positive samples showed positive test for culture, thereby indicating its high sensitivity as diagnostic tool.

The detailed information on diagnosis of dermatophytosis and comparison of the efficacy of common methods employed in mycology laboratory is very valuable in establishing a reliable method for early information on diagnosis of dermatophytosis that may be crucial for determining appropriate therapy for the successful treatment. However, the outcome of the present study may enlighten the importance of rapid microscopic method in early diagnosis of fungal infections.
2.6 QUALITATIVE AND QUANTITATIVE SCREENING OF PROTEASE ENZYMES

After the deposition of viable arthrospores or hyphae on the surface of the susceptible individual, the dermatophytes secrete an arsenal of proteases which digest the keratin network into assimilable oligopeptides or amino acids. The penetration of arthrospores is usually accompanied by dermatophytes excreting sulphite as a reducing agent. In the presence of sulphite, disulphide bounds of the keratin substrate are directly cleaved to cysteine and S-sulphocysteine, and reduced proteins become accessible for further digestion by various endo- and exoproteases secreted by the fungi. Sulphitolysis is likely to be an essential step in the digestion of compact keratinized tissues which precedes the action of all proteases (Tainwala and Sharma, 2011 and Monod, 2008). These enzymes induces inflammatory reactions such as redness (ruber), swelling (induration), heat and alopecia (loss of hair). Inflammation causes the pathogen to move away from the site of infection and take residence at a new site resulting in the classical ringed lesion (Lakshmipathy and Kannabiran, 2010). Since the proteolytic activities of dermatophytes have been a subject of interest to understand the pathogenicity of infection, many studies have reported the screening of these protease in vitro and many efforts have been accomplished to characterize secreted dermatophytic proteases at the molecular level, only punctual insights have been afforded into other aspects of the pathogenesis of dermatophytosis.

Proteolytic activities of dermatophytes have been a subject of interest to understand the pathogenicity of infection. Lee et al., 1987 demonstrated that 21 fungal isolates of dermatophytes and other moulds were able to hydrolyze gelatin but with variable capabilities and observed that T. rubrum was the most active protease producer. Tsuboi et al., 1989 demonstrated kertinolytic proteinase that hydrolyzed a synthetic chymotropsin substrate Suc-Ala-Ala-Pro-Phe respectively. Wawrzkiewicz et al., 1991 showed that the preparation of keratin substrate constituted a convenient model for a preliminary estimation of fungal keratinolytic activity and it can be a source of information about the localization of these enzymes. Muhsin et al., 1997 screened a total of 123 isolates of 14 species of dermatophytes and yeasts were for the activity of five extracellular enzymes including elastase, keratinase, protease (gelatinase), lipase and phospholipase, by using solid media. Muhsin and Aubaid, 2000 purified an exocellular keratinase from
T. mentagrophytes var. erinaci isolated from patients infected with tinea cruris and estimated its molecular weight as 38 kDa by SDS-PAGE. Mahmood et al., 2009 isolated T. mentagrophytes that have ability to produce extracellular protease when grown on 0.2% soy protein liquid media which was indicated by proteolysis activity 111.9 U/ml against casein when added as substrate to the culture filtrates of T. mentagrophytes with specific activity 2238 U/mg. Ferreria-Nozawa et al., 2009 showed that the growth of T. rubrum synthesized and secreted almost the same levels of an alkaline phosphatase with an apparent optimum pH ranging from 9.0 to 10.0 when grown on both low- and high- phosphate medium suggesting that this enzyme was encoded by an alkaline gene, i.e., a gene responsive to ambient pH signaling. Venkatesan et al., 2010 elucidated the keratinase activity profile among the three ecological groups viz. geophilic, zoophilic and anthropophilic of dermatophytes on mineral medium consist of human hair, human nail and chicken feather individually and stated that the enzyme moderation could be an attribute for obligate anthropization in certain dermatophytes.

Sharma et al., 2011 evaluated the in vitro biodegradation of keratin by clinical isolates of dermatophytes and some soil fungi and found that M. gypseum and T. verrucosum were highly degraded (49.34%) the animal hairs. Leng and Wei, 2011 presented the first qualitative proteomics study on secretome (more than 90 proteins) of T. rubrum grown in keratin or elastin containing medium, which to some extent mimic in vivo through some spectroscopic analyses. Gokulshankar et al., 2011 compared the protease activity of different ecological group of dermatophytes in their vegetative and sporulation phase and revealed that all the isolates of anthropophilic dermatophytes like T. rubrum, T. mentagrophytes, T. tonsurans, T. violaceum and E. floccosum recorded reduced protease activity during artificially induced sporulation phase in comparison to their vegetative growth phase. Ademola et al., 2013 elucidated the keratinase activity profile among the 343 clinical isolates of dermatophytes namely, T. mentagrophytes (228), T. rubrum (58), T. schoenleinii (36), M. audouinii (14) and E. floccosum (7) grown on SD broth were inoculated on mineral medium consisting of human hair, horse hair, cow hair, chicken hair and fowl scales individually and observed that keratinolytic activity were significantly increased in feather when compared to other keratin substrates. Singh, 2014 screened qualitatively and quantitatively seven species of Microsporum, four of Trichophyton and one of Epidermophyton for extracellular
keratinase on solid milk agar plates and chicken feather containing liquid medium and observed that the keratinase of *Microsporum* species ranged from 50-300 KU/ml and of *Trichophyton* 100-140 KU/ml while that of *Epidermophyton* was 20 KU/ml.

The proteases of dermatophytes playing an essential role on the pathogenesis of dermatophytes. The previous studies revealing that the virulence of dermatophytes was primarily based on the ability of secretion of these enzymes. However, such enzymes were not exclusively studied in India, therefore, studying the enzymatic activity along with the genome which code for these enzymes in the pathogenic strains of dermatophytes is absolutely essential to determine their biological significance. Thus investigation of the enzymatic activity from the clinical strains and determination of genes, which code the enzyme, are the subject of the present study. Hence this aspect has been addressed in this thesis.

**2.7 GENOTYPIC SEQUENCING OF THE DERMATOPHYTIC STRAINS**

Molecular biology based techniques have solved problems concerning the morphology-based identification of dermatophytes and have improved our knowledge on the epidemiology of dermatophytosis. Further development of molecular diagnosis of dermatophytosis requires the investigation of additional molecular markers for diagnostic tools targeting multiple loci as well as the improvement of techniques (Kanbe, 2008). In contrast, in all but a few cases distinction between dermatophyte strains has failed, which has hindered the development of molecular based epidemiological investigations (Kac, 2000).

Makimura *et al.*, (1999) demonstrated the mutual phylogenetic relationships of dermatophytes of the genera *Trichophyton*, *Microsporum* and *Epidermophyton* were by using ITS1 region ribosomal DNA sequences and also determined the ITS1 sequences of 11 clinical isolates to identify the species. Turenne *et al.*, 1999 insisted that sequence variability of the ITS2 region of fungi was potentially useful in rapid and accurate diagnosis of clinical fungal isolates. Jackson *et al.*, 1999 used RFLPs identified in the rDNA repeat for molecular strain differentiation of the dermatophyte fungus *T. rubrum* by using the universal primers ITS1 and ITS4. Nascimento and Martinez-Rossi, 2001 analyzed the 18S-rDNA gene sequence combined with morphological and biochemical criteria in order to detect genetic
differences between seven *Trichophyton* isolates and estimated their phylogenetic relationships.

Walberg *et al.*, 2006 studied clinical specimens from 346 patients with suspected onychomycosis, analyzed by 18S PCR (detection) followed by sequencing and subsequent database search (identification) in parallel with routine culture on agar. Yang *et al.*, 2008 introduced a nested PCR assay that can be directly applied to clinical specimens and heightened the detection of sensitivity based on the ribosomal DNA (rDNA) gene complex in fungi denoting gene order and the position of the internal transcribed spacer (ITS). Frealle *et al.*, 2007 differentiated the isolates by DNA sequencing of the variable ITS regions flanking the 5.8S rDNA and of the housekeeping gene encoding the manganese containing superoxide dismutase (MnSOD) and analyzed the relationships between ITS/MnSOD sequences and host origin, clinical pattern and phenotypic characteristics. Kong *et al.*, 2008 sequenced the ITS region (ITS1, 5.8S and ITS2) for 42 dermatophytes belonging to seven species and developed a novel padlock probe and rolling-circle amplification based method for identification of single nucleotide polymorphisms (SNPs) that could be exploited to differentiate between *Trichophyton* spp., and observed that there was good agreement between ITS sequencing and the rolling circle amplification (RCA) assay. Li *et al.*, 2008 evaluated the feasibility of using sequencing of the ribosomal ITS1 and ITS2 regions for identification of 17 dermatophytes species and insisted that ITS sequencing provided a very accurate and useful method for the identification of dermatophytes. Mirhendi *et al.*, 2008 studied the use of PCR followed by enzymatic digestion for differentiation of *T. rubrum* and *T. mentagrophytes* through the amplified products of ITS1-5.8SrDNA-ITS2 regions. Mirzahoseini *et al.*, 2009 designed a PCR-RFLP analysis of the PCR-amplified ITS region of rDNA to rapid identification of dermatophytes in clinical specimens and also showed that this technique was a rapid and reliable tool in the identification of major pathogenic dermatophytes. Malinovschi *et al.*, 2009 designed the PCR reactions to use a common reverse primer and 2 specific forward primers to be efficient under the same PCR reactions allowing the detection of two fungi from one reaction volume.

Rezaei-Matehkolaie *et al.*, 2012 amplified the ITS1-5.8S-ITS2 region of rDNA from various reference strains of dermatophyte species using the universal fungal primers ITS1 and ITS4 and digested the PCR followed by Mval-RFLP was a
useful and reliable schema for identification and differentiation of several pathogenic species and could be used for rapid screening of even closely related species of dermatophytes in clinical and epidemiological settings. Bergman et al., 2013 developed and validated a rapid and sensitive real-time PCR method for detection of all known species of dermatophytes, including identification of *T. rubrum* and *T. interdigitale*. Elavarshi et al., 2013 performed a PCR-RFLP using pan fungal primer targeting the ITS region for the identification of dermatophytes species and strains directly from clinical material. Mohammadi et al., 2015 study inter- and intraspecific genomic variations for identification of clinically important dermatophyte species obtained from clinical specimens in Isfahan, Iran using PCR-RFLP. ITS1-5.8S-ITS2 region of rDNA was amplified using universal fungal primers.

In India, there are very few reports pertaining to the genotypic identification of dermatophytes. Bagyalakshmi et al., 2008 developed and optimized PCR based RFLP targeting 18S rDNA and ITS region of fungi for rapid detection and identification of dermatophytes. Garg et al., in 2007 and 2009 evaluated nested PCR using primers targeting dermatophyte specific sequence of CHS1 gene. in skin, hair and nail samples. The review of literature on the genomic sequencing on the dermatophytes revealed a very few reports in India and no reports from Tamil Nadu. This lacuna in the literature has been addressed.

### 2.8 THE DETERMINATION OF METALLOPROTEASE (*MEP1-5*) AND SUBTILISIN (*SUB1-7*) GENES

During the course of infection, the severity of infection requires the release of specific enzymes that facilitate the penetration into the host tissue as these secreted keratinolytic enzymes were potential fungal virulence factors whose molecular characterization would be an important step towards the understanding of dermatophytic infection pathogenesis. Descamps et al., 2002 first isolated the family encoding potential virulence related factors in *M. canis* which secreted a 31.5 kDa keratinolytic subtilisin-like protease and using a probe corresponding to a gene's internal fragment, the entire gene encoding this protease named *SUB3* was cloned from a *M. canis* IEMBL3 genomic library. These results showed that *SUB1*, *SUB2*, and *SUB3* encode a family of subtilisin like proteases and strongly suggested that these proteases were produced by *M. canis* during the invasion of keratinized structures. Descamps et al., 2002 first isolated three genes (*MEP1*, *MEP2*, *MEP3*) encoding metalloprotease-like enzymes in *M. canis*.
MEP2 and MEP3) from *M. canis* genomic library using *A. fumigatus* metalloprotease genomic sequence (MEP) as a probe and successfully expressed an active recombinant enzyme keratinolytic metalloprotease in *Pichia pastoris*. Vermout *et al.*, 2004 purified and tested the recombinant keratinolytic metalloprotease (rMEP3) as a subunit vaccine in experimentally infected guinea pigs in order to identify protective immunogens against *M. canis* infection. Jousson *et al.*, 2004 showed that two subtilisins, Sub3 and Sub4, were detected in culture supernatants and both recombinant enzymes produced in *P. pastoris* were highly active on keratin azure suggesting that these proteases play an important role in invasion of keratinized tissues by the fungus. In an another study, Jousson *et al.*, 2004 isolated a five-member MEP family from genomic libraries of *T. rubrum* and the phylogenetic analysis of genomic and protein clearly indicated that the multiplication of MEP genes in dermatophytes occurred prior to species divergence.

Vermout *et al.*, 2007 first evaluated the utility of RNA mediated silencing as a reverse genetic tool in dermatophytes using target genes namely, SUB3 and DPPIV coding for a subtilisin and a dipeptidyl peptidase and found that inhibition was globally more efficient for SUB3 than for DPPIV concluding that RNA silencing can be used for functional genomics in *M. canis*. Giddey *et al.*, 2007 suggested that a switch of habitat could be related to a differential expression of genes encoding homologous secreted proteins as they investigated the possibility that closely related dermatophyte species with different behaviours secrete distinct proteins when grown in the same culture medium. Baldo *et al.*, 2008 developed a model to study the adherence of *M. canis* to feline corneocytes through the use of a reconstructed interfollicular feline epidermis (RFE) and observed that chymostatin and two mAbs inhibited *M. canis* adherence to RFE suggesting that subtilisins and Sub3 in particular were involved in the adherence process. Moallaei *et al.*, 2009 determined the proteases activity of *T. vanbreuseghemii* soil and clinical isolates of *Microsporum* and characterized their genome and observed the intra-strain differences in production of serine proteinases. Staib *et al.*, 2010 revealed 23 distinct protease genes expressed in vivo protease gene in the fungal cells, which was surprisingly different from the pattern elicited during in vitro growth on keratin by the use of a cDNA microarray. Lemsdekk, 2010 assayed for the presence of fungalysin and subtilisin proteases by PCR amplification and observed a higher incidence in anthropophilic species when compared to geophilic and zoophilic
species. Preutt et al., 2010 performed qRT-PCR to analyze transcript expression in 7 SUBs and 5 MEPs and examined genes that account for differences in host selection and observed that initial expression of SUB7 and MEP2 was higher in T. tonsurans when compared to T. equinum suggested that SUBs 1, 5, 6, 7 and MEPs 2, 3 may be contribute to host selection.

Understanding the pathophysiological mechanisms underlying an infection is the rational basis for the development of therapeutic and prophylactic strategies. In the case of dermatophytosis, the almost exclusive localization of the causative agents in keratinized tissues and their ability to secrete keratinolytic activity in vitro have focused research primarily on fungal secreted proteases. Consequently, as there were no reports has been evidenced in studying the SUB and MEP genes encoding proteases, an endeavor has been made in this study to tackle the lacuna in the literature.

2.9 BIOINFORMATIC ANALYSIS

Sequencing genomes of different pathogenic fungi produced plethora of genetic information. This "omics" data might be of great interest to probe strain diversity, identify virulence factors and complementary genes in other fungal species and importantly in predicting the role of proteins specific to pathogenesis in humans. The recent availability of genome sequence information and improved genetic manipulation have enabled researchers to begin to identify and study the role of virulence factors of dermatophytes. Ranganathan and Garg, 2009 also stated that identifying secretory proteins involved in pathogen infection will lead to the discovery of potential drug targets and biomarkers for diagnostic applications. Choi et al., 2010 constructed Fungal Secretome Database (FSD) for the prediction of secretory proteins and insisted that FSD could serve as an integrated platform supporting researches on secretory proteins in the fungal kingdom. Gudimella et al., 2010 proposed a component called "fungome" for those fungal proteins implicated in pathogenesis which will allow researchers to improve the annotation of fungal proteins. Priebe et al., 2011 determined a software tool named Fungifun which assigns functional annotations to fungal genes or proteins and also performs gene set enrichment analysis including the FunCat categorization. Lum and Min, 2011 annotated the Fungal Secretome Knowledgebase (FunSecKB) which provides a resource of secreted fungal proteins, i.e. secretomes, identified from all available fungal protein data in the NCBI RefSeq database. The secreted proteins were
identified using a well evaluated computational protocol which includes SignalP, WolfPsort and Phobius for signal peptide or subcellular location prediction, TMHMM for identifying membrane proteins and PS-Scan for identifying endoplasmic reticulum (ER) target proteins, thus insisting FunSecKB will be a valuable resource for exploring the potential applications of fungal secreted proteins. Achterman and White, 2012 summarized the current understanding of dermatophyte virulence factors and discussed future directions for identifying and testing virulence factors.

2.10 OBJECTIVES FRAMED TO ADDRESS THE LACUNA IN THE LITERATURE

After a thorough persual of the literature pertaining to dermatophytosis, an attempt has been made in this thesis to study certain aspects of dermatophytosis which are of considerable diagnostic and therapeutic significance that have lacuna hitherto not been adequately investigated. Initially, the prevalence of different clinical pattern, aetiological agents and predisposing factor of dermatophytosis seen at Salem zonal hospitals, Tamil Nadu was determined. The efficacy of potassium hydroxide (KOH) mounts in relation to the culture for the rapid diagnosis of dermatophytosis was studied. The qualitative and quantitative estimation of proteases on media containing different protein substrate viz., casein, gelatin-peptone and keratin media was investigated. The important aspect of this thesis was 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. A unique aspect of this thesis was the prediction of protein structures to assess the function through bioinformatic tools and the identification of novel protease inhibitors against metallo and serine proteases through virtual screening of drug molecules.