CHAPTER VI

DISCUSSION

A total of 138 specimens (129 skin scrapings and 9 nail clippings) of clinically suspected dermatophytosis, fresh cases who had not been administered antifungals were selected from the Dermatology Outpatient Department of a tertiary care hospital, Sri Ramachandra Medical Center, Porur, Chennai, India, during the study period between January and December 2010.

Distribution of gender

In the present study, dermatophytosis was more common among female (63.04%) than male (36.95%) population which was similar to the earlier epidemiological studies with female predominance in Europe and America (Simonnet et al., 2011; Costa-Orlandi et al., 2012). The increase in the number of females may be due to the decrease in personal inhibitions, breaking the trend of social restrictions and being more conscious of their well being.

On the contrary, based on the earlier epidemiological surveys on dermatophytosis in India, documented that the dermatophytosis were common among men in South India with M:F ratio 2.7:1 and 1.6:1 respectively (Doddamani et al., 2013; Mahale et al., 2014). Similarly, from North India, reported that the dermatophytic infections were common among men with M:F ratio 63:11 and 4:1 respectively (Bhatia and Sharma, 2014; Bhagra et al., 2014).

Gelotar et al., 2013 documented that the fingernail infections were dominant among female (80%) than male (20%) whereas, the toenail onychomycosis was more common among male with M:F ratio 6.7:1 (Yadav et al., 2015).
Bose et al., 2011 reported that the scalp infections was more common among male than female population with M:F ratio 2.8:1. Oke et al., 2014 reported that the males (40.6%) were frequently exposed to scalp infections than females (29.1%) and similarly males (63.7%) were dominant among school children in chief town of the Adamawa region of Cameroon (Kechia et al., 2014). The greater prevalence of females was reported from scalp infections with M:F ratio of 1:1.4 among pediatric tinea capitis in India (Grover et al., 2012) and similarly Jain et al., 2014 documented that greater prevalence of tinea infections was found to be among males, except tinea capitis where females preponderance was found, while on contrary, Jha and Murthy, 2013 recorded a male preponderance.

**Distribution of age**

In our study, majority of the patients were between 31-40 years age group (32.6%), followed by 41-50 years age group (23.91%). On the contrary, Vyas et al., 2013 reported that 75% (45/60) of dermatophytic infections were from 5-10 years age group and 50% (30/60) of the dermatophytes were isolated from tinea capitis clinical type. Malik et al., 2014 and Bhagra et al., 2014 from North India reported that the dermatophytic infections were common among 11-20 years (35.1%) and 21-30 years age group (28%) respectively. Mahale et al., 2014 from South India reported that both the 1-10 years and 41-50 years age group each with 23.1% have been reported to have infection with dermatophytes. Gupta et al., 2014 reported that the older age groups more than 60 years (32%) were commonly infected with dermatophytes.

Although, all age groups are vulnerable to acquire dermatophytic infections, among the earlier epidemiological studies from India, the 21-30 years age group was more commonly infected with the etiological agents of dermatophytosis (Lyngdoh et
al., 2013; Bhagra et al., 2014; Jain et al., 2014) and onychomycosis (tinea unguium) (Reddy et al., 2013; Lone et al., 2013).

Socio-economic category

The patients were grouped based on the socio-economic category - very low, low and middle economic class. In the present study, 13.76% belonged to the very low class, 62.31% of low class and 23.91% of middle socio-economic category. Poor hygienic conditions, overpopulation, high physical activities with excessive sweating, irregular bathing, sharing clothes and hair accessories and using air-tight foot wear were the major risk factors of dermatophytosis in these groups.

Madhavi et al., 2011 documented that the lower economic group were highly susceptible to dermatophytosis, as they were unaware of these fungal skin infections, single or multiple lesions develop and delayed treatment led to the development of chronicity. Sajjan and Mangalgi, 2012 recorded that the low economic group were more in number causing tinea capitis clinical type among children and similarly, Lyngdoh et al., 2013 reported that the majority of the patients belonged to the low economic class. Sahai and Mishra, 2011 reported that 90% of the patients were from sub-urban background.

On the contrary, Jha and Murthy, 2013 reported that the lower class from urban region were commonly affected with dermatophytic infections while the labor groups from rural region were less comparatively with 58.9% and 41.1 % respectively. Since urban people are highly conscious of their health, they frequently visit the clinics for treatment, whereas the rural people try self-mediation and intake inappropriate antifungals was reported from rural regions (Jha and Murthy, 2013).
Similarly, Singla et al., 2013 reported that the urban areas (53%) were more susceptible to dermatophytic infections.

**Clinical patterns of dermatophytosis**

Majority of the patients were reported with dermatophytic skin infections (93.47%) in our study, but very few with fungal nail infections (0.06%). Tinea corporis (57.97%) was the commonest clinical type in the present study. Similarly, Jain et al., 2014 reported that tinea corporis (35.2%) was the commonest clinical type, followed by tinea cruris (22.4%), tinea capitis (11.2%), tinea mannum (7.1%) tinea pedis and tinea unguium each with 6.6% prevalence rate. The present study report was in accordance with the earlier epidemiological studies from South India (Balakumar et al., 2012; Jha and Murthy, 2013) and North India (Malik et al., 2014; Bhatia and Sharma, 2014). In our study the frequency of tinea corporis was found to be greater in females than male population. In India, tinea corporis is the most significant clinical type of dermatophytosis, whereas the prevalence was comparatively low in America and Africa with 0.07% and 0.6% respectively (López Martínez et al., 2010; Oke et al., 2014).

Tinea cruris (24.63%) was the predominant clinical type next to tinea corporis in the present study. Similarly, several workers had documented that the tinea cruris was the second most predominant clinical type in India (Komathi et al., 2010; Hanumanthappa et al., 2012; Bhagra et al., 2014; Surendran et al., 2014; Jain et al., 2014). On the contrary, one study from Patiala, reported that tinea cruris was the dominant clinical form with 40% prevalence rate (Singla et al., 2013). The frequency of tinea cruris was found to be low in females; which was in accordance with the earlier study (Doddamani et al., 2013).
The other clinical patterns of dermatophytosis such as tinea unguium (6.52%), tinea faciei (2.17%) and tinea pedis (0.72%) were less frequently reported in the present study. On the contrary, tinea pedis (26.6%) was found to be frequently reported in Meghalaya, East India (Lyngdoh et al., 2013). Similarly, one report from Hyderabad documented that the tinea pedis (17%) was found to be the second most frequently observed clinical pattern of dermatophytosis (Madhavi et al., 2011). The prevalence of tinea unguium was found to be predominant in North India (Gupta et al., 2014) and South India (Reddy et al., 2013) with prevalence rate of 52% and 78.35% respectively.

Tinea unguium (14.4%) was the most significant clinical type among Europeans (Drakensjö and Chryssanthou, 2011). This may be due to the fact that the people use communal swimming pools, extended sports activities with occlusive footwear. Other risk factors included were the high prevalence of diabetics and vascular disorders (Vena et al., 2012). Among the dermatophytic nail infections, toenails were more commonly affected than the fingernails (Simonnet et al., 2011) and particularly, the distal and lateral subungual onychomycosis (94%) was frequently reported from toenail infections (Yadav et al., 2015). The incidence of tinea unguium and tinea pedis is increased remarkably in Italy (Vena et al., 2012). Vasconcellos et al., 2013 estimated that the onychomycosis was often restricted to the elderly group than the young age groups.

In the present study, few individuals had mixed infections of tinea corporis with cruris (7.97%) clinical type, which was similar to the earlier epidemiological study (Vyas et al., 2013; Mistry et al., 2014; Jain et al., 2014; Madhavi et al., 2011; Surendran et al., 2014). Tinea pedis associated with tinea unguium was frequently
reported from Japan, particularly in summer season (Sei, 2012) and Turkey (Akçaglar et al., 2011). Vena et al., 2012 reported that gender predominance was observed among women there was a combination of tinea unguium with corporis and for men it was tinea pedis with cruris. Few workers documented that there was few more combinations such as tinea mannum with unguium (Mistry et al., 2014), tinea corporis with mannum (Jain et al., 2014; Surendran et al., 2014), tinea corporis with capitis (Madhavi et al., 2011) and tinea unguium with pedis and mannum (Bhagra et al., 2014).

Interestingly, Surendran et al., 2014 documented a variety of new combinations of clinical patterns such as tinea corporis with cruris and mannum (4.34%), tinea corporis with barbae (4.34%), tinea corporis with faciei (2.17%), tinea corporis with pedis (2.17%), tinea corporis with cruris and unguium (2.17%), tinea cruris with barbae (2.17%), tinea unguium with mannum (2.17%), tinea cruris and unguium (2.17%). The principal cause of these combinations may be due to poor hygienic conditions and the infection was transmitted from one region to another, particularly through direct contact with the skin lesions.

In the present study, 3 patients (2.17%) were infected with dermatophyte species on their face. Jha and Murthy, 2013 reported tinea mannum, faciei and barbae with 51 (5.5%), 32 (3.4%), 21 (2.2%) patients infected with dermatophytes respectively.

Lyngdoh et al., 2013 reported the presence of tinea incognito (6.2%) in Meghalaya, East India, which is an infection produced as a result of inappropriate treatment with topical corticosteroids. Surekha et al., 2015 reported tinea imbricata, which is a rare form of tinea corporis observed in chronic cases with concentric rings
dispersed throughout the body. Bhatia and Sharma, 2014 reported tinea gladiatorum which is an infection of athletes. These tinea infections are not found in the present study.

The scalp infections were not seen in the present study, as the incidence of tinea capitis is found to be low in India as compared to Africa (Oke et al., 2014). The low frequency of scalp infections may be due to the fact that the Indians frequently use hair oils, most importantly mustard oils, which play a major role against dermatophytic infections in-vitro (Mistry et al., 2014). However, one report from Jaipur, North India documented that tinea capitis was the predominant clinical type with 50% prevalence rate (Vyas et al., 2013). Few workers reported that tinea capitis was the second most dominant clinical type from North India (Sahai and Mishra, 2011; Pandey and Pandey, 2013) and South India (Jha and Murthy, 2013).

Tinea capitis was not observed among adults and it was often restricted among the prepubertal children (83%) (Simonnet et al., 2011). Tinea capitis was observed frequently among children below 10 years (94.1%) (Hanumanthappa et al., 2012) and 15 years age group (72.7%) (Lyngdoh et al., 2013), whereas in Northern California, the incidence of tinea capitis has decreased over the years from 1998 to 2007 among children below 15 years age group (Mirmirani and Tucker, 2013).

Tinea capitis (26.9%) was the most significant clinical pattern in Africa (Oke et al., 2014). The scalp lesions were varied from typical alopecia to postulation in chronic infections, inflammatory to non-inflammatory type and few patients reported with numerous small plaques of alopecia like “trichophytic” type (64.2%) and large plaques of alopecia like “microscopic” type (34.6%) and few reported with non-specific lesions (6.2%) (Kechia et al., 2014). The major predisposing risk factors of
scalp infections were documented by Ayanlowo et al., 2014 they are sharing hair accessories, carrying objects on the head and overcrowding. The prevalence of tinea capitis (22.3%) was the most significant clinical type observed in Saudi Arabia (Khaled et al., 2015).

There was a seasonal predominance of clinical patterns, where tinea corporis and capitis were observed in winter, while tinea unguium and mannum in summer and autumn respectively. The frequency rate of tinea infections decrease in summer (27.9%), winter (27.3%), autumn (26.2%) and spring (18.6%) (Sepahvand et al., 2009).

Microscopy and culture

In the present study, among 138 clinical specimens, direct microscopy and culture was positive for dermatophytosis in 48.55% (67/138) and 62.31% (86/138) respectively. Most of the epidemiological studies from India, observed that the direct microscopy showed high positivity rate than culture. The positivity rate of direct microscopy ranged between 38% and 96%, while it was as low as 38.2% from Meghalaya, East India (Lyngdoh et al., 2013) and as high as 96% from Mysore, South India (Surendran et al., 2014). Several workers reported that the high positivity rate of direct microscopy ranged between 61.2% and 91.34%.

Among the epidemiological studies with high positivity rate of direct microscopy, the culture positivity rate ranged between 29% and 87%, with a low of 29.3 % (Lyngdoh et al., 2013) and a high of 87.43% (Ghosh et al., 2014). The reason for low positivity rate in culture may be due to prior treatment with antifungal drugs
(Mahale et al., 2014). Hence, selection criteria of patients and proper and adequate quantity of specimen should be collected while sampling (Bhagra et al., 2014).

Madhavi et al., 2011 documented high culture positivity rate of 58% more than direct microscopy (43%). Surprisingly, the present study report was similar to very few earlier epidemiological studies with high culture positivity rate from South India (Madhavi et al., 2011; Balakumar et al., 2012) and North India (Jain et al., 2014). The reason for low positivity rate in direct microscopy may be probably due to severe inflammatory reaction which blinds the fungal elements (Bhagra et al., 2014). Eventually, the importance of culture method plays a significant role in the laboratory diagnosis of infections.

**Etiological agents of dermatophytosis**

In the present study, among 129 skin scrapings, 81 were culture positive, among them *T. rubrum* (43) was the most frequently isolated dermatophyte species, followed by *T. mentagrophytes* (23), *E. floccosum* (9) and *Candida* species (6). Among nine nail clippings 5 were positive on culture, among the dermatophytes, *T. rubrum* (1) and among non-dermatophytes *Candida* species (2) and *Fusarium* species (2) were isolated.

Tinea corporis followed by tinea cruris were the predominant clinical type documented in our study. Majority of patients were infected with the anthropophiles *T. rubrum*, followed by *T. mentagrophytes*. Similarly, Mistry et al., 2014 reported that *T. rubrum* (53.9%) followed by *T. mentagrophytes* (27.9%) were the commonest dermatophyte species isolated in Rajkot. While, Bhatia and Sharma, 2014 reported vice versa, that *T. mentagrophytes* (63.5%) were predominant and *T. rubrum* (35.1%)
was the second most common species in Himachal Pradesh. *T. rubrum* (50%) and *T. mentagrophytes* (47.36%) were the commonest species isolated from scalp infections (Sajjan and Mangalgi, 2012; Bose *et al*., 2011). The present study report is in accordance with the earlier epidemiological studies from South India (Doddamani *et al*., 2012; Surendran *et al*., 2014) and North India (Singla *et al*., 2013; Prasad *et al*., 2013; Malik *et al*., 2014). Several workers documented that these two dermatophyte species were frequently isolated from all tinea infections in India.

Infections with *E. floccosum* (11.11%) were not so common in our study and it was observed to be high compared to the earlier epidemiological studies. The prevalence of *E. floccosum* ranged between 1% and 8%, while it was as low as 1.47% (Bhagra *et al*., 2014) and as high as 8.33% (Doddamani *et al*., 2013). On the contrary, *E. floccosum* was the most frequently isolated dermatophyte species in Iran (Sepahvand *et al*., 2009; Bassiri-Jahromi and Khaksari, 2009).

Though *T. tonsurans* was not reported in the present study, the prevalence is comparatively high in few parts of India and America. Jha and Murthy, 2013 reported that *T. tonsurans* (22.3%) was isolated from 206 patients and this dermatophyte was the second most predominant species in Mysore, South India. Similarly, Mirmirani and Tucker, 2013 reported that *T. tonsurans* (91.8%) was frequently isolated species from scalp infections in Northern California. *T. tonsurans* is an anthropophilic dermatophyte species frequently isolated from tinea capitis and corporis Jha and Murthy, 2013. Several workers documented the distribution of *T. tonsurans* to be about 1-2% from tinea faciei, mannum, pedis and unguium (Lyngdoh *et al*., 2013; Gupta *et al*., 2014).
Though few *Trichophyton* species such as *T. violaceum*, *T. verrucosum*, *T. soudanense* and *T. schoenleinii* were not isolated in our study, these dermatophytes were isolated in very few geographical regions in India with high prevalence. *T. violaceum* (88.6%) was the most predominant dermatophyte species frequently isolated from scalp infections in India (Grover *et al.*, 2010). *T. violaceum* was frequently isolated from tinea capitis and corporis and similarly, few workers reported that *T. violaceum* was found to cause infection on face, beard, nails, groin and feet. Similarly, Marwa *et al.*, 2011 reported that the prevalence of *T. violaceum* (40.3%) was found to be high among pediatric tinea capitis in Ismailia, Egypt.

*T. soudanense* was the commonest dermatophyte species frequently isolated from scalp infections in Australia, Europe and Africa (McPherson *et al.*, 2009; Vena *et al.*, 2012; Kchia *et al.*, 2014), while it was less frequently isolated from scalp and nail infections in Kolkata, East India (Ghosh *et al.*, 2014). *T. verrucosum* was the most predominant dermatophyte species frequently isolated from onychomycosis (Ghosh *et al.*, 2014) and scalp infections (Mahale *et al.*, 2014), while very few workers reported that this dermatophyte was less frequently isolated from skin infections. Several workers reported that *T. schoenleinii* was found to be low in India (Prasad *et al.*, 2013; Jain *et al.*, 2014), which was in accordance with the earlier epidemiological study from France (Simonnet *et al.*, 2011).

*Microsporum* species was not documented in the present study and moreover they were less frequently isolated in India (Hanumanthappa *et al.*, 2012; Balakumar *et al.*, 2012; Prasad *et al.*, 2013). Few rare *Microsporum* species were isolated such as *M. nanum* and *M. cookie* (Vyas *et al.*, 2013; Mistry *et al.*, 2014). *M. ferrugineum* is an anthropophilic non-endemic pathogen in India, which was isolated from 13 cases
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(9.75%) in Lucknow (Sahai and Mishra, 2011). On the contrary, the prevalence of *Microsporum* species is high in Africa, where Oke *et al.*, 2014 reported that the *M. audouinii* (28%) was the most predominant dermatophyte species isolated among children causing scalp infections in South-Western Nigeria. Similarly, *M. canis* (30.8%) and *M. gypseum* (17%) were repeatedly isolated dermatophytes species next to *T. violaceum* from scalp infections (Marwa *et al.*, 2011).

A variety of rare dermatophyte species such as *T. concentricum* (Tinea corporis and cruris), *T. megninii* (tinea corporis and cruris), *M. fulvum* (tinea corporis, capitis and barbæ), *T. terrestre* (tinea corporis, mannum and capitis), *T. equinum*, *T. simii* (tinea corporis and pedis) and *M. praecox* (Tinea corporis and capitis) were isolated from North India (Grover *et al.*, 2010; Pandey and Pandey, 2013; Ghosh *et al.*, 2014; Jain *et al.*, 2014). Simonnet *et al.*, 2011 from Europe reported that the *M. pracex* and *M. langeronii* were rarely isolated from skin and scalp infections respectively, whereas the latter was isolated from a patient who came back after his holidays from sub-Saharan Africa. Similarly, *M. fulvum* was isolated from skin lesions and *T. terrestre* from foot and nail mycosis (Drakensjö and Chryssanthou, 2011; Vena *et al.*, 2012).

In the present study, among the non-dermatophytes, *Candida* (5.79%) species was isolated from tinea corporis and unguium and *Fusarium* (1.44%) was isolated from nail infections. In general, onychomycosis is a common fungal infection, which accounts for up to 50% of the fingernail and toenail infections (Faergemann and Baran, 2003). Weinberg *et al.*, 2003 reported that the non-dermatophytes constituted approximately 10% of the causative agents of onychomycosis, whereas El Batawi *et
al., 2007 reported about 68.75% were non-dermatophytes and 0.1% was dermatophytes that caused onychomycosis.

*Candida* and *Aspergillus* species were frequently isolated from trunk, groin, scalp and nail infections (Vyas et al., 2013; Jain et al., 2014). Few workers reported a variety of non-dermatophytes isolated from onychomycosis such as *Fusarium* species (5 cases), *Penicillium* species (2 cases), *Scopulariopsis* species (3 cases), *Helminthosporium* species (11 cases), *Trichosporon* species (1 case) (Vyas et al., 2013; Mahale et al., 2014; Malik et al., 2014). *Paecilomyces* species (0.6%) was isolated from groin and *Curvularia* species (2.5%) was isolated from trunk, groin and nail infections (Jain et al., 2014). *Phialophora* species (12.5%) was isolated from dermatophytic infections and *Alternaria* species (6.25%) was isolated from nails and scalp infections (Malik et al., 2014).

**Molecular identification of dermatophytosis**

In the present study, we compared two PCR based typing methods for the direct identification of dermatophytes from skin and nail specimens. Among the 66 skin scrapings and 3 nail clippings, the pan fungal primer which targeted the ITS region, amplified all the fungal DNA, including both the dermatophytes and non-dermatophytes. Hence, the application of the pan fungal primer on the direct clinical specimens may not be specific in identification of dermatophytes. The dermatophyte specific primer which targeted the 18S rDNA region specifically amplified the dermatophyte DNA and it did not amplify the other fungi. This shows that the dermatophyte specific primer is better and helps in accurately identifying them directly from clinical specimens.
In the present study, when compared with the culture for 66 skin scrapings, the sensitivity of the smear was 75%. The diagnostic sensitivity of PCR using pan fungal primer and dermatophyte specific primer was 100% and 95.45% respectively. The PCR using dermatophyte specific primer identified only the dermatophytes and not the non-dermatophytes, whereas PCR using pan fungal primer identified both dermatophytes and non-dermatophytes. In our study, the diagnostic specificity of PCR was 77.27%. The diagnostic accuracy using pan fungal primer and dermatophyte specific primer was 92.42% and 89.39% respectively. Furthermore, the dermatophytes that were positive on culture were also positive by PCR targeting the dermatophyte specific primer and pan fungal primer and were highly reproducible. It can be concluded that PCR detected all the true positives with a 100% correlation.

Among the 47 PCR positive cases using dermatophyte specific primer, five cases were positive by PCR alone, and these patients responded well to the antifungal treatment. The reason for the culture negativity in the PCR positive cases could be due to inadequate fungal load in the specimen and therefore, there was no growth in culture.

Therefore, molecular techniques can be used as an epidemiological tool for the detection of dermatophytes. In the identification of dermatophytes, molecular methods have an edge over the conventional procedures, which are slow to grow and lack enough sensitivity and specificity (Garg et al., 2009). They may not detect all the true positives. The standard laboratory identification can easily identify dermatophytes up to the genus level, but for the identification of the species and strains, it is difficult as these fungi show variations and do not follow the typical characteristic colony morphology. Therefore, an accurate identification of der-
matophytes at the species or the strain level can be done best by using molecular methods for the epidemiological surveys. Moreover, genotypic methods are dependent on the genetic makeup which would be more accurate than the conventional confirmations.

Genotypic methods such as arbitrarily primed PCR (AP-PCR) (Liu et al., 1996), random amplified polymorphic DNA (RAPD) (Howell et al., 1999; Baeza and Giannini, 2004), repetitive sequence PCR (rep-PCR) (Pounder et al., 2005), restriction analysis of the mitochondrial DNA (Mochizuki et al., 1990; Kawasaki et al., 1992), semi-nested PCR (Yang et al., 2008), nested PCR (Garg et al., 2007), multiplex PCR (Kim et al., 2011) and single-strand conformation polymorphism (SSCP) analysis (Cafarchia et al., 2009), are the available techniques for the identification of dermatophytes. However, few methods have reported a low sensitivity and specificity in the identification of the dermatophytes species.

Recently, a number of genetic advances in dermatophytes have been reported, which include - targeted gene inactivation, gene silencing and transcriptional profiling methods (Grumbt et al., 2011). Whole genome sequencing (Martinez et al., 2012) was also developed to study the future outbreaks on the biology, virulence, pathogenicity and the host specificity of the clinically important dermatophytes. Molecular typing is essential for the identification of the fungal isolates upto the genus, species and the strain levels for epidemiological purposes.

**RFLP analysis**

When the RFLP pattern was studied using Mva I, Dde I and Hae III restriction enzymes it was found that using the Mva I (Jackson et al., 1999; Shin et al., 2003) and the Dde I (Shin et al., 2003) restriction enzymes produced unique band profiles.
consistently and reproducible for differentiating species of dermatophytes, but Hae III restriction enzyme was not suitable for the identification of the dermatophyte species as they produced similar band patterns. The application of the Mva I and Dde I enzymes by using the ITS amplicons helped in the easy identification of the dermatophyte species. Whereas in case of the dermatophyte specific primer using the Mva I and Hae III restriction enzymes, showed an identical band length consistently with different dermatophyte species and with the Dde I enzyme, it showed no recognition site on the dermatophytes tested and hence all three cannot be used for the speciation of dermatophytes.

Moreover, by using the pan fungal primer and the dermatophyte specific primer with these three restriction enzymes, it was not possible to detect any strain variations among the dermatophytes. This shows that the recognition site for the identification of strain variations was not located in the ITS and 18S rDNA region. As described in earlier studies, the strain variations can be identified by targeting the ribosomal DNA of the Non-Transcribed Spacer (NTS) region (Mochizuki et al., 2003).

**DNA sequencing**

Among 47 *T. rubrum* isolates, DNA sequencing confirmed ten isolates as *T. rubrum* var. *raubitschekii*, which were identified phenotypically as urease positive (unlike *T. rubrum* which is urease negative). Since *T. rubrum* var. *raubitschekii* possess minor variations in morphological and physiological features, it is now considered as a synonym of *T. rubrum*.

*T. rubrum* var. *raubitschekii* was initially found in indigenous parts of Africa, Asia, and South America. First report of ten cases of *T. rubrum* var. *raubitschekii* was
identified from South India. Even though the organism is now synonymized as *T. rubrum*, isolation of *T. rubrum* var. *raubitschekii* from clinical specimens was not reported from India. A brief clinical review of isolation of *T. rubrum* var. *raubitschekii* reported until date is listed in Table 7.

*T. rubrum* complex is the most common causative agent of dermatophytosis. The most predominant species of the complex is *T. rubrum* worldwide. Gräser et al., 2000 compared the conidial morphology and physiologic features of 15 species and varieties (*T. circonvolutum*, *T. fischeri*, *T. fluviomuniense*, *T. glabrum*, *T. gourvilii*, *T. kanei*, *T. kuryangei*, *T. megninii*, *T. pedis*, *T. raubitschekii*, *T. rodhaini*, *T. rubrum* var. *nigricans*, *T. soundanense*, *T. violaceum* var. *indicum* and *T. yaoundei*) with the DNA sequence targeting the ITS region of rRNA, PCR fingerprinting and amplified fragment length polymorphism (AFLP) assay (Gräser et al., 2000). Eventually they were reclassified or reported as a synonym of *T. rubrum* or *T. violaceum* (Gräser et al., 2000).

In the past, *T. raubitschekii* was identified as a distinct species from the more common *T. rubrum* and *T. mentagrophytes* and the fungus is significantly associated with tinea corporis (Kane et al., 1990). In our study, we isolated the fungus from patients having tinea corporis and tinea cruris. The reddish pigment on the reverse of the colony and negative for in-vitro hair perforation test are highly suggestive of *T. rubrum*, while granular appearance, rounded microconidia, urease positive are suggestive of *T. mentagrophytes*. 
Table 7 Review on clinical literature of *Trichophyton rubrum* var. *raubitschekii*

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<td>Costa <em>et al.</em>, 2003</td>
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<td>Brazil</td>
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<td>Tietz <em>et al.</em>, 2002</td>
<td>4</td>
<td>Germany</td>
<td>Ghana (1), Cameroon (3)</td>
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<td>van Gelderen de Komaid and Borges de Kestelman, 2001</td>
<td>2</td>
<td>Argentina</td>
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<td>Taplin, 2001</td>
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<td>Vietnam</td>
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<td>Lacaz <em>et al.</em>, 1999</td>
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<td>Caiuby <em>et al.</em>, 1996</td>
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<td>Asia (23), Southern Europe (7), Northeast Europe &amp; North America (8)</td>
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<td>Kane <em>et al.</em>, 1990</td>
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*Animal dermatophytosis, isolated from a dog.*
In the present study *T. rubrum* var. *raubitschekii* possessed minor differences in laboratory features like urease positive, produced rounded microconidia a feature uncommon in *T. rubrum*. As described by Ishizaki *et al.*, 1993 using restriction fragment length polymorphism (RFLP), *T. rubrum* var. *raubitschekii* produced the same pattern as *T. rubrum* suggesting conspecificity with *T. rubrum*. Our study results correlated with Gräser *et al.*, 2000 showing that the gene sequence of the clinical strain of *T. rubrum* var. *raubitschekii* shared sequence similarities of ITS region of rRNA with the ATCC strain of *T. rubrum* (Gräser *et al.*, 2000).

Among 25 *T. mentagrophytes*, 3 were identified as *T. mentagrophytes* var. *interdigitale* by DNA sequencing. The previous report on *T. interdigitale* from India was made in 1996 (Ranganathan *et al.*, 1996) and the present report is the second one from India. Recently, Yadav *et al.*, 2015 reported that the *T. interdigitale* was found to be the most predominant dermatophyte species isolated from toenail mycoses.

*Trichophyton mentagrophytes* complex are worldly accepted to be one of the most common agent of dermatophytosis, next to *Trichophyton rubrum*. *T. rubrum*, *T. interdigitale* and *E. floccosum* are the important causative agents of tinea pedis (Singh and Srivastava, 1994). *T. interdigitale* produce numerous sub-spherical or pyriform microconidia, occasionally spiral hyphae and multi-septate macroconidia present in few isolates. *T. mentagrophytes* var. *mentagrophytes*, a zoophilic dermatophyte produce abundant single-celled spherical or subspherical microconidia in clusters, multi-celled macroconidia and occasionally spiral hyphae would be present. Therefore, these strains exhibit similar kind of colony morphology and make the identification difficult by phenotypic methods in standard laboratory diagnostics.
Genotypic methods help to overcome these problems in identification of these strains for epidemiological research. As described by Jackson et al., 1999 T. interdigitale showed identical band pattern of T. mentagrophytes with ITS amplified product using Mva I restriction enzyme in the present study. PCR targeting the ITS region using Mva I restriction enzyme was not helpful in discrimination of these strains. Mochizuki et al., 1996 used 29 isolates of T. mentagrophytes var. interdigitale and performed restriction enzyme analysis of mitrochondrial DNA. The band pattern obtained showed identical to A. vanbreuseghemii and therefore, discussed T. mentagrophytes var. interdigitale were phylogenetically close to A. vanbreuseghemii. Mochizuki et al., 2003 described that the RFLP profile targeting the NTS region was helpful in differentiation of T. mentagrophytes var. mentagrophytes strains than among T. mentagrophytes var. interdigitale strains.

Epidemiological data showed that overall the isolation of T. interdigitale is uncommon in India. Our isolation of T. interdigitale will help in bringing more awareness in looking for T. interdigitale using molecular techniques for epidemiological survey

**Molecular strain typing**

In our study, we identified strain variants using a simple repetitive oligonucleotide (GACA)$_4$ primer, which was found to be useful in the rapid identification of dermatophyte isolates and T. mentagrophytes strain variants. We successfully amplified all the clinical isolates and the band pattern obtained gave a classic picture among dermatophyte species.

A well-marked similarity was recognized between the band profiles of T. rubrum and T. rubrum var. raubitschekii isolates in the present study. The latter could
not be differentiated from \textit{T. rubrum} strains, which are being reported for the first time from south India. Since these two species are closely related, it must be considered under \textit{T. rubrum} complex (Gräser \textit{et al.}, 2000). Species-specific band pattern was observed among the \textit{E. floccosum} isolates. However, no intra-specific variation was recognized among the \textit{T. rubrum} and \textit{E. floccosum} isolates. This shows that the band profiles obtained for \textit{T. rubrum} and \textit{E. floccosum} isolates are in accordance with the earlier study (Faggi \textit{et al.}, 2001).

The \textit{T. mentagrophytes} isolates produced three distinct band profiles, of which the second and third band profiles differed from the first profile by single band length approximately at 800 and 650 bp respectively. The band patterns of \textit{T. mentagrophytes} isolates consisted of three or four bands, which are surprisingly different from the earlier studies, producing complex band profiles of the \textit{T. mentagrophytes} variants (Faggi \textit{et al.}, 2001; Shehata \textit{et al.}, 2008).

In general, dermatophytes easily lose their species specific morphological features in culture. Recently, few methods like real-time PCR and MALDI-TOF mass spectrometry have been optimized for the rapid identification of the dermatophyte species (Yüksel and Ilkit, 2012; Nenoff \textit{et al.}, 2013). The advantage of these methods are comparatively more than the phenotype identifications, however, the main disadvantage is the higher cost and may not be affordable for the routine laboratory settings.

The identification of the genotype variations using the short (GACA)$_4$ primer gives a classic discrimination between the \textit{T. rubrum}, \textit{T. mentagrophytes} and \textit{E. floccosum} by a single-step PCR. As reported in earlier studies, PCR-based fingerprinting using (GACA)$_4$ primer is simple and rapid in identification of
dermatophyte species and *T. mentagrophytes* strain variants (Faggi *et al.*, 2001; Shehata *et al.*, 2008).

**Virulence activity of dermatophytes**

The virulence activities of dermatophytes such as phospholipase, lipase, proteinase, gelatinase and hemolytic activity were analyzed by gel diffusion assay. The phospholipase hydrolyzes phospholipids into fatty acids and other lipophilic substances. Likewise the lipase hydrolyzes lipids. The proteinase has proteolytic activity wherein it breaks down the proteins into polypeptides and amino acids. The gelatinase hydrolyze gelatin into sub components such as polypeptides, peptides and amino acids. The gelatinase activity of dermatophyte species was analyzed for the first time in India. The medium enriched with specific substrates showed rapid growth of dermatophytes within 3-5 days of incubation showing that they have the ability to breakdown the substrate present in the skin of patients with dermatophytosis for their growth. The virulence enzymes were produced by all the dermatophyte species which was similar to the earlier virulence study (Muhsin *et al.*, 1997) and hence acts as virulence markers for dermatophytes.

Few researchers have analyzed the production of these virulence enzymes by dermatophyte species experimentally *in-vitro*. The dermatophytes initially break the lipid surface layer during the first phase of growth and subsequently, colonize the stratum corneum of the skin (Hellgren and Vincent, 1981). Hence, lipase enzyme plays a vital role during dermatophytic infections. A variety of analytical methods were employed for the determination of lipase activity such as volumetry, spectrometry, radioactive assay, immunoassays, conductimetry, chromatography and biosensors (Stoytcheva *et al.*, 2012). These emerging techniques can be developed for
the quantification of lipolytic activity of dermatophyte species. Hellgren and Vincent, 1981 reported that the lipolytic activity was found to be high in old cultures of *E. floccosum*. The level of proteolytic activity of *T. rubrum* isolates was varied based on the parameters such as pH, temperature, incubation period and substrate concentration. The proteolytic activity plays a major role in the penetration and pathogenesis during dermatophytic infection (Kadhim *et al*., 2015).

The dermatophytes usually colonize the epidermal layer of the skin and they are unable to penetrate the deeper tissues of an immunocompetent individual. The immunological response of the host to the metabolic products released by the fungus is directly proportional to the severity of the infections, which may result from mild to acute inflammation. The humoral and cellular immunity involves activation of the lymphocytes, macrophages, neutrophils and mast cells at the site of infection. The hemolysins are the lipids and proteins which are toxic to these cells in many bacterial infections. Likewise the hemolysins produced by dermatophytes may also act similarly and decline the immune response of the host (Schaufuss and Steller, 2003). The Columbia blood agar base with sheep blood showed slow growth for about 20 days of incubation at 37°C and observed single complete zone of hemolysis on *T. rubrum* complex, *T. mentagrophytes* complex and *A. grubyi*. This non-enzyme virulence activity with a variety of dermatophyte species was analyzed for the first time in India.

Schaufuss and Steller, 2003 reported bizonal hemolytic activity of *T. rubrum* and *T. equinum* where a complete zone of hemolysis was surrounded by a small incomplete zone demonstrating that two different cytolytic activities were expressed,
whereas \textit{T. mentagrophytes} and \textit{T. verrucosum} produced single complete zone of hemolysis observed around the colony.

Dermatophytes initially colonize the outer layer of the epidermis, which is rich in keratinous material such as skin, hair and nail. Therefore, the keratinase is the major virulence enzyme produced during infection. Keratinase is produced only in the presence of keratin. They mainly attack the disulfide (d-d-) bond of the keratin. The keratinase enzyme is produced by keratinophilic bacteria and fungi. Many researchers have worked with the bacterial strains in the production of keratinase using poultry feathers (Kumar \textit{et al.}, 2011; Mazotto \textit{et al.}, 2011). Similarly the keratinase activity of non-dermatophyte molds was also analyzed using poultry feathers (Sharaf and Khalil, 2011; Awasthi and Kushwaha, 2011; Kazi \textit{et al.}, 2013).

In the present study native poultry feathers were utilized as a keratin substrate to determine the keratinase activity of dermatophyte species. Minimum amount of nutrient is required for the initial growth of dermatophytes and hence low concentration of 0.5\% of Soyabean casein digest broth was used as nutrient supplement by providing amino acids and long chain peptides for the growth of dermatophyte species. \textit{M. gypseum}, \textit{M. canis}, \textit{A. grubyi}, \textit{T. mentagrophytes}, \textit{T. interdigitale} completely degraded the keratin substrate within 12-14 days of incubation whereas others such as \textit{T. rubrum}, \textit{T. rubrum} var. \textit{raubitschekii}, \textit{T. tonsurans}, \textit{E. floccosum} partially degraded the substrate even after longer exposure of 30 days of incubation, which was analyzed for the first time in South India.

Wawrzkiewicz \textit{et al.}, 1991 observed the keratinase activity of \textit{Trichophyton gallinae} using soluble keratin substrate, where the poultry feathers were initially treated with chemicals to break the disulfide bond of the keratin protein which gives
stability to the protein. Similarly, Sharma and Sharma, 2011 utilized the processed poultry feather keratin and analyzed the keratinolytic activity of dermatophyte species by gel diffusion assay. They observed that all dermatophytes species tested produced keratinase, except M. canis, whereas in the present study M. canis completely degraded the native feather keratin and produced keratinase.

The keratinase activity was determined by spectrophotometry, which showed higher keratinase activity from the culture filtrate of T. interdigitale and T. mentagrophytes. This property of the keratinase enzyme can be extrapolated in the leather industry for de-hairing process of the raw animal skin.

The quantitative estimation of free amino acids was analyzed from the culture filtrate of T. interdigitale and T. mentagrophytes produced many essential and non-essential amino acids. The determination of amino acids after biodegradation of poultry feathers by dermatophyte species was analyzed for the first time in India. Thus the poultry feathers can be recycled using these dermatophytes and produced amino acids which in turn can be utilized as animal feed supplement.

The keratinolytic protein of dermatophytes was demonstrated using molecular methods such as PCR targeting this protein for the first time in India. We observed that the keratinolytic protein was detected from all the dermatophyte species. Hence, the keratinase activity using poultry feathers as keratin substrate, quantification of amino acids released after biodegradation of poultry feathers and PCR in detection of the keratinolytic protein from keratinophilic fungi, we experimentally confirm that the keratinase act as a virulence marker for dermatophytes.