5.0 DISCUSSION

The present study highlights the clinical pattern and prevalence of different dermatophyte species implicated in different tinea/ringworm infections in Shimla, Solan and Parwanoo geographic areas of Himachal Pradesh. The climatic conditions of this state are favourable for the development of superficial mycoses (Deshmukh et al., 2010). In general, hot and humid environment of the tropical and sub-tropical regions are best suited for the dermatophytic infections which have been reported from different parts of India. There is a huge variation in the climatic conditions of Himachal Pradesh due to variation in altitude (450–6500 meters). The climate varies from hot and sub-humid tropical (450–900 meters) in the southern low tracts, warm and temperate (900–1800 meters), cool and temperate (1900–2400 meters) and cold glacial and alpine (2400–4800 meters) in the northern and eastern high elevated mountain ranges. Shimla lies in the south-western ranges of the Himalayas. It is located at 31.61°N 77.10°E with an average altitude of 2397.59 meters (7866.10 ft) above mean sea level. Solan city is located at 30.92°N 77.12°E. It has an average elevation of 1502 meters (5249.34 ft). Parwanoo is located adjacent to Kalka, Haryana (30.83°N 76.95°E) which is quite hot during summer and humid during rainy season. Being hilly state, the three cities chosen for the study have relatively higher population density consisting primarily of farmers and a large proportion of the construction workers/laborers particularly in Parwanoo which is an industrial area. Besides the suitable climatic conditions, other factors such as the migration of laborers, workers and tourists frequently visiting this region, overcrowding, unhygienic life style of the community with low socio-economic background might contribute to the development of dermatophytosis in this region of the state. All the 202 cases of superficial skin infections tested positive for fungi in KOH mounts, only 74 (36.63%) were positive for dermatophyte species on culturing in SDA. However, 85 samples were found positive for the non-dermatophytic yeasts and moulds alone while 32 samples were found culture negative as no growth was observed on culturing these samples even though they were found KOH positive. The reasons for not recovering the fungi in culture may be due to several factors; i. the amount of fungus may be quite less, and suppressed by the cycloheximide and chloramphenicol in the SDA medium used for recovery of dermatophytes, hence not recoverable ii. It is also possible that some topical formulation of antifungal drugs might have been applied or some oral drug taken, a fact not disclosed to the physician by the patient while the clinical history was being extracted. *Aspergillus flavus*, *A.*
*fumigatus*, *A. niger*, *Penicillium* spp. were the common non dermatophytic moulds associated with these infection while *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. guilirmondii* and *C. parapsilopsis* were the common yeasts isolated from KOH positive samples in another study in our laboratory. Several researchers have reported the association of non-dermatophytic and other fungi with dermatophytosis world over (Havlickova *et al*., 2008; Enemuor and Amedu, 2009; Prasad *et al*., 2013). The association of the dermatophytes and other fungi moulds as well as yeasts may be important from the view that the invasive infection by the non-dermatophytic fungi (moulds as well as yeasts) might take place and there may be disseminated infection. Disassociation might also lead to chronicity due to failure of the antifungal therapy to ward of these agents. Another possibility of concurrent infections with the bacterial agents constituting the normal flora as well as from environment might also a result due to the combined infections of dermatophytes with other fungi.

85.1% culture positive cases of dermatophytosis of the affected males and rest females. The higher prevalence in males has been reported in India as well as other countries of the world by several researchers (Singh and Beena, 2003a; Balakumar *et al*., 2012). This may be due to the differences in occupational exposure of both the sexes as males are more involved in construction and other works requiring physical activity. Further, although patients of all ages were susceptible to dermatophytosis but most (64.9%) belonged to the age group of 21-50 years. Other researchers have reported highest prevalence in the age group of 21-30 years (Sarma and Borthakur, 2007; Patel *et al*., 2010). The probable reason for higher prevalence in this group could be due to the fact that the individuals in this group are often most active because of their involvement in the outdoor activities such as studies, jobs etc.

Various tinea conditions in the present study were diagnosed by the clinician himself based on the clinical presentation. Tinea corporis was the most common clinical condition observed in which exposed parts of the body are mainly affected followed by tinea cruris in which groin and adjoining areas are affected (Fig.4.1). Similar observations have been made by Venkatesan *et al*., 2007. Tinea conditions are consequence of exhaustive physical work and prolonged exposure to the sun leading to excessive sweating. In addition, the tight fittings and synthetic clothing particularly in males provide damp, sweaty and warm skin conditions. All these factors favour the growth of the dermatophytes (Ranganathan *et al*., 1995; Singh and Beena, 2003b). Tinea
pedis and tinea unguium might result from wearing of socks and shoes for longer periods providing damp conditions especially in the inter-digital spaces.

*Trichophyton mentagrophyte* was the predominant dermatophyte implicated in 63.5% cases of dermatophytosis followed by *T. rubrum* (34.6%) while *M. gypseum* was involved only in 1.35% cases. We, however, did not observe any involvement of *Epidermophyton* spp. in the present study. Interestingly, we found *T. mentagrophyte* as predominant species followed by *T. rubrum*. This finding is contrary to the observations made of others in which a reverse trend has been recorded (Patel *et al*., 2010; Balakumar and Rajan, 2012; Pandey and Pandey, 2013). The plausible explanation for this can be seen in the fact that *T. rubrum* is generally linked to chronic dermatophytosis (Aya, *et al*., 2004). However, we do not have exact data about the chronic cases of dermatophytosis as they were excluded on the basis of history obtained from the patients. Therefore, the low proportion of *T. rubrum* may represent implication of this organism in acute superficial mycosis. This organism is a slow growing one, and there is a possibility that other dermatophyte species might overgrow or mask the growth of *T. rubrum* while attempting isolation. PCR amplification directly from the samples might prove a better tool to exclude this possibility. Besides, the use of effective and prolonged antifungal therapy to treat the patients might have reduced the incidence of *T. rubrum* in this region.

*Tinea corporis* was the most frequently encountered tinea condition in this geographical region of the state followed by tinea cruris. *T. mentagrophyte* was implicated as the predominant species followed by *T. rubrum* and *M. gypseum* as discussed earlier. Unhygienic conditions among low socio-economic group, frequent migration of laborers, workers, frequent visits of tourists to this region may be some of the contributing epidemiological factors. Although, the present study is a random study that focuses primarily on the occurrence of different dermatophyte species in the state of Himachal Pradesh, more systematic study covering larger population and over a longer period of time would give a better insight into the epidemiology of dermatophytosis in the state.

Being most common superficial mycoses in humans and domestic animals (Chinelli *et al*., 2003), a number of topical as well as systemic antifungal agents have been introduced for treating this condition and more are underway (Chadeganipor *et al*., 2003). The dermatophyte species differ in their susceptibility to different antifungal agents and resistant strains of dermatophytes to a particular antifungal agent have been reported (Fernandez-Torres *et al*., 2001). The introduction
of wide range of new antifungal agents and the recovery of clinical isolates exhibiting resistance to antifungal agents such as amphotericin B,azole group etc. makes the susceptibility testing more important particularly for surveillance of resistant strains in epidemiological studies. The detection of resistant strains might help better management of infections due to such strains by selecting appropriate therapy which would prevent further spread of the resistant strain in the community.

For determining susceptibility of dermatophytes which was intended for filamentous fungi, some researchers followed the protocol M38-A of CLSI, 2002 (Mota et al., 2009). The document was later modified to M38-A2 by CLSI in 2008. This document provides the protocol for dermatophytes also which has been followed by us for determining the MIC values of itraconazole, terbinafine and ketoconazole against different dermatophyte species. The unavailability of such reference method previously perhaps presented difficulty in the standardization of some parameters such as temperature, incubation time, selection of growth medium etc. for different species of dermatophytes (Jessup et al., 2000). Prior to CLSI guidelines of 2008, there was no uniform or standard prescribed procedure was in place for determining the susceptibility of dermatophytes to antifungal agents. Different researchers followed different protocols for this purpose which resulted in variability in determining MIC values. But with the protocol M38A2 of CLSI, it is now possible to obtain uniformity of results in determining MIC values of antifungal drugs which in turn, would be useful to select appropriate amounts of antifungal agents that would ensure effective treatment. Several methods have been used for susceptibility testing e.g. disk diffusion method, broth macro and microdilution method, colorimetric microdilution method, E-test etc. (Perea et al., 2001; Fernández-Torres et al., 2003; Karaca and Koc, 2004; Santos and Hamdan, 2005). We used broth micro-dilution method for determining MIC values of itraconazole, terbinafine and ketoconazole against dermatophyte species recovered from human patients.

We incubated T. rubrum, T. mentagrophyte and M. gypseum at 35°C as mentioned in the M38-A2 protocol. Some researchers obtained better growth at 28°C (Pujol et al., 2002; Barros MES et al. 2007; Araujo et al., 2009). The cultures of T. mentagrophyte and M. gypseum were incubated for 4 days and T. rubrum for 5 days as good growth was observed after the specified periods which were used for susceptibility testing. Good inhibitory activity of all the three antifungal
agents against *T. mentagrophyte*, *T. rubrum* and *M. gypseum* was observed in the present study (Table. 4.10). Itraconazole and ketoconazole had the lower mean MIC values as compared to terbinafine. This suggests more effectiveness of both the drugs as compared to terbinafine. Low MIC values for these antifungal agents have also been reported by others (Fernandez-Torres *et al.*, 2001; Ghannoum *et al.*, 2004).

*T. mentagrophyte* isolates were found more susceptible to both itraconazole and ketoconazole as compared to terbinafine since lower MIC$_{50}$ values of itraconazole (0.125μg/ml) and ketoconazole (0.0625μg/ml) against this dermatophyte species were observed whereas this value was recorded at 0.5μg/ml for terbinafine. The values are comparable to those reported by others in respect of *T. rubrum* and *T. mentagrophyte* (Silva *et al.*, 2006; Adimi *et al.*, 2013,). As no significant difference in MIC$_{50}$ values of itaconazole and ketoconazole against *T. rubrum* and *T. mentagrophyte* isolates was observed by us, both the species exhibited similar susceptibility to terbinafine also. The MIC$_{50}$ and MIC$_{90}$ values of this drug were recorded at 0.5μg/ml and 2μg/ml respectively. These values are higher than those reported by other workers (Favre *et al.*, 2003; Mota *et al.*, 2009). These results are substantiated by the findings of Gupta *et al.*, (1998) and Roberts in 1997. These researchers reported that the oral drug formulations of terbinafine and itraconazole were also required in more extensive and severe fungal infections. Ketoconazole was found most effective against a single isolate of *M. gypseum* tested in our study. In order to obtain a better picture of susceptibility pattern of *M. gypseum* isolates, a large number of isolates are required to be tested before making definitive conclusion about MIC values of different antifungal agents.

On comparative analysis of the effectiveness of the three antifungal drugs by statistical t-test, it was revealed that ‘itraconazole and terbinafine’ and ‘terbinafine and ketoconazole’ were found independent based on the P value of < 0.005 in case of *T. mentagrophyte* isolates. However, similarity existed between MIC values of ‘itraconazole and ketoconazole’ against *T. mentagrophyte* as the P value of > 0.005 was recorded in this case. The MIC values of ‘itraconazole and terbinafine’ against *T. rubrum* were found independent on the P value of < 0.005. However, similarity existed between MIC values of ‘itraconazole and ketoconazole’ and ‘terbinafine and ketoconazole’. The MIC$_{50}$ and MIC$_{90}$ observed in the present study based on standard protocol M38-A2 of CLSI 2008 might serve as index values for further studies covering...
large number of isolates from different geographic regions of the state over a longer period. Also, such studies might reflect on the acquisition of drug resistance among isolates of dermatophyte species based on MIC values.

Isolation of the causative agents from a disease condition is considered to be the gold standard in diagnosis. In the present study, we have used conventional methods of isolation of dermatophyte species and other associated fungi and their identification by microscopic examination of the LCB stained preparations besides the colony characteristics typical of dermatophyte species and other tests such as urease test and hair perforation test in case of *M. gypseum*. The recovery of dermatophytes from the tinea infections takes at least three weeks to achieve diagnosis as these organisms are slow growing. In order to check the environmental contamination with fungi, SDA media containing cyclohexamide and cloramphenicol have been used. Also, the plate containing this medium was exposed in the laminar flow during the process of recovery in order to rule out the possibility of contamination of the culture from the environment. We did not observe any growth on such plate kept as uninoculated control and incubated along with the sample inoculated plates. The molecular methods overcome the limitation of the conventional methods in that they are rapid, hence less time consuming and also do not require the skilled personnel to examine the isolate. However, the false positive and false negative results in the PCR assay are to be taken due care of. In the present study we amplified the ribosomal RNA using primers derived from the internal transcribed spacer region (ITS). The size of the amplicons of the *T. mentagrophyte*, *T. rubrum* and *M. gypseum* as identified by conventional methods ranged in size between ~700-850 bps.

On nucleotide sequencing of the amplicons of the specified dermatophyte species and by multiple sequence alignment with the published sequences of the NCBI, all ten *T. mentagrophyte* isolates were identified as *T. mentagrophyte* var. *interdigitale*, *T. rubrum* isolates (three in number) as *T. rubrum* and a single isolate of *M. gypseum* isolate *Arthroderma gypseum*. *T. mentagrophyte* var. *interdigitale* is implicated in tinea infections in human beings while *T. mentagrophyte* var. *mentagrophyte* is of zoophilic origin. Although we amplified and sequenced only fourteen isolates of dermatophyte species the present study, as against 74 examined by conventional methods. All the 14 isolates turned out to be the same as identified by conventional
methods. Thus, PCR assay served as perfect adjunct to the conventional diagnostic methods, we did not observed any disconcordance between the results of these methods.

Furthermore, with an increasingly aging population and the increasing occurrence of immunocompromised patients, many previously unknown fungal infections, including dermatophytosis, have emerged as important causes of morbidity. The application of chemotherapy has also contributed to the occasional modification and alteration of the morphological characteristics of dermatophytes, resulting in atypical colonial growth and appearance and complicating the laboratory identification procedures based on phenotypic features. Therefore, the availability of improved laboratory methods is essential for rapid and accurate detection and differentiation of the dermatophytes involved, so that appropriate treatment and preventive measures can be adopted to control spread of dermatophytosis.

Nucleotides sequence homology studies of ITS gene amplicons of rRNA to the published NCBI sequences revealed that the *T. mentagrophyte* isolates showed 98 to 99% homologies with *T. interdigitale* isolate 490 (JN133999), *T. interdigitale* strain WM10.87 (HQ014707), *T. interdigitale* isolate 3 (KC595992), *T. interdigitale* strain IFM41050 (AB193720), *T. mentagrophyte* (HQ223449) and *T. interdigitale* strain YY01667760 (KC833525) while *T. rubrum* isolates showed 97 to 99% homologies with *T. rubrum* strain ATCC28191 (KF278457), *T. rubrum* strain IFM45885 (AJ270805), *T. rubrum* isolate Z09071149 (JX122344), *T. rubrum* isolate Z10031064 (JX122347), *T. rubrum* strain CBS392.58 (Z97993) and *T. rubrum* strain CBS202.88 (AJ270804). A single isolate of *M. gypseum* recovered from tinea corporis condition showed 95 to 96% homologies with *Arthroderma gypseum* isolate CBS100.64 (JX101934), *A. gypseum* isolate bM130 (JX101934) and *A. gypseum* (AJ970150). The genera *Arthroderma* has now been named as *Microsporum gypseum* but the previously published sequences are still being referred to as *Arthroderma*.

Internal transcribed spacer (ITS) refers to a piece of non-functional RNA situated between structural ribosomal RNA (rRNA) on a common precursor transcript. Read from 5' to 3', this polycistronic rRNA precursor transcript contains the 5' external transcribed sequence (5' ETS), 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA and finally the 3' ETS. During rRNA maturation, ETS and ITS pieces are excised and as non-functional maturation by-products are rapidly degraded. Genes encoding ribosomal RNA and spacers occur in tandem repeats that are
thousands of copies long, each separated by regions of non-transcribed DNA termed as non-transcribed spacer (NTS). Sequence comparison of the ITS region is widely used in taxonomy and molecular phylogeny because i. it is easy to amplify even from small quantities of DNA (due to the high copy number of rRNA genes) ii. It has a high degree of variation even between closely related species. The ITS region is the most widely sequenced DNA region in molecular ecology of fungi (Peay et al., 2008) and has been recommended as the universal fungal barcode sequence (Schoch et al., 2012). It has typically been most useful for molecular systematic at the species level, and even within species (e.g., to identify geographic races). Because of its higher degree of variation than other genetic regions of rDNA (for small and large subunit rRNA), variation among individual rDNA repeats can sometimes be observed within both the ITS and NTS regions. In addition to the standard ITS1 and ITS4 primers used by various workers (White et al., 1990), several taxon-specific primers have been described that allow selective amplification of fungal sequences. Sequence analysis of the variable regions of rDNA, especially the ITS regions, has defined the nucleotide sequences of these regions and demonstrated the species–specific structure of dermatophytes. According to phylogenetic studies using these data, dermatophytes can be considered to be homogenous group with recent evolutionary divergence, since all data obtained from molecular analysis showed low genetic diversity between species belonging to one genus (Kanbe, 2008).

The ITS sequence data on dermatophyte confirm that ecologically and phenotypically strongly separated species may have only small numbers of differences even in this normally highly variable genetic region (Summerbell et al., 1999). Makimura et al., (1999) analyzed the nucleotide sequences of ITS1 region of pathogenic dermatophytes and reported that the nucleotide sequences of ITS1 region were reasonably useful, not only for understanding the phylogenetic relationships among dermatophytes, but also for identification of dermatophytes at the species level. The amplification of the ITS region of rDNA have been used by various other workers for identification of the dermatophyte species. Li et al., (2008) identified 17 dermatophyte species using PCR amplification of ITS regions of rRNA. The results of their study indicated that most of the T. mentagrophyte isolated from the human patients were T. interdigitale. Similar results were observed by nucleotide sequence homology of ITS region in our study also, where all the 10 isolates of T. mentagrophyte were identified as T. interdigitale.
Two dermatophyte species *M. canis* and *T. tonsurans* were successfully identified using universal ITS1 and ITS4 primers (Malinovschi et al., 2009). Makimura et al., (1999) demonstrated by using ITS1 of rRNA that various species of *Arthroderma vanbereuseghemii- Arthroderma simii* groups containing various dermatophyte species have been over classified and belonged to *Arthroderma benhamiae* group. They also identify 11 clinical isolates of dermatophytes successfully up to strain level by comparing base sequences of ITS1 DNA with sequence data base. Besides the sequencing of 18S rRNA, 5.8S rRNA, 28S rRNA other molecular methods such as PCR-RFLP, PCR fingerprinting have also been used by several workers for identifying of dermatophyte species. Graser et al., (1999) used sequencing of ITS regions, PCR fingerprinting and RFLP techniques to define the precise structure of *T. mentagrophyte* and *T. tonsurans* groups which were as identified by conventional methods. They suggested that all the 24 species of these groups should be reduced to only five. Ghannoum et al., (2013) amplified the ITS regions of different dermatophytes and digested the amplicon using *Mval* enzyme. The restriction enzyme patterns of species specific band were observed. *T. mentagrophyte* exhibited a set of 3 band of sizes 200 bp, 400bp, 700 bp while in case of *T. rubrum* bands of sizes 380, 430 and 550 bp were observed. PCR- based identification of common dermatophyte species using primer sets specific for the DNA topoisomerase II genes has been described by Kanbe et al., in 2003. They designed the species specific primer sets for common dermatophyte species and used these primer mixtures for nested PCR reaction. On amplification the bands of different sizes were obtained for different dermatophyte species by these researchers. They also used PCR as well as PCR-RFLP targeting the DNA topoisomerase II gene using four restriction enzymes (*afl, Hinc II Hinf I and PflM I*) and successfully identified even those species of dermatophytes which were not identified using PCR technique alone. Liu et al., (2000) used arbitrarily primed PCR for rapid identification of dermatophyte species. The amplification was achieved by using short random primers (10 nucleotides long) at relatively low temperature by these workers. Maximum 20 dermatophyte species out of 25 analyzed by AP-PCR showed distinct DNA patterns with a random primer, while amplification was also observed with other random primers also.

Gultzmer et al., (2006) used light cycler PCR and RFLP systems for the rapid detection/identification of isolates of dermatophytes and other pathogenic fungi in clinical specimens.
directly. Almost all studies of species identification of dermatophytes have shown that PCR-RFLP targeting ITS regions is a valuable tool. The use of this tool directly on the clinical samples will give rapid and accurate detection and identification of the various fungi involved. Although nucleic acid based methods are rapid, more sensitive and useful for strain differentiation but it may not be possible to perform these tests for routine diagnostic of the dermatophyte species. However, the variation in phenotypic characteristics such as variation in colony morphology of dermatophyte species which may be due to certain environmental factors such as change in temperature, growth medium or may be induced by the therapy. Such variations may not result due to change in the nucleotide sequences. In order to co-relate the phenotypic variation the genotyping is essentially required. The primary focus of the present study was to establish a mycology laboratory within the Department of Microbiology where the human clinical cases of dermatophytosis and other fungal infections of humans could be undertaken. Using the conventional methods as well as PCR amplification of ITS region we could successfully identify the dermatophyte species implicated in dermatophytosis in patients of the geographical region comprising of Solan, Parwanoo and Shimla of Himachal Pradesh. Also, in this laboratory the studies on candidacies of human patients have also been carried out and the study reflected that non-albican Candida species such as C. dubliniensis, C. parapsilosis, C. krusei, C. tropicalis, C. glabrata, C.guilliermondii etc. are emerging pathogens in blood stream and superficial mycoses.

5.1 Future projections
The present study highlights certain epidemiological factors associated with dermatophytosis in human patients at Shimla, Solan and Parwanoo towns of Himachal Pradesh. The study has been carried out on samples collected randomly during a period of two years. The study can be extended to other parts of the state covering a large population. This would give a better picture in respect of the entire state and also more epidemiological factors including socioeconomic problems might be involved in the prevalence of dermatophytosis in the state. Studies on dermatophytosis of animal species can be undertaken for better correlation of transmission and hence prevalence of dermatophytes in this geographic region. Employing conventional and molecular methods for identification we could successfully identify different dermatophyte species and see the nucleotide sequence homology among the rRNA amplicons of different
dermatophyte species isolated in the study with the corresponding published sequences. This further suggests that the amplification of ITS regions directly from the samples could be useful tool in the diagnosis of dermatophytosis. By use of this tool, it is very likely that the identification could be made in a short time frame with enhanced sensitivity as compared to the conventional methods of isolation and identification. This would in turn help in early management of the dermatophytosis. Further studies are however, required to be conducted on a larger number of samples. Also, the genus specific and species-specific primers can be used for this purpose in PCR assays which would obviate the necessity of nucleotide sequencing of the amplicons as the identity of the amplified products can be established by restriction enzyme (RE) analysis, alternatively the probing of the amplified product can be undertaken. In the present study we have tested the susceptibility of the dermatophyte isolates to antifungal agents (itraconazole, ketoconazole and terbinafine) and determined the MIC_{50} values of these agents. However, more agents can be evaluated in a similar manner and over a large number of isolates collected over a longer period of time covering larger population so as to have a better clinical correlation in terms of effectiveness of these antifungal drugs. All these factors would give a better insight into the epidemiology of dermatophytosis as well as MICs of the antifungal agents in this part of the country. The constant surveillance of the dermatophyte species and their susceptibility to the antifungal agents is of utmost importance as it is likely that the resistance against the antifungal agents might develop with prolonged use of such drugs over a prolonged period of time. The studies on the concurrent/ secondary infections could be more useful with regard to the appropriate therapy and management of such infections. We have undertaken such studies in our laboratory and observed the association of non-dermatophytic fungi in the cases of dermatophytosis. It would be interesting to investigate the multi factorial etiology although the superficial mycosis is not a serious condition however; it might result into greater degree of stress to the patient and might present difficulty in treating such infections. The studies can be extended to investigate various virulence factors of different dermatophytic species which could further help in the better understanding the pathogenesis of infections due these agents.