3.0 RESEARCH METHODOLOGY

The composition of media, chemicals and reagents, statistical test, equipment and glassware used in the study are given in Annexture-I, II, III and IV respectively.

3.1 Collection of samples

The samples were collected from the individuals who visited the skin outpatient department (Skin OPD) at Indira Gandhi Medical College (IGMC), Shimla, Regional Hospital (RH) at Solan, Employee’s State Insurance (ESI) Hospital at Parwanoo and Harihar Hospital at Mandi in the state of Himachal Pradesh. The samples were abbreviated as VBS for samples from Shimla, VBSo for samples from Solan, VBP and VBPo for samples from Parwanoo and VBM for samples from Mandi.

Samples in the form of skin scrapings, hair follicles and nail scrapings (202 in number) were collected randomly in batches from outpatients with different tinea conditions; Tinea corporis, Tinea capitis, Tinea cruris, Tinea pedis, Tinea unguium, Tinea faciei, Tinea manuum and Tinea gladiatorum. Samples collected from these conditions were diagnosed by the clinician himself while examining the patients and the same were processed in the Microbiology Laboratory at Shoolini University for recovery of dermatophyte species. For obtaining the samples aseptically, the infected areas or lesions were wiped with 70% ethanol in order to remove the dirt and environmental contaminants. Skin and nail scrapings, hair along with follicles were collected with the help of sterile scalpel/ tweezers from advancing margins of the lesions in sterile plastic containers (Sterile Uricol: Himedia, Fig. 3.1). The information about the applications of remidal antifungal therapy was obtained through inquiry from the patients since the clinician asked them to produce the outpatient card in case any treatment was taken by them during past 2–3 months. Also, the information regarding immunosuppressive/ immunocompromised state including co-infection with HIV and other conditions such as diabetes was also retrieved by the clinician. In addition, the information regarding sex, age and profession of the patients were noted down. The samples were transported to Microbiology Laboratory of the Shoolini University at Solan for isolation of the etiological dermatophytic species and further analysis.
3.1.1 Inclusion and exclusion criteria

The following inclusion and exclusion criteria were adopted while collecting samples. The patient visiting the outpatient department in the hospital settings showing lesions typical of dermatophytosis as adjudged by the clinician were eligible to participate in this study. Age limit and sex bias were not used and patients of all age groups and both the sexes were included. The exclusion criteria included: use of antifungal therapy (oral as well as topical) within 2–3 months prior to the commencement of the study; presence of serious underlying systemic conditions as adjudged inappropriate by the clinician for inclusion in the study; other infections bacterial as well as fungal in the skin folds and nails (paronychia) etc. were excluded.

The research project SUIEC/12/04 entitled “Isolation and identification of dermatophyte species recovered from human patients in Himachal Pradesh by conventional and molecular methods and their susceptibility to common antifungal agents” was approved by the Institute Ethics Committee of Shoolini University as communicated through its letter no. SUBMS/IEC/12/45, dated 19th March, 2012.

Fig. 3.1 Sterile plastic containers heaving samples of a. skin scrapings b. nail scrapings c. hair follicles, recovered from patients suspected of dermatophytic condition.

3.2 Examination of direct KOH mounts of the samples

Hair and skin samples were treated with 10% KOH and nails with (40% KOH) for 10 min., mounted on glass slide and examined under light microscope for the presence of fungi under low
power of magnification. The KOH clears the keratinaceous material by digesting proteinaceous debris, bleaches the pigment and loosens the sclerotic material without damaging the fungus. The samples positive for fungi were processed for the isolation of the dermatophyte species on Sabouraud’s dextrose agar.

3.3 Isolation and identification of dermatophytes

3.3.1 Isolation on Sabouraud’s dextrose agar
The samples were cultured on the Sabouraud’s dextrose agar containing cyclohexamide (0.05%) and chloromphenicol (0.004%) (SDA, Himedia) under sterile conditions. The plates were incubated at 30°C for four weeks and monitored for the growth of dermatophyte. With the help of L-shaped inoculating needle a portion of the dermatophytic growth was picked up and streaked on SDA slants. The colonies on the slants were examined for their morphology, texture and pigmentation both on obverse and reverse sides of the slants. The confirmation was done by microscopic examination of the stained preparations as described below under section 3.3.3.

3.3.2 Culturing on dermatophyte test media (DTM)
The colonies from SDA slants were subcultured on the dermatophyte test medium. This medium is a selective one for the growth of dermatophyte species. The slants were incubated at 30°C for 1 to 2 weeks, and examined for the growth and the color change, from pink to red in case of dermatophytic growth in the medium.

3.3.3 Microscopic examination
For identifying the dermatophyte species, colony of each isolate was stained in Lactophenol Cotton Blue (LCB) and observed under low as well as high power of light microscope. The identification was based on features such as organization of hyphae (pencil shaped, spiral, pyriform, septations etc.), microconidia and macroconidia (tear shaped, drop like, spherical, in bunches, abundance or rare etc.). T. rubrum (ATCC-28188), T. mentagrophyte (ATCC-18748) and M. gypseum (ATCC-24102) obtained from Post Graduate Institute of Medical Education and Research (PGIMER) Chandigarh, were included as standard strains in the study.

3.3.4 In vitro hair perforation test
Sample of healthy human hairs, 1-2 cm long, preferably from a child or blonde were collected and sterilized by autoclaving at 121°C for 15 minutes. The sterile pieces of hair were placed in
sterile petri plate containing 25ml of sterile water and 2-3 drops of 10% yeast extract. The plates were inoculated with small fragments of test fungi, incubated at temperature ranging from 25°C - 30°C. The single strand of the hair was removed after two to three days and thereafter, at regular intervals and stained in lactophenol cotton blue/congo red and observed under microscope for perforations in the hairs. In this manner, the hairs up to 4 weeks before declaring them negative in test.

3.3.5 Urease test
For this test, the isolates were streaked on the surface of urea agar base slants, incubated at 30°C for 48 hours. The hydrolysis of urea was indicated by change of color from red to pink due to rise in pH of the medium using phenol red as pH indicator (Kwon-Chung *et al.*, 1992).

3.4 Determination of *in vitro* antifungal susceptibility of dermatophytes using broth micro-dilution method.

3.4.1 Dermatophyte species tested
A total of 53 isolates of dermatophyte species: *T. mentagrophyte* (34 in number), *T. rubrum* (18) and *M. gypseum* (1) were tested for their susceptibility to itraconazole, terbinafine and ketoconazole. The isolates were maintained in sterile distilled water and cultured on potato dextrose agar (PDA) medium at 30°C for 5-7 days before testing them for their susceptibility to the antifungals. *Candida parapsilosis* strain ATCC- 22019 and *C. krusei* strain ATCC- 6258 were included as reference strains in the test.

3.4.2 Antifungal agents used in assay
Three antifungal drugs, itraconazole (Metro Chem API Pvt. Ltd. Erragadda, Hyderabad, India), ketoconazole (Aarti drugs Ltd., Thanne, Maharashtra, India) and terbinafine (Shreeji Pharma International, Sarabhi, Vadodara, Gujarat, India) in powdered form were used in the susceptibility assays.

3.4.3 Broth micro-dilution method
Protocol M38-A2 of CLSI (2008) for filamentous fungi was followed for determining susceptibility of dermatophyte species, to antifungal agents in broth micro-dilution assay.
3.4.4 Drug dilutions
Stock dilutions of itraconazole, ketoconazole and terbinafine were prepared in dimethylsulfoxide DMSO (Hi-media) following the standard protocol. The dilutions were prepared as given in Annexure-II. Two fold dilutions of each stock solution were prepared in RPMI 1640 medium (Hi-Media) without sodium bicarbonate and containing L glutamine. The dilutions were used in the test at a pH of 7.0±0.1 with 3-(N-morpholino)propanesulfonic (MOPS) buffer (Hi-Media) along with 1N NaOH. As given in the annexure, the different concentrations used ranged 0.0078μg/ml to 128μg/ml.

3.4.5 Preparation of inoculums of dermatophyte species
Cultures of dermatophyte species (7-8 days old) grown on Sabouraud’s dextrose agar (SDA) slants at 30°C were used to prepare inoculums. The fungal growth was covered with 5ml of sterile normal saline and suspensions prepared by scraping the growth from the surface of the slants with a sterile swab. The scrapped portion contained conidia and hyphal fragments. The heavy particles were allowed to settle down for 10 to 15 min. The upper portion of the suspension was transferred to fresh tube and its optical density was set equivalent to 0.5 Mcfarland standards. The final cell density was set between 2x10³ to 6x 10³ colony forming units per ml. for use as inoculums in the assay.

3.4.6 Test procedure of broth micro-dilution test
Flat- bottomed, 96 well micro-titer plates (Costar-3596) having 8 rows and 12 columns were used in the susceptibility assay. Test organisms in a volume of 100μl each were placed in the wells of 8 rows of the plates (one test organism in each row). The drug dilutions in a volume of 100μl each were added to each well of ten columns of the plate from left to right. The concentration of the drug was highest in the first column and decreased thereafter. The 11th and 12th columns contained control containing inoculums and medium without antifungal agent and un-inoculated negative control respectively. The contents were incubated at 35°C for 4 to 5 days.

3.4.7 Quality control reference strains
C. parapsilosis strain ATCC-22019 and C. krusei strain ATCC-6258 were used as quality control reference strains in each assay. These strains have been approved by the CLSI as quality control strains for testing susceptibility to itraconazole and ketoconazole. The susceptibilities of
these strains to itraconazole, terbinafine and ketoconazole were also determined. The micro-titer plates containing these strains were incubated at 28°C for 48 h as recommended by CLSI.

3.4.8 Determination of MIC values of antifungal agents

The Minimum inhibitory concentration (MIC) value of a drug is defined as the lowest antifungal concentration at which growth is not visible in the wells when detected visually (80 to 100% inhibition). These values for each drug were recorded.

3.4.9 Data analysis

The mean values, MIC range, MIC$_{50}$ and MIC$_{90}$ values for all the three antifungal agents, used in the assay, were determined following standard procedure. The statistical analysis was done by t-test using IBM-SPSS20 software in order to find the independence of the variables or whether they were having similarity in their MIC values at the significance level of P values <0.005. The p values of t-test are presented in annexure-III.

3.5 Molecular Identification

The isolates VBSo-3, VBSo-5, VBSo-6, VBSo-17, VBSo-18, VBSo-29, VBSo-30, VBSo-62, VBM-3 and VBS-3 identified as *T. mentagrophyte*, isolates VBSo-13, VBso-20, VBP-24 identified as *T. rubrum* and isolate VBS-32 identified as *M. gypseum* by conventional methods were further identified by amplification of ITS1 and ITS2 regions of rRNA of these isolates followed by nucleotide sequencing of the amplified products, and their identification by homology with available sequences from National Centre for Biotechnology Information (NCBI) data base. The various steps involved in the process of molecular identification are detailed below.

3.5.1 DNA extraction

The selected dermatophyte species were cultured in glucose yeast extract peptone (GYEP) broth for 5-7 days at 30°C. A small amount of hyphal growth was picked up, dried, and crushed in pestle and mortar. Extraction buffer (1ml) was added to it and crusted again. The crushed semisolid liquid was then transferred into microfuge tubes and 150 µl of 10% SDS added to it. The microfuge tubes were incubated at 65°C for 30 minutes, centrifuged at 10,000 rpm for 10 min. The supernatant was collected in fresh microfuge tube while the pellet was discarded. Ice cold solution of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the supernatant in
an equal amount and tubes were again centrifuged at 10,000 rpm at 4°C for 10 min. The top aqueous layer was transferred into fresh microfuge tube, and the centrifugation carried out with phenol: chloroform: isoamyl alcohol. This process was repeated twice. Finally, equal volume of 100% ice cold ethanol was added to the top aqueous layer collected in fresh microfuge tube. For DNA precipitation, the tubes were incubated overnight at -20°C. Centrifugation was done at 12,000 rpm at 4°C for 10 min. The supernatant was discarded and the pellet was washed in 70% ethanol, centrifuged again at 12,000 rpm for 10 min. The pellet was suspended in 100 µl of TE buffer. The quantity of DNA was assessed by its electrophoresis on 1% agarose gel.

3.5.2 Polymerase chain reaction (PCR) assay
Amplification of internal transcribed spacer regions 1 and 2 (ITS1 and ITS2) was performed in the PCR assay. The nucleotide sequences of the primer pair used in the PCR assay are as follows:

ITS1 (Forward) 5’:TCCGTAGGTGAACCTGCGG-3’
ITS4 (Reverse) 5’:TCCTCCGCTTATTGATATGC-3’ (White et al. 1990)

The PCR reaction mixture (25µl) contained: 12.5 µl of 2X PCR master mix (MBI fermentas) [contained 0.05 U/µL Taq DNA polymerase, reaction buffer, 4 mM MgCl2, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP)], 1µl primers (10 pmole/µl each of ITS1F and ITS4R), template DNA (3.0 µl) and molecular grade water (7 µl). The initial denaturation of the template DNA was carried out at 95°C for 3 min. followed by 32 cycles of amplification with a denaturation step at 94°C for one min, primer annealing at 55°C for 1 min and extension step at 72°C for 1 min, followed by final extension of strands at 72°C for 5 min.

3.5.3 Gel electrophoresis of amplicons
All the PCR products were electrophoresed in 1% agarose gel containing 0.2 µg/ml ethidium bromide (EtBr) at constant current of 90 mA. The PCR products were loaded in the wells using gel loading dye. DNA bands were visualized by gel documentation system.

3.5.4 Nucleotide sequencing of amplicons
The nucleotide sequencing of the amplicons of ITS-1 and ITS-2 segments of rRNA of dermatophyte isolates (VBSo-3, VBSo-5, VBSo-6, VBSo-13, VBSo-17, VBSo-18, VBSo-20,
VBSo-29, VBSo-30, VBSo-62, VBS-3, VBP-24, VBM-3 and VBS-32) was done commercially by Xcelaris genomics laboratory, Ahmadabad, India.

3.5.5 Determination of homology of the sequences of amplicons with published standard strains
The sequences of amplicons of ITS region were compared with each other and homology was determined with published nucleotide sequences of standard strains using the nBLAST tool of National Centre for Biotechnology Information (NCBI). The sequences of amplicons of ITS regions of each isolate were compared with the sequences of ITS regions of the standard isolates/strains using CLUSTAL W2.1 tool online. The sequence homology among the isolates of the same dermatophyte species was also determined in case of *T. mentagrophyte* and *T. rubrum*.

3.5.6 Determination of the phylogenetic relationship
For determining the phylogenetic relationship of our isolates, the phylogenetic tree was constructed by employing neighbor-joining method derived from analysis of the ITS region of dermatophyte isolates and related sequences obtained from NCBI using MEGA4 software.