MATERIALS & METHODS

Ashokan K Kuttyil “Study on the prevalence of subcutaneous mycoses in north Kerala”, Department of Microbiology, Medical College Calicut, University of Calicut, 2006
Patients getting treatment from Dermatology and Surgery Departments of Calicut Medical College for chronic sub cutaneous infections, referred patients from in and around Calicut, Wynad, Malapuram and Cannanore districts to the above Departments were taken for the study. Since Calicut Medical College is a referral hospital for the above districts, all suspected fungal infections from the peripheral hospitals are referred to this medical college for diagnosis and treatment. So, any study conducted in the Dermatology and other departments of Calicut Medical College would give a correct picture of subcutaneous fungal disease prevalent in this area. Wynad is a hilly area with high altitude and various types of plantations. The tribals are an important population in Wynad. Patients having subcutaneous skin lesions from all age groups of both sexes of varying economic status were included in this study.

The study group consisted of 161542 patients with various skin lesions who attended the dermatology, surgery and ENT departments of Calicut Medical College, for a period of five years from January 2000 to January 2006.

For each case, history, duration of symptoms, clinical features, physical signs, associated diseases like tuberculosis, syphilis, drug addiction etc were noted in detail. Treatments taken for the present illness and history of drugs taken in the past were noted. History of intake of prolonged antibiotics, antituberculous drugs, corticosteriod, and antimalignancy therapy were also considered. X-ray findings, detailed laboratory investigation like total Leucocyte count, Differential leucocyte count, VDRL etc were noted.
COLLECTION, TRANSPORT, PROCESSING, AND EXAMINATION OF SPECIMEN

Specimen collection, transport and storage are extremely important components in the provision of accurate results for the diagnosis and management of subcutaneous mycoses. Specimens must be collected under aseptic conditions or after appropriate hygienic preparation to optimize the significance of mycologic results. The specimens most frequently submitted for the recovery of fungi causing subcutaneous mycoses includes aspirate, biopsy samples, skin scrapings and surgical tissue. Swabs are not an effective means of specimen collection and should be avoided when possible. Portion of suspicious necrotic, purulent, or caseous specimens should be examined microscopically and inoculated onto culture media. Tissue specimens should be minced with scalpels into 0.5 to 10 mm pieces and inoculated directly to culture media. Tissue homogenizers should not be used, because some moulds do not have regularly septate hyphae and thus can be easily killed by homogenization.

The specimens included tissue from the infected sites. Biopsies were taken from the lesion for:

- Microscopical examination
- Histopathological examination
- Fungal culture
- Routine microbiological culture
- Anti fungal sensitivity testing of the isolated fungi
MICROSCOPICAL EXAMINATION

**Wet-Mount or Tease-Mount Technique.** The wet preparations are used for diagnosis of fungal infections from clinical specimens or to study the morphological features of the fungal isolates. Wet mount is a rapid method of preparing fungal colonies for microscopic examination. A bent needle or spade of heavy gauge wire is used to remove colony fragments from the culture. When possible, the surface spore material should be removed by gently scraping the surface of the culture toward the center (oldest portion) of the colony.

**Wet mount with Normal Saline:** This preparation is used for observing pigmented fungi and its structures.

**Hydroxide Mounts:** The aqueous potassium hydroxide digests protein debris and dissolves the cement substance which holds the keratinized cells together. It is prepared from the following ingredients:

- Potassium Hydroxide : 10 gm
- Glycerol : 10 ml
- Distilled Water : 80 ml

Place a small part of the biopsy tissue or scrapings on a clean glass slide. Pour a drop of 10% KOH on the specimen and place a cover glass over it. Heat the slide gently over the flame and examine under microscope after a few minutes. If the specimen is not properly dissolved, it may be kept for some more time in a wet Petri dish and examined.

**Calcofluor White Stain (CFW):** Calcofluor White is a water soluble colorless textile dye and fluorescent whitener. This selectively binds to the cellulose and chitin of the fungal cell wall and visualized when exposed to long wavelength of visible light. It fluoresces light blue when exposed to UV light (346-365 nm). Recently it has become
a very popular staining method because of its more sensitivity than other conventional staining techniques.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>CFW M2R</td>
<td>100 mg</td>
</tr>
<tr>
<td>Evans Blue</td>
<td>50 mg</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
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</table>

Mix well and store at room temperature in a dark room. The calcofluor solution is prepared by dissolving calcofluor white powder in distilled water with a final concentration of 0.01%. Evan’s Blue is also added (0.1%) in order to reduce non specific background fluorescence and reveal the surrounding tissue. Evan’s Blue counter stain also produces a contrasting orange to ruby-red background, thereby enhancing detection of fungi. This stain gives apple green fluorescence of the fungi. UV light of wavelength between 300 and 412 nm are used for visualizing the fungal structures under fluorescent microscope. It is difficult to differentiate hyphae from collagen fibers and other artifacts by conventional KOH wet mounts. Therefore CFW staining techniques is far superior to the conventional staining techniques for the detection of fungi in clinical specimens. It is technically simple, quick and highly reliable to identify fungi.

**PHOL Stain:**

The acronym PHOL is derived from the initials of surnames of four scientists i.e. Pal, Hasegawa, Ono, Lee. This is used similar to the LCB stain for the examination of fungal isolates. It contains formalin instead of phenol and methylene blue in place of cotton blue of LCB stain.

**Neutral Red Stain:** Is useful and easily an applicable method for the evaluation of the viability of the fungal elements. This stain is used as a vital stain.
DIFFERENTIAL STAINS:

1. **Grams stain**: Grams stain is effective for detection of some of the fungal pathogens. Brown and Brenn modification of grams stain is used for Nocardia and Actinomyces species in tissues. In general, the procedure is more suited to sections than to smears. The yeast cells usually show up well stained morphology but filamentous fungi in smears become desiccated and their morphological characteristics are usually lost. The fungi are usually gram positive and seen as violet colored in the stained smear. Fix the smear by passing over a flame. Place 0.5 % aqueous crystal violet solution on the slide for 20 seconds. Wash the smear gently under tap water. Apply grams iodine solution over the slide for 20 seconds. Wash with water and decolorize quickly with solution of equal parts of acetone and 95 % ethanol and wash immediately in the running tap water. Counter stain with 0.5 % aqueous safranine for 10 seconds and again wash with water, air dry. Then observe under a microscope.

2. **Modified Acid-Fast Stain**: the aerobic bacteria like Nocardia species and aerobic Actinomycetes species are difficult to be differentiated because both are filamentous gram positive bacteria. Therefore the smear should be stained with modified acid fast stain (Kinyoun’s method) as nocardia is weakly acid fast giving pink or red color to the bacilli.

   Make a smear and fix it by passing over the flame. Flood the slide with Kinyoun carbol fuschin for 3-5 minutes. Then pour off the excessive stain and flood the slide with 50 % alcohol and immediately with water. Decolorize with 1 % aqueous sulfuric acid. Wash with tap water. Counter stain with methylene blue for 1 minute. Rinse with water and examine under oil immersion.
Hematoxylin and Eosin Stain:

Hematoxylin and Eosin (H & E) Staining is one of the basic stains used in many of the diagnostic settings. It is used to stain the nuclei by oxidized hematoxylin (hematin) through mordant (chelate) bonds of metals such as aluminum followed by counterstaining by the xanthene dye- eosin, which colors in varying shades the different tissue fibers and cytoplasm. A general tissue demonstration picture is produced and serves as the main diagnostic technique.

(a) Staining solutions

<table>
<thead>
<tr>
<th></th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematoxylin</td>
<td>5 gm</td>
</tr>
<tr>
<td>Ethyl alcohol</td>
<td>50 ml</td>
</tr>
<tr>
<td>Potassium alum</td>
<td>100 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>950 ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>40 ml</td>
</tr>
</tbody>
</table>

(1) Eosin 1 % aqueous ws yellowish

(2) Differentiator 1 % HCL in 70 % alcohol

(3) Bluing agent 2% aq.sodium bicarbonate

(b) Staining technique

- Bring sections to water
- Stain with hematoxylin solution for the requisite time
- Wash briefly in water and differentiate in acid alcohol
- Wash well in water and blue for 10-30 seconds.
- Wash in water and stain with eosin solution for 3 minutes
- Wash quickly in water, differentiate and dehydrate in alcohol. Clear and mount as desired.

Results:

- Keratohyalin, nuclei, cytoplasmic RNA, some calcium salts, bacteria - Blue
Muscle, keratin coarse elastic fibers, fibrin, fibrinoid - Bright red

Collagen, reticulin, myelinated nerve fibers, amyloid - Pink

Red blood cells - orange

**GIEMSA STAIN**

This is a compound stain formed by interaction of methylene blue and eosin. On exposure to acids, alkali and ultraviolet light, a number of oxidation products (methylene azures) are formed from methylene blue which give contrast staining. The modified method *ie* May-Grunwald Giemsa (MGG) technique is commonly used.

Solutions: Grind 0.3 g of May-Grunwald dye in a little methanol, decant and add more methanol and grind until the dye is in solution and make up to a final volume of 100 ml and filter.

Dilute 20 parts of May-Grunwald solution with 30 parts of pH 6.8 phosphate buffer.

**Staining Procedure**

Fix the smears in methanol for 5 to 10 minutes.

Stain in dilute M-G solution for 10 minutes.

Rinse in pH 6.8 buffer.

Stain in Giemsa solution for 30 minutes.

Wash and differentiate in pH 6.8 buffer for 5-20 minutes until the desired color balance is achieved. Air dry and see under a microscope.

**Results**

<table>
<thead>
<tr>
<th>Element</th>
<th>Color</th>
</tr>
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<tbody>
<tr>
<td>Nuclei</td>
<td>purple</td>
</tr>
<tr>
<td>Cell cytoplasm</td>
<td>blue to mauve</td>
</tr>
<tr>
<td>Red blood cells</td>
<td>pink</td>
</tr>
</tbody>
</table>
PERIODIC ACID SCHIFF STAIN

This stain is very useful for demonstrating fungi in tissues that are usually stained darker than the surrounding tissues. The disadvantages are that many components of tissues which are carbohydrate in composition, are stained by PAS stain. Moreover, actinomycetes such as Nocardia are not stained by the PAS method but are stained by the methenamine stain. The principle of PAS stain is based on Feulgen reaction is that hydrolysis with HCL liberates aldehydes which recolor Schiff’s reagent. The polysaccharides of fungi and bacteria are oxidized by periodic acid to form aldehyde groups that yield red colored compounds with Schiff’s fuchsin sulphite. The protein and nucleic acids remain unstained. The nuclei stain blue, fungi magenta or red and background is light green.

Solutions

1% aqueous periodic acid Schiff’s reagent:

Basic fuchsin : 1 gm
Distilled water : 200 ml
Sodium metabisulphite : 2 gm
Analar conc. hydrochloric acid : 2 ml
Decolorizing charcoal : 2 gm

Staining procedure:

- Bring the sections to water
- Oxidize with periodic acid solution for 5 minutes
- Rinse well in distilled water
- Treat with Schiff’s reagents for 15 minutes
- Wash in running water for 5 to 10 minutes
- Stain nuclei with Harris’ hematoxylin solution
- Dehydrate in alcohol, clear in xylene and mount in a synthetic resin medium
Result:

PAS positive substances : Magenta or red
Nuclei : Red

Gridley’s Fungal Stain: In the Gridley’s fungal stain, the mycelia, yeasts, elastic tissue, mucin are stained as purple and background as yellow. This is like PAS staining but chromic acid is used as the oxidizing agent. The aldehyde that is produced recolor Schiff’s reagent, giving the fungi purple color. The elastic fibers and some connective tissue mucin stain purple, making fungus demonstration more difficult in tissues such as skin.

GOMORI’S METHENAMINE –SILVER STAIN FOR FUNGAL HYphaE.

This stain works on the principle of liberation of aldehyde groups and their subsequent identification by reduced silver method. It is used for the demonstration of polysaccharide content of the fungus in tissue sections. The aldehydes reduce the methenamine silver nitrate complex, resulting in the brown- black staining fungal cell wall due to deposition of reduced silver wherever aldehydes are located. Grocott’s modification of Gomori’s methenamine silver stain is usually used. Tendolkar and colleagues have devised simplified Grocott’s silver staining by use of running water for washing, avoiding use of alcohol, xylene and expensive gold chloride which do not affect the staining character of fungi.

The fungi and bacteria are stained black, mucopolysaccharide dark grey and cytoplasm old rose and tissue pale green. The GMS stain is better than other fungal stains as:

I. It stains both live and dead fungi as compared to PAS which stain only live fungi
II. It also stains the filamentous higher bacteria of Actinomycetes (Actinomyces, Nocardia, Steptomyces and Actinomadura) which are not stained by other fungal stains
Fixation:- 10% Formalin

Technique:- Paraffin section

Solutions: 1) 5% Chromic acid

2) 5% Silver Nitrate Solution

3) 3 % Methenamine solution

4) 5% Borax solution

5) 1% Sodium Bisulfite Solution

6) 0.1% Gold Chloride

7) 2 % Sodium Thiosulfate(Hypo) Solution

8) Stock Methenamine – Silver Nitrate Solution

Silver Nitrate,5 % Solution

Methenamine,3 % Solution

9) Working Methenamin-Silver Nitrate Solution

Borax, 5% solution-----2.0 ml

D.water------------------25.0 ml

Mix and add

Methenamine-Silver Nitrate stock solution---25 ml

10) Stock Light Green

Light Green------0.2 gm

D.Water----------100 ml

Glacial Acetic Acid—0.2 ml

11) Working Light Green

Light Green (Stock)----10 ml

Distilled Water--------50 ml
**Staining Procedure:**

1. De-parafinize sections through 2 changes of xylene, absolute alcohols to distilled water as usual.
2. Oxidize in 5% chromic acid solution for 1 hour.
3. Wash with running tap water for few seconds.
4. Rinse in 1% solution of sodium bisulfite for 1 minute to remove any residual chromic acid.
5. Wash in running tap water for 5 to 10 minutes.
6. Wash with 3-4 changes of distilled water.
7. Place in working methenamine-silver nitrate solution in an oven at 58 to 60 °C for 30 to 60 minutes until sections turns yellowish-brown. Dip slide in distilled water and check for an adequate silver impregnation with a microscope.
8. Rinse 6 times in distilled water.
9. Tone in 0.1 % gold chloride solution for 2 to 5 minutes.
10. Rinse in distilled water.
11. Remove unreduced silver with 2 % sodium thiosulfate for 2 to 5 minutes.
12. Wash thoroughly in tap water.
13. Counter stain with light green for 30 to 45 seconds.
14. Dehydrate with 2 changes of 95 % alcohol, absolute alcohol, clear with 2 to 3 changes of xylene and mount in permount.

**RESULT:**

- Fungi- sharply delineated in black
- Mucin- taupe to dark gray
- Inner part of mycelia and hyphae- old rose
- Back ground – pale green.
MAYER’S MUCICARMINE STAIN

This is used for staining of Cryptococcus and Rhinosporidium species. Cryptococcus stains deep rose red, nuclei black, tissue yellow. In case of rhinosporidiosis, the sporangium and the endospores are stained by mucicarmine stain.

Staining Procedures

- Bring sections to water
- Stain the nuclei with an alum hematoxyli solution.
- Stain with mucicarmine solution for 20 minutes
- Wash in water, dehydrate, clear and mount as desired

Masson- Fontana Silver Stain

The Masson-Fontana Silver Stain (MFSS) is used to identify phaeoid (dematiaceous) fungi. The histopathological examination of tissue is one of the most accurate means of documenting invasive fungal infection. Despite these advantages, histopathological stains are non specific and they do not provide identification of a fungal pathogen. The phaeoid fungi are now among the emerging fungal pathogens. These organisms are classified as phaeoid because they have melanin in their cell wall. MFSS specifically stain melanin of the phaeoid fungi.

- Bring sections to distilled water
- Treat with ammoniacal silver solution in a dark container
- Wash well with several changes of distilled water
- Treat with 0.5% sodium thiosulphate for 2 minutes
- Wash, counter stain in the neutral red solution for 3-5 minutes
- Wash, dehydrate, clear and mount.

Results: Melanin, argentaffin, chromaffin, some lipofuscin pigments stain black and nuclei stain red.
TISSUE PROCESSING AND STAINING

The tissue taken from the lesion (biopsy) is collected in 10% formalin, which acts as a preservative and sent for tissue processing and staining. The following steps are performed for tissue processing. Molten wax is used for Impregnation.

DEHYDRATION: - As paraffin wax is immiscible with water, removal of water from the tissue is required, for this ascending grades of alcohol is used.

CLEARING: - Alcohol used in the first step is also immiscible with paraffin wax. So in the next step, removal of alcohol from the tissue is necessary. This is performed by adding a solvent which is miscible with molten wax. For this procedure chloroform is used.

IMPREGNATION WITH WAX: - After clearing, the tissue is infiltrated with molten paraffin wax, for preserving the cellular morphology and integrity.

EMBEDDING OR BLOCKING: - The tissue is finally transferred from the paraffin wax bath to the molten wax containing mold with a pair of warm forceps. Allow to solidify and the block may be removed.

SCHEDULE FOR PARAFFIN WAX PROCESSING

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Time required</th>
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<tbody>
<tr>
<td>70 % alcohol</td>
<td>1 hour</td>
</tr>
<tr>
<td>80 % , ,</td>
<td>1 hour</td>
</tr>
<tr>
<td>90 % , ,</td>
<td>1 hour</td>
</tr>
<tr>
<td>100 % alcohol</td>
<td>1 hour</td>
</tr>
<tr>
<td>100 % , ,</td>
<td>1 hour</td>
</tr>
<tr>
<td>100 % , ,</td>
<td>1 hour</td>
</tr>
</tbody>
</table>
Chloroform------------------------------- 1 hour
Chloroform------------------------------- 1 hour
Chloroform------------------------------- 1/2 hour

Paraffin Wax (molten)----------------- 1 ½ hour
Paraffin Wax(molten)------------------ 1 ½ hour
Paraffin Wax(molten)------------------ 30 minute

in vacuum

Sections are made from the paraffin embedded tissue by an instrument called “MICROTOME”. The section thickness should be between 4 to 5 μm.

STAINING AND MOUNTING

Staining and mounting of paraffin section is as follows

1. Removal of wax with xylene
2. Hydration through alcohol
3. Staining
4. Dehydration with alcohol
5. Clearing with xylene
6. mounting under a cover slip

PROCEDURE FOR HEMATOXILIN AND EOSIN STAINING

1. Xylene 1 minute
2. Xylene 1 minute
3. Xylene 1 minute
4. Absolute alcohol 1 minute
5. 90% alcohol 1 minute
6. 80% alcohol 1 minute
7. 70% alcohol 1 minute
8. Distilled water 1 minute
9. Harris Hematoxillin 5 minute
10. Wash in water
11. Differentiation in acid/alcohol for 30 minute
12. Blue in tap water
13. Eosin 1 minute
14. Dehydration, 70% alcohol 1 minute
15. 80% alcohol 1 minute
16. 90% alcohol 1 minute
17. Absolute alcohol 1 minute
18. Xylene 1 minute
19. Xylene 1 minute
20. Mount in DPX

H&E staining is to examine the tissue form of the fungus eg. Sclerotic / muriform bodies. In order to see the fungal hyphae in tissue, Gomori’s Methenamine Silver Nitrate Stain (Grocott’s Application to Fungi) is used.

Simultaneous routine bacterial cultures were put up followed by inoculation into LJ media and RCM to detect other bacterial infections.

**CULTURE**

Common culture media used for culture were:

1. Sabouraud’s Dextrose Agar (SDA):- This is the most commonly used medium in the diagnostic mycology laboratory. The Sabouraud dextrose agar
(SDA) is the name recommended for the present day versions of the medium originally designed by the French dermatologist Raymond Sabouraud. The ingredients of this medium are as follows:

Peptone : 10 gm
Dextrose : 40 gm
Agar : 20 gm

Distilled water: 1000 ml

Autoclave the ingredients at 121 °C for 15 minutes and adjust the final pH to 5.6. Sometimes, saprobic fungi grow rapidly on this medium and often overgrown obscuring the true pathogen.

2. Neutral Sabouraud’s Dextrose Agar (SDA) [Emmons modification]

The Emmons’ modification differs from the original Sabouraud’s formulation with lower concentration of glucose and a neutral pH. It contain 2 % dextrose and neopeptone with a final pH of 6.8-7.0.

Neopeptone : 10 gm
Dextrose : 20 gm
Agar : 20 gm

Distilled water : 1000 ml

3. Sabouraud’s Dextrose Agar with antibiotics (mg/ml Chloramphenicol) by dissolving 50 mg of chloramphenicol in 10 ml of 95 % Ethyl Alcohol and the adding to the boiling medium (1 litre) and Cycloheximide (Actidione) 0.5mg/ml by dissolving 500 mg of cycloheximide in 10 ml of acetone then adding to boiling medium (1 litre).

4. New media for fungal isolation and its storage

A novel media was prepared from indigenously available ingredients for the
isolation and maintenances of fungal culture. It has an added advantage over the conventional SDA as it is cheap and the ingredients are easily available. It gives a luxuriant growth of all types fungi. Compared to SDA, it is a good media for the storage of different fungal cultures.

The ingredient of this special media is

- Bengal gram : 1%
- Green gram : 1%
- Sodium Chloride : 0.5%
- Glucose : 2%
- pH : 6.5

Before adding glucose, mix the media by boiling and filter to make it transparent. Add glucose sterilized at 10 pound pressure and dispense in test tubes similar to SDA.

5. Cornmeal Agar/ Cornmeal Tween agar: It is a nutritionally deficient media hence suppress the vegetative growth and stimulates sporulation in fungi

- Cornmeal : 5 gm
- Agar : 4 gm
- Distilled water : 200 ml
- Tween 80 (1%) : 2 gm

6. A new preparation for keeping permanent Wet mount for long periods

- Glycerol : 2 ml
- Lactic Acid: 1 ml
- Phenol : 0.5 ml

Glycerol prevents drying, phenol kills the fungus and lactic acid preserve the fungus. Mainly this is useful for keeping the undisturbed fungal structure from a slide culture and is also useful for dematiaceous fungi.
STUDY OF THE FUNGAL CULTURES

GROSS MORPHOLOGY:

Fungal growth on SDA were observed. The important factors to be noted are

1. Rate of growth: fungi develop varying characteristics in different media, so it is important to describe the characteristics on standard medium, such as Sabouraud’s Dextrose Agar. Virtually all the medically important fungi are normally described by their appearance on SDA, which is one reason this medium continues to be used as the primary isolation medium. A rapidly growing fungus develops characteristic morphology within 2 to 5 days. Whereas a slow-growing colony may take 2 to 3 weeks: intermediate growers mature within 6 to 10 days. The growth rate will vary with media and temperature changes.

2. General topography, whether flat, hemispherical, or raised, folded, verrucose, cerebriform and heaped margins regular or irregular.

3. Texture, whether yeast-like, glabrous, powdery, granular, velvety or cottony.

4. Surface pigmentation.

5. Pigmentation on the reverse.

MICROSCOPY

Growth from the culture tubes were examined microscopically after placing a small portion of the growth on a glass slide, teasing it with two sterile needles after adding lactophenol cotton blue/normal saline in case of pigmented fungi, and then a coverslip.

Under microscope the following features were noted:

Mycelium- Whether true or pseudomycelium

Hyphae- Whether septate or non-septate
Whether branching or not

Whether pigmentation present or absent

**Spore Bearing Structures:-** Determine how the spores are attached. Do they develop directly from the hypha, as arthrospore and chlamydospores, or from specialized structures known as conidiophore. Are the spore bearing structures simple, such as a short unbranched stalk, or are they more complex with branching and/or whorls.

**Conidia:-** size, shape, and arrangement of conidia

whether smooth or rough

Also noted the presence of any granule from the lesion, color, shape and size of the granules. Cultures were examined at regular intervals and sub culturing was done if contaminants threatened to overgrow the suspected pathogen. All the cultures were retained 2 months before discarding as negative. The major disadvantage of wet mount is that the characteristic arrangement of spores is disrupted when pressure is applied to the coverslip.

There are two commonly used methods for examining the undisturbed microscopic morphology of fungi and they are (1) Adhesive Tape method and Microslide culture method.

**LACTOPHENOL COTTON BLUE:**

**Lactophenol Cotton Blue (LCB)** is used to study the morphological features of the fungal isolates.

1. **Plain LCB:**

   Melted Phenol : 20 ml
   Lactic Acid : 20 ml
   Glycerol : 40 ml
Cotton Blue : 0.05 gm
Distilled Water : 20 ml

The small amount of fungal growth is transferred to a drop of Lactophenol Cotton Blue (LCB) on a clean glass slide and teased apart with dissecting needles. The lactophenol cotton blue kills, preserves, and stains the fungal specimen. A cover slip is applied and the specimen is examined microscopically under low magnification and then to high magnification. The method is limited in usefulness because the spores are often separated from the spore bearing structures, and this makes identification difficult for a number of fungi. This stain is useful for studying the morphology of fungus which are hyaline. The LCB preparation can be permanently preserved if Polyvinyl alcohol is used. It contains the following ingredients:

Polyvinyl alcohol power : 15 gm
Distilled water : 100 ml

Mix the powder at 80° C in a beaker placed in water bath and filter through double-layered cloth.

Staining solution:

PVA stock solution : 56 ml
Melted Pheno : 22 ml
Lactic acid : 22 ml
Cotton Blue : 0.05 gm.

SLIDE CULTURE TECHNIQUE

Adhesive (Scotch) tape preparation:

The transparent adhesive tape preparation allows one to observe the microorganism microscopically approximately the way it sporulates in culture. The spores are intact, and the microscopic identification of an organism can be made easily.
1. Touch the adhesive side of a small length of transparent tape to the surface of the colony.

2. Adhere the length of tape to the surface of a microscope slide to which a drop of lactophenol cotton blue has been added.

3. Observe microscopically for the characteristic shape and arrangement of the spore.

**Microslide culture**

This method might appear to be the most suitable for making the microscopic identification of an organism because it allows one to observe microscopically the fungus growing directly underneath the coverslip. This technique was used to study the undisturbed relationship between reproductive structures and mycelium and also the sporulation characteristics of the organism. Microscopic features should be easily discerned, structures should be intact, and representative areas of growth are available for observation.

1. Cut a small block of suitable agar medium in 4x4 mm thickness.

2. Place the agar block over a sterile glass slide in a Petri dish.

3. With a right ankled wire, inoculate the four quadrants of the agar block with the organism.

4. Apply a sterile coverslip onto the surface of the inoculated agar block.

5. Add small amount of sterile distilled water and incubate at 30 °C.

6. After a suitable incubation period, remove the coverslip and place it on a microscope slide containing a drop of lacto phenol cotton blue/ normal saline in case of dematiaceous fungi.

7. Observe microscopically for the characteristic shape and arrangement of spores.
INVITRO ANTIFUNGAL SUSCEPTIBILITY TESTING:

Antifungal susceptibility tests are designed to provide information that will allow the physician to select the appropriate antifungal agent useful for treating a specific infection. Compared to antibacterial susceptibility test, antifungal susceptibility testing is in a primitive form. Invitro antifungal susceptibility testing is influenced by a number of variables, including Inoculum size and preparation, medium formulation and pH, duration and temperature of incubation, and the criterion used for MIC endpoint determination. In addition, antifungal susceptibility testing is complicated by problems unique to fungi, such as slow growth rates (relative to bacteria) and the ability of certain dimorphic fungi to growth either as a unicellular yeast form that produces blastoconidia or as a hyphal or filamentous fungal form that may produce asexual spores, depending on pH, temperature and medium composition Mc Ginnis, M.R; and M.G.Rinaldi (1986).

The basic properties of antifungal agents themselves, such as solubility, chemical stability, mode of action, and the tendency to produce partial inhibition of growth over a wide range of concentrations, must be taken into account. All variables have been standardized, and efforts are under way to develop interpretative guidelines for different antifungal agents. Numerous antifungal agents have been developed, and the newer agents are on the horizon.

The antifungal susceptibility testing is performed to provide information that allows the clinician to select an appropriate antifungal agent useful for treating a particular fungal infection. The agents may appear resistant in vitro and still have clinical efficacy. Sometimes, there has been no correlation between clinical response and susceptibility with in a fungal species. Unfortunately, this testing has not progressed as far as tests used for deciding the susceptibility of bacteria to the antimicrobial agents. There is no standard method used by all laboratories and there is
disagreement concerning specific conditions of incubation and other variables necessary for performing the test. The problems associated with antifungal susceptibility, which are given below.

1. Problems in relation to the fungal organism
   a. Slow growth rates in relation to bacteria.
   b. Dimorphism in fungal growth as yeast-mold.

2. Problems in relation to the antifungal agents
   a. Solubility in aqueous media.
   b. Stability of the antifungal agent.
   c. Partial inhibition of growth.
   d. Mechanism of action

3. Test conditions that affect the MIC of antifungal agent
   a. Composition and pH of medium
   b. Inoculum preparation and size.
   c. Incubation temperature and time
   d. Endpoint criteria

4. Lack of correlation between results of antifungal susceptibility testing and the clinical outcome.

Despite of all the difficulties, these tests are important for selection of appropriate antifungal agent and as a method to detect the development of resistance in certain organisms during the antifungal therapy.

Classification of Antifungal Drugs

A. ANTIFUNGAL ANTIBIOTICS

1. Polyene Antibiotics
   a) Amphotericin B
1) Conventional amphotericin B.

Amphotericin B deoxycholate

2) Liposomal formulations of Amphotericin B

Amphotericin B lipid complex

Amphotericin B colloidal dispersion

Liposomal- encapsulated Amphotericin B

Nystatin

Pimaricin

Hamycin

2) Other Antibiotics

Griseofulvin

Pradimicin

B. SYNTHETIC ANTIFUNGAL AGENTS

1. Thicarbamates

Tolnaftate

2. Allylamines and Benzylamines

Naftifine

Terbinafine

Butenafine

3. Azoles

1) Imidazoles

Bifonazole

Clotrimazole

Fenticonazole

Miconazole

Oxiconazole

Butoconazole

Econazole

Ketaconazole

Omoconazole

Sulconazole
B. MISCELLANEOUS ANTIFUNGAL AGENTS

- Flucytosine
- Ciclopiroxolamine
- Amorofine
- Whitifield’s ointment
- Potassium iodide
- Selenium sulfide
- Undecylenic acid
- Haloprogin
- Triacetin
- Echinocandin
- Nikkomycin
- Gentian violet paint

The commonly used antifungal agents for treating subcutaneous fungal infections are 

**Polyene Macrolide Antifungals:** The polyenes are water insoluble and are inactivated by heat, light, and acid. The medium should be well buffered (pH 7.0), and the test solutions should be protected from light. These consist of Amphotericin-B, Nystatin, 5-Flurocystosine, and Griseofulvin.

**Amphotericin-B.** Is produced by actinomycete Streptomyces nodosus. It binds the ergosterol component of the fungal cell membrane and alter the selective permeability of this membrane. However, other sterols, including those present in mammalian cell membranes, are also bound. The most important adverse reaction associated with amphotericin B is renal insufficiency. A newer agent, liposomal amphotericin B, reportedly diminishes, this adverse reaction. Conventional and liposomal formulations of amphotericin B are recommended for eumycetoma caused by Madurella and Fusarium species. Before introduction of azoles, amphotericin B was the drug of choice for treatment of relapsed lymphocutaneous infection, pulmonary infection and other unifocal deep form of sporotrichosis.
**Azole Antifungal agents:** The azole group of antifungal agents consists of the imidazoles and triazoles. The important group in imidazoles is ketoconazole and is useful for sporotrichosis. The triazoles group of antifungal agents like itraconazole, posaconazole and voriconazole are used for treating various subcutaneous fungal infections. These agents disrupt the integrity of the fungal cell membrane by interfering with the synthesis of ergosterol. On the other hand, the azoles, except fluconazole, have relatively good chemical stabilities but shows poor solubility in aqueous media.

**Fluconazole:** is a triazoles, which is exceptionally soluble in water. This can be used for oral and intravenous administration. Therapeutic levels are easily reached in the central nervous systems. Side effects of fluconazole therapy are usually minimal. This drug is useful for treating histoplasmosis, blastomycosis, coccidiodomycosis, aspergillosis and for cryptococcal meningitis in AIDS patients.

**Itraconazole:** It is a triazoles antifungal agent having a broad spectrum antifungal activity against most pathogenic fungi except zygomycetes. It has a good response against disseminated aspergillosis, blastomycosis, coccidiodomycosis, paracoccidioidomycosis, phaeohyphomycosis, eumycetoma and chromoblastomycosis caused by cladosporium species.

**Posaconazole:** It is also a triazoles antifungal agent and is useful for various systemic and subcutaneous mycosis.

**Allylamine and Benzylamine:** Allylamine is a newly developed class of synthetic antifungal agents with activity against wide range of fungi. These agents selectively inhibit the key enzyme, squalene epoxidase, which is required for fungal ergosterol biosynthesis. This inhibition is not mediated through cytochrome p-450, consequently, accumulation of squalene weakens the cell membrane leading to fungal cell death. This group consist of Naftifine, Terbinafine and Butenafine
Terbinafine:- It is useful for very useful for various systemic and subcutaneous mycosis

Miscellaneous Antifungal agents:

Flucytosine:- Flucytosine (5-Fluorocytosine) is a synthetic fluoropyrimididine and mainly used in the treatment of infections caused by yeasts and phaeoid fungi. This is a water soluble drug and can be administered orally. It is converted by fungal cytosine deaminase to antimetabolite, 5-fluorouracil, which inhibit thymidylate synthetase and consequently DNA synthesis. Flucytosine and amphotericin B act synergistically and are useful for treating various fungal infections as combination therapy.

Potassium Iodide:- Is the therapy of choice for cutaneous/lymphatic sporotrichosis.

Diaminodiphenylsulphone (dapsone-DDS) This drug is widely used for treating leprosy and is also useful for treating rhinosporidiosis.

Some of the antifungal susceptibility testing methods are described below.

a) Macro and Microdilution methods for Yeast NCCLS (M27-A) The US National Committee for Clinical Laboratory Standards (NCCLS) has released the approved version (M 27-A) of standardized broth macrodilution and microdilution methods for the antifungal susceptibility testing of yeasts in 1997. The M 27 document was proposed in 1992. After two multicentric studies this method has been approved as the standard method for antifungal susceptibility testing for yeasts. This document describes a broth macrodilution and microdilution modifications, specifies a defined culture medium as the standard medium(RPMI 1640 broth buffered to pH 7.0), as well as an inoculum standardized by spectrophotometric reading approximately 1000 cells/ml and visual determination of MIC endpoint determination after incubation at 35° C for 48 hours to 72 hours.
Macro and Microdilution methods for Filamentous Fungi NCCLS(M38-P)

Although the number of serious infections caused by the filamentous fungi is lower than the number of yeast infections, antifungal susceptibility testing of these opportunistic pathogens is important in the clinical laboratory. The determination of MICs for filamentous fungi can be facilitated with a method that overcomes observer's bias and quantifies the hyphal growth of molds. The NCCLS has also proposed the antifungal susceptibility testing of conidia forming filamentous fungi in 1998 which can be performed as per the M38-P document on a similar pattern as that of yeasts. Both these documents, M27-A and M38-P are currently being used worldwide for antifungal susceptibility testing for yeasts and molds, respectively. As turbidity measurements and colony counts are not useful in the case of filamentous fungi, colorimetric methods based on the measurement of metabolic activity may facilitate determination of MIC.

There have been several multicenter studies involving filamentous fungi which have been used in the development of the National Committee for Clinical Laboratory Standards (NCCLS) reference method for broth dilution antifungal susceptibility testing of conidium forming filamentous fungi. The test employs a methodology similar to that for yeasts but requires spectrometric inoculum determination based on conidial size. Interlaboratory agreement is high for the broth dilution, thus making it suitable as a reference standard., Espinel-Ingroff A et al (1997). The inoculum for each isolate is prepared by first growing the fungus on potato dextrose agar slants for 7 days at 35° C.

A conidial suspension is prepared by flooding each slant with approximately 2 ml sterile 0.85 % saline. The resulting mixture is withdrawn, and the heavy particles are allowed to settle for 3 to 5 min. The upper homogenous suspension containing the
conidia is mixed for 15 second with a vortex. The turbidity of the mixed suspension is measured by using a spectrophotometer at 530 nm and adjusted to a specific final transmission range for each species tested. Only conidial suspensions of approximately $0.05 \times 10^4$ to $5 \times 10^4$ CFU/ml have been evaluated for antifungal susceptibility testing of mold by this method. The QC organism used for yeast testing plus an isolate of *Paecilomyces variotti* ATCC 22319 may be tested in the same manner as the other isolates and should be included each time an isolate is evaluated with any antifungal agent.

All tubes and microdilution trays are incubated at $35^\circ$C and observed each for the presence of growth, when growth is visible in the growth control, each tube is vortexed for 10 seconds. Immediately prior to being scored, this allows the detection of small amounts of growth. The growth in each tube and well is compared with that of the growth control (drug free) and given a numerical score as follows: 0, optically clear or showing no growth; 1, approximately 75 % reduction in growth; 2, approximately 50 % reduction in growth; 3, approximately 25 % reduction in growth; 4, no reduction in growth. The MIC endpoint criterion for molds is the lowest drug concentration that inhibits approximately $\geq 75$ % of the growth of the fungus being tested compared with the control. Espinel-Ingroff A et al (1993). However, it is somewhat cumbersome to perform and not likely to be used in clinical microbiology laboratories.

Thus modifications of the reference method are acceptable and expected. With this in mind, several modifications of the macrobroth reference method of antifungal susceptibility are currently under investigation. They offer promise as alternative approaches that may better serve practical clinical laboratory needs. To improve objectivity and speed of current antifungal susceptibility testing, the yeast Rapid Susceptibility Assay (RSA) was adapted for *Aspergillus fumigatus* Tracy J. Wetter et al (2003).
This method is based on glucose utilization in the presence of an antifungal drug. *Aspergillus fumigatus* conidia were incubated in 0.2 % glucose RPMI 1640 containing 0.03 to 16 micro gram of amphotericin B or itraconazole/ml. Drug-related inhibition of glucose utilization correlated with suppression of conidial germination. Following incubation of conidia with various concentration of antifungal drug, the percentage of residual glucose in the growth medium was determined colorimetrically and plotted against drug concentration to determine the MIC. National Committee for Clinical Laboratory Standards (NCCLS) M 38-P testing was also performed to obtain NCCLS MIC’s for direct comparison. Result of this study showed that the mold RSA provides a more objective and rapid method for aspergillus species susceptibility testing than the NCCLS M 38-P assay.

A simple screening semisolid agar antifungal susceptibility test (SAAS) method was developed by Cigdem etal (2004). They compared MIC results of the NCCLS M38-P broth dilution method with SAAS screening test for four antifungal agents tested against 54 clinical isolates of filamentous fungi. The antifungal agents used for the study were amphotericin B, amphotericin B lipid complex, itraconazole, posaconazole. The SAAS test supported the growth of all filamentous fungi tested. They found excellent concordance of results for all four drugs tested and found that the SAAS test compared favourably to NCCLS broth micro dilution test for molds and might be useful preliminary screening test for molds. This test for filamentous fungi uses inocula prepared from a colony swab, without the need for special equipment.

Carmen Castro, M etal 2004 compared the susceptibilities of 63 isolates of *Aspergillus* species to voriconazole by a modified NCCLS M38-A method and Sensititre Yeast One Colorimetric method. The overall agreement was 82.5%, ranging
from 100% for *Aspergillus niger* and *Aspergillus terreus* to 62.5% for *Aspergillus flavus*. This test is a commercial colorimetric panel that consists of a disposable tray which contains dried serial dilutions of five antifungal agents in individual wells. The wells also contain an oxidation-reduction indicator (Alamar blue) to generate clear-cut endpoints based on a visually detectable color change. The MIC obtained by this method was compared to those obtained by the modified reference broth micro-dilution method. Sanchez Sousa A et al (1999).

**The interpretative guidelines as defined by NCCLS are as follows**

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>Susceptible concentration</th>
<th>Resistant concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole</td>
<td>≤ 8.0 μg/ml</td>
<td>≥64.9 μg/ml</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>≤ 0.125.0 μg/ml</td>
<td>≥0.1 μg/ml</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>≤ 4.0 μg/ml</td>
<td>≥32.0 μg/ml</td>
</tr>
</tbody>
</table>

Amphotericin B susceptibility or resistance cannot be distinguished using the NCCLS method. It is suggested that an MIC of at least 1.0 μg/ml be considered as resistant; however, this information is tentative. Ketoconazole susceptibility testing has suggested that isolates with an MIC between 0.313 and 16 μg/ml be considered as susceptible.

**b) Disk Diffusion Method:**

This method has wide-spread use for antibacterial drug testing. The agar diffusion testing has limited application in antifungal drug susceptibility testing. This method is useful for testing the antifungal action of flucytosine. In this method a disk containing the antifungal agent which diffuses in the surrounding medium, inhibit the growth of fungi and measurements of zone of inhibition are taken.

**c) Etest:**

The Etest is a patented commercial method for determination of MIC. It is set
up in a similar method as the disc diffusion test except the disk is replaced by a calibrated plastic impregnated with a concentration gradient of the antifungal agent.

d) Fungitest:

This is an alternative to the NCCLS reference procedures in which growth of isolates is measured in cultures containing just one or two antimicrobial drug concentrations that distinguish resistant from susceptible strains.

e) Spectrophotometric Methods

This method is used to determine MIC end points more objectively by reading broth micro dilution plates with spectrophotometer. However, the determination of spectrophotometric MIC requires the selection of a level of inhibition and different studies have employed different endpoint definitions.

f) Flowcytometry

During the last decades, flowcytometry has been developed as a powerful tool in many diagnostic and research laboratories. A rapid assay of antifungal activity has been developed by utilizing flowcytometry to detect accumulation of a vital dye in drug damaged fungal cells. It has been suggested that flowcytometry may provide an improved, rapid method for determining and comparing the antifungal activities of compounds with different mode of action.

**ANTIFUNGAL SUSCEPTIBILITY TESTING USING AGAR DILUTION**

In the present study agar dilution method is used for invitro antifungal activity of terbinafine. Serial dilutions of drug are prepared in Sabouraud’s agar and poured into tubes. Conidial suspension of the test fungus was prepared in BHI broth at a concentration of $10^6$ cells/ml. 0.1 ml of the above conidial suspension was inoculated into the sabourud’s tube with serial dilutions of antifungal agent. The tubes were incubated at room temperature and observe for growth. Control tubes without antibiotics were also included.