Laboratory Diagnosis of Mycoses

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Introduction:

Diagnosis in the laboratory is made by demonstration of the fungus in the skin, exudates or deeper tissues. Isolation of the organism in culture is usually necessary to confirm this and to identify the specific causative fungus.

The detection of antibodies and antigens in patients' serum is necessary in the systemic infections, particularly when an early diagnosis is desired, but these tests are generally carried out in specialised laboratories.

The diagnosis of a superficial fungus infection is made by the observation of fungal elements in infected keratin. This can be carried out in a few minutes while the patient is attending the clinic and enough information is usually obtained for treatment to begin.

The production of fluorescence under a Wood’s lamp (wavelength 365 A°) will also aid in the detection of certain infected hairs and in some other skin conditions, e.g. pityriasis versicolor.

Experiment 1. Collection of cutaneous and infected nails specimens:

Skin

Skin lesions are sampled by scraping with a blunt scalpel and collecting the scales onto clean glass slides (with a spreading lesion the active periphery is selected).

1. Wipe the infected area (lesions) with 70% alcohol to remove bacterial contaminants.
2. Scrape the site of infection (with a spreading lesion, the active periphery is selected) gently with the side of a blunt scalpel blade or the edge of a glass microscope slide. Where vesicles are present, the roof of the blister, cut off with fine pointed scissors, will often reveal abundant hyphae.
3. Collect scrapings (scales and hairs) either in a Petri dish or in a paper envelop, or onto clean glass slides for further processing. Vellus hairs from the limbs or face will often show mycelium in the follicle when it may be scanty elsewhere.

Hairs:

A pair of flat-ended forceps is used to remove scalp or body hairs but, if they are infected, hair stumps can be removed easily by scraping with a scalpel.

The morphology of the spore arrangement may be more readily preserved if the hairs remain embedded in a scale.

1. Using a pair of flat-ended forceps remove scalp or body hairs. Scrape infected with a scalpel. The morphology of the spore arrangement may be more readily preserved if the hairs remain embedded in a scale.
2. Collect hairs either in a Petri dish or in a paper envelop, or onto clean glass slides for further processing.
3. Scalps may be sampled for culture using a brush technique which is a useful method to survey siblings and contacts of infected children and also suspected pets in the household.

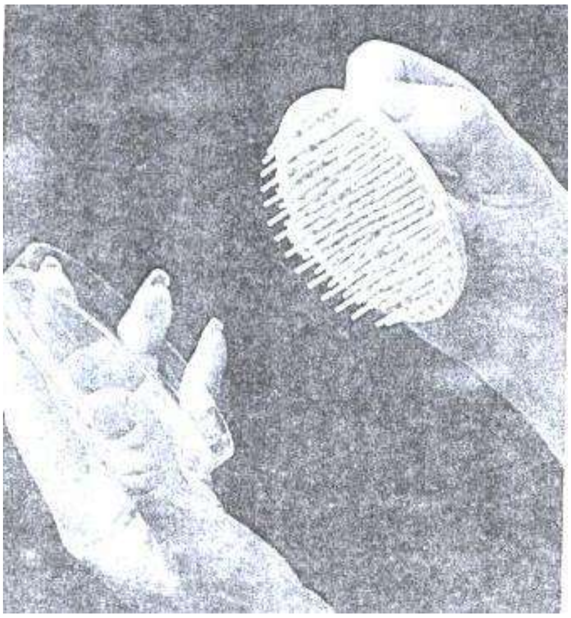


Figure 1. A commercially available massage brush is pressed into a petri dish containing medium after vigorous brushing of the scalp.

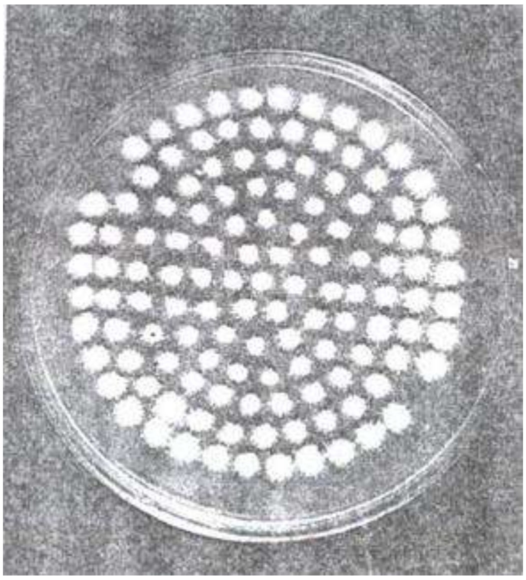


Figure 2. Clinically infected children produce fungal colonies from many points of the brush.

Nails*:*

Nails are often thickened when infected and clippers are essential to cut off the whole thickness, although submerged debris, removed with a scalpel or probe, may contain fungal elements.

1. Using clippers, cut off the whole thickness of the infected nails. Remove submerged debris, with a scalpel or probe.
2. Collect nail clippings and submerged debris in a Petri dish or in a paper envelop, or a sterile tube for further processing.

Mucosae*:*

Mucosae are sampled with cotton swabs. These are placed in transparent medium if there is to be any delay before processing.

Sample with cotton swabs, and place the swabs in transparent medium if there is to be any delay before processing.

Sputum, body fluids, biopsies, etc.

Collect in sterile containers.

Labeling of collected specimens: label's information may include, subject's name, sex, age, profession, infected part, infection type, contact with domesticated animals, medication, and other important notes.

Record your observations.

Experiment 2. Microscopic examination of clinical specimens.

Direct microscopy

Samples of skin, hair or nail are placed in a drop of 30% potassium hydroxide (KOH) directly on a microscope slide; after placing a coverslip in position, the specimens can be examined immediately.

Softening of the tissue can be hastened by heating gently but hairs should he handled with particular care and allowed to soften without heat so that the arrangement of the spores will not be destroyed.

The incorporation of dimethyl sulphoxide (DMSO) in the potassium hydroxide (DMSO 40ml; distilled water 6Oml; KOH 30g) may also help to clarify the specimens.

When examining specimens it is important to ensure that the material has softened adequately and that the intensity of light passing through is not too strong. It is also necessary to alter the focus while scanning the slide.

Unstained preparation are generally satisfactory for the demonstration of fungi in keratin, but clorazole black E may be added to DMSO to aid in the differentiation of hyphae from common artefacts such as cotton fibres.

If hairs are infected the size of arrangements of the spores, together with the ability to fluoresce under a Wood's lamp will help towards the identification of dermatophytes species involved.

Table Hair invasion by dermatophytes.

Species	**Spore arrangement**	**Size (microns)**
Microsporum		
M. canis	Ectothrix *	2-3
M. gypseum	Ectothrix*	2-3
M. audouinii	Ectothrix*	2-3
M. ferrugineum	Ectothrix*	2-3
Trichophyton		
T. violaceum	Endothrix	4-8
T. tonsurans	Endothrix	4-8
T. soudanese	Endothrix	4-8
T. schoenleinii	Endothrix	
T. mentagrophytes	Ectothrix	3-5
T. verrucosum	Ectothrix	5-10

*Fluorescence under Wood's lamp

Parker's stain, (equal parts of 30% KOH and Parker's blue-black ink) is particularly useful to demonstrate the fungus in scales from pityriasis versicolor as the organism takes up this stain *immediately*. It may also be helpful to distinguish some non-dermatophyte infections of nails but dermatophytes will only take up the blue colour after several hours in the stain.

Smears from mucosae may also be examined as unstained wet preparations in saline or KOH but heat fixed preparations may be stained by Gram or by periodic acid Schiff (PAS).

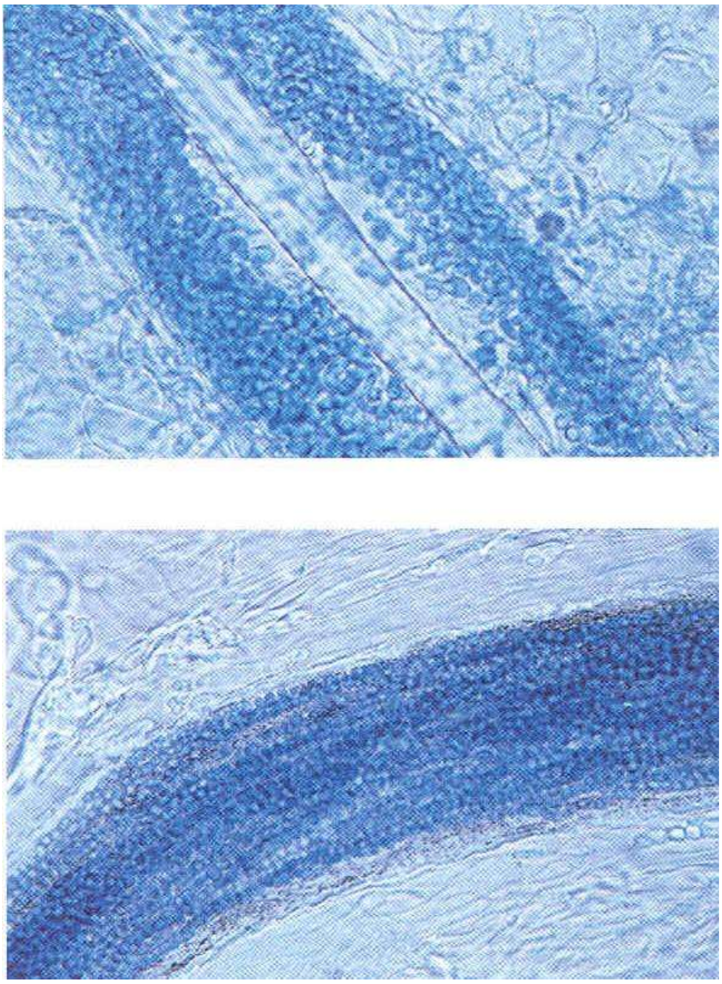


Figure 3. Ectothrix (top) hair invasion showing the formation of arthroconidia on the outside of the hair shaft. The cuticle of the hair is destroyed. Endothrix (bottom) hair invasion showing the development of arthroconidia within the hair shaft only. The cuticle of the hair remains intact.

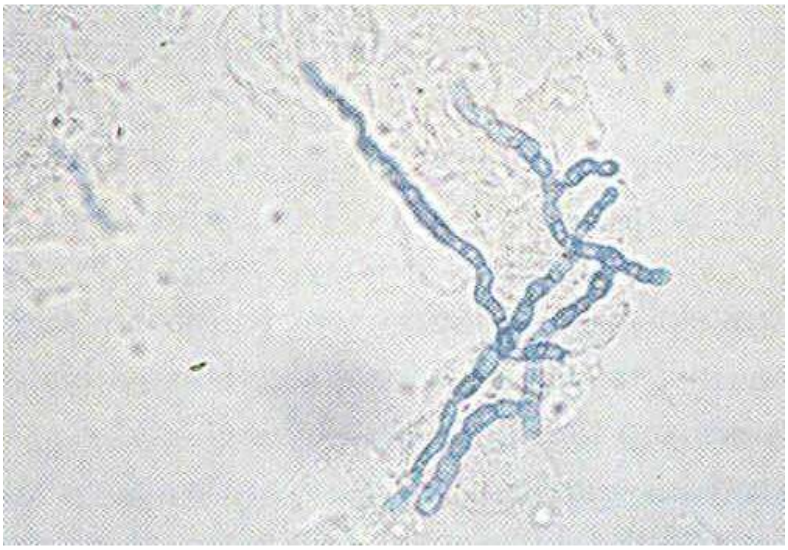


Figure 4. Skin scraping showing hyaline, septate hyphae diagnostic for a dermatophyte.

Pus, exudates and body fluids may he spun down and the deposit examined for the presence of yeasts, filaments or spherules either unstained or after staining by Gram, PAS, methenamine silver, Gemsa or mucicarmine according to the disease suspected.

For cryptococcosis, and particularly when any CSF material is being examined, an India ink preparation should he included

Sections from biopsy specimens should be stained using the above stains as appropriate.

Potassium Hydroxide (KOH) Mounting Fluid:
Used to search for fungus in skin scrapings and hair. Twenty percent KOH causes keratin to swell and clear, and provides an advantageous refractive index to reveal the fungus hyphae.

With DMSO		Without DMSO	
KOH crystals	20 gm	KOH crystals	20 gm
Dimethyl Sulfoxide (DMSO)	40 ml	Glycerin	20 ml
Distilled water	60 ml	Distilled water	80 ml

Use of KOH is one of the most rapid methods for direct examination of clinical specimens. The rate of clearing can be increased by gently heating the slide. If used without DMSO, the slide must be slightly warmed to promote clearing. Addition of DMSO permits quick examination often without warming. Addition of DMSO or glycerin prevents rapid drying of the fluid, permits observation of the slide for up to 24 or 48 hours. Slides may be ringed with fingernail polish to save for longer periods. To provide a semi-permanent preparation, the KOH must be replaced by glycerin by capillary displacement. Blot at one side of slide and slowly add glycerin from the other side.

Prepare a temporary mount as follows.

a. Mix a small portion of the specimen (scales, hair or nail) in a drop of water, physiologic saline, or 10 - 30 % KOH containing 10 % glycerin on a microscopic slide. The incorporation of dimethyle sulfoxide (DMSO) in KOH (DMSO 40 ml; distilled water 60 ml; KOH 30 g) may also help to clarify specimens.
b. Add a coverslip over the drop.
c. If KOH is used without DMSO, warm the slide on an electric hot plate or a very low Bunsen burner flame until the tissue has cleared. …
d. Gently press the coverslip to make the specimen almost transparent. Hairs should be handled with particular care and allowed to soften without heat so that the arrangement of the spores will not be destroyed.
e. Let the mount stand at room temperature for approximately 30 minutes if the specimen is thick or viscous.
f. Examine microscopically for the presence of hyphae or other fungal structures.

Illustrate and record your observations.

Experiment 3. Ultraviolet (Wood's Light) examination for fluorescent hairs

Hairs infected with the parasitic *Microsporum* species may be detected by the yellow green fluorescence in 365 A ultraviolet. The skin itself does not fluoresce. In veterinary medicine the Wood's light is useful to detect *M. canis* infections in cat, dogs, and other small animals.

Illustrate and record your observations.

Experiment 4. Culturing of fungi:

Petri dishes are very satisfactory for the culture of fungi but cotton wool plugged tubes may also be used.

Screw-capped bottles are essential when hazardous species such as *Histoplasma capsulatum* and *Coccidioides immitis* are suspected but they do not allow for the maximum development of spores and pigments which are necessary for the identification of many species.

Sabouraud Dextrose Agar (SDA) is the medium most commonly used (Peptone-preferably mycological, 1 %, Dextrose 4%, Agar 1.5%, pH 5.6).

A broad spectrum antibacterial antibiotic, e.g. chloramphenicol (0.005 %) is incorporated and the medium can be made selective for dermatophytes by adding cycloheximide (0.05%) to inhibit contaminating fungi, however, when attempting to isolate yeasts or any other non-dermatophytes, cycloheximide should be excluded or a duplicate series of plates used. Both chloramphenicol and cycloheximide may be added to the medium before sterilising.

Sabouraud Dextrose Agar is available commercially in dehydrated form from several sources, but as the morphology of the fungi, particularly the production of pigments, will vary according to the source of the medium. This also applies to the nature of the peptone if the medium is prepared from individual ingredients.

A medium which will aid a non-specialist laboratory to differentiate dermatophytes from other fungi is Dermatophyte Test Medium (DTM).

A change in pH produced by the proteolytic activity of dermatophytes is demonstrated by the incorporation of phenol red in the medium. A change from yellow to red indicates the presence of a dermatophyte.

The medium can be used for primary cultures if antibiotics are included and has a particular use in identifying dermatophyte isolations in large field studies. It is available commercially.

A richer medium such as Brain Heart Infusion Agar may be necessary to isolate fungi causing deep mycoses, particularly for the yeast phases of the dimorphic species.

The medium should be poured to give thick plates (25ml / plate) and allowed to dry by leaving at room temperature overnight, so minimising drying out of the agar throughout the long incubation time.

Hairs, small fragments of skin, or nails clipped into as small pieces as possible are placed on the surface of the agar with the aid of a straight needle and pressed into the surface to make a good contact.

Biopsy material should be ground or cut up into small pieces to give numerous inocula, and for the culture of blood, vented blood culture bottles with biphasic media are recommended.

Incubation at room temperature is adequate for the isolation of dermatophytes but 26^o C to 28^o C is preferred. Cultures may be identified after 10 to 14 days' incubation.

Yeasts and aspergilli may be isolated at 37^oC on plates incubated for 2 to 10 days. When a dimorphic fungus is suspected, cultures should be placed at both 26^o C and 37^o C in order to isolate the filamentous and yeast phases of the organisms, respectively.

Plates or bottles of culture from deeper tissues should be incubated for up to 3 to 4 weeks according to the disease suspected.

Scalps may be sampled for culture using a brush technique which is a useful method to survey siblings and contacts of infected children and also suspected pets in the household.

Dermatophyte Test Medium (DTM)

Dermatophyte Test Medium (DTM) is a specialized agar used in medical mycology. It is based on SDA with added cycloheximide to inhibit saprotrophic growth, antibiotic to inhibit bacterial growth, and phenol red a pH indicator. The pH indicator is useful in distinguishing a dermatophyte fungus, which utilizes nitrogenous material for preferred metabolism, producing alkaline by-products, imparting a red color change to the medium. Typical saprotrophic fungi utilized carbohydrates in the medium producing acidic by-products and no red color change.

This selective medium excludes most bacteria and many contaminant fungi. It is combined with phenol- red (a yellow → red pH indicator) which is quickly affected by dermatophytes and related fungi to permit their early recognition.

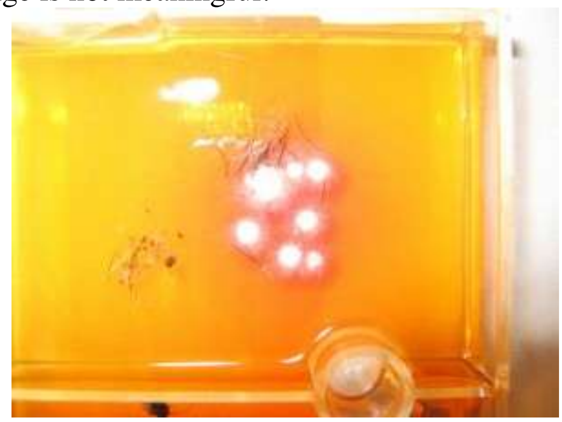
Phytone (BBL)	10 gm
Dextrose	10 gm
Agar Agar	20 gm
Phenol Red solution	40 ml
HCl (0.8 N)	6 ml
Cycloheximide (Actidion)	0.5 gm
Distilled water	1000 ml
Gentamicine sulfate	0.1gm of active drug (= 100 µg / ml)
Chlortetracycline HCL	0.1 gm (= 100 µg / ml)

1. Add phytone, dextrose and agar to 1000 ml distilled water, boil to dissolve.
2. Add 40 ml of phenol red solution while stirring [phenol red solution, 0.5 gm phenol red dissolved in 15 ml 0.1 N NaOH, made up to 100 ml with distilled water.
3. Add 6 ml 0.8 N HCL with stirring.
4. Dissolve 0.5 gm cycloheximide in 2 ml acetone , add it to hot medium while stirring
5. Dissolve gentamicin sulfate powder in 2 ml Distilled water, to give a final concentration in the medium of 100 µg / ml of active drug.
6. Autoclave at 121 °C for 10 minutes, cool to 47 °C.
7. Dissolve 0.1 g chlortetracycline HCL in 25ml of sterile distilled water, then added to the medium while stirring.
8. Pour in 30 ml culture tubes (as slants) or in plates, cool.
9. Store under refrigeration.
10. After inoculation leave caps loose to allow adequate growth and to develop color change. Incubation is at 28 °C.

11. Color change of indicator (in case of dermatophyte growth) should be interpreted not later than two weeks after inoculation. A change in pH produced by the proteolytic activity of dermatophytes is demonstrated by Phenol red indicator that is added to the DTM, changing from yellow to red indicates dermatophyte presence.

The DTM incorporates antibiotics (gentamicin and chlortetracycline) to suppress bacterial growth, cycloheximide to suppress saprophytic fungal growth, and phenol red as a color indicator. It provides both carbohydrate and protein nutrients. Dermatophytes (fungi belonging to the genera *Microsporum*, *Trichophyton* and *Epidermophyton*) preferentially use protein as an energy source, whereas most saprophytic fungi use carbohydrates first. This is the key to interpreting the color change that may be seen with DTM.

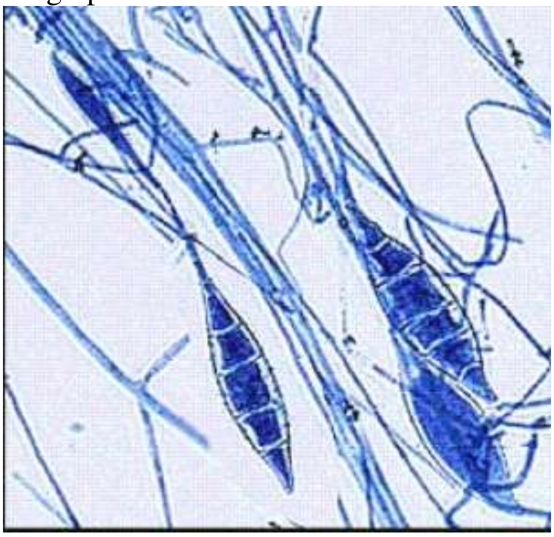
The early growth of dermatophytes will usually cause the DTM to change from yellow to red, due to the production of alkaline by-products of protein metabolism. Saprophytes may do the same, but usually only when the colony is much larger. This is why it is important to check the fungal cultures daily—if a large colony has already grown over the DTM, a red color change is not meaningful.



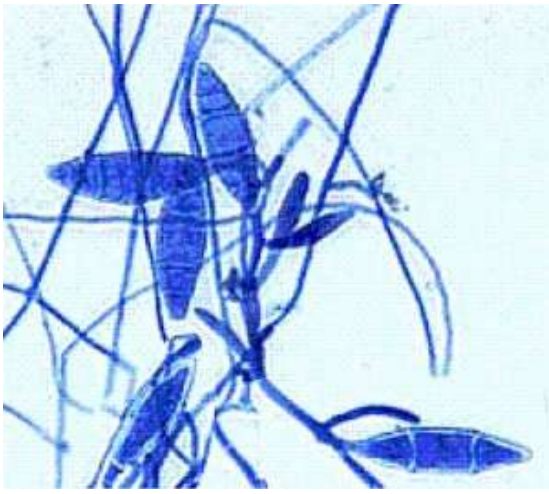
Trichophyton growth on DTM

In addition to observing for a red color change, the interpretation of DTM growth should include an assessment of macroscopic and microscopic colony morphology. Dermatophytes grown on DTM are generally lightly pigmented (white, buff, tan or cinnamon-colored), whereas common saprophytes are often darkly pigmented. The colony surfaces of the dermatophytes of medical importance are often described as powdery, granular or cottony.

A slide for microscopic examination of suspect colonies is prepared by lightly touching a piece of clear cellophane tape to the surface of the colony, then placing it on the slide with a drop of the lactophenol cotton blue. Macroconidia of *M. canis* and *M. gypseum* are usually easy to find, but *T. mentagrophytes* often doesn't produce macroconidia on DTM. *M. canis* macroconidia typically have six or more cells, thick cell walls and knobby ends. *M. gypseum* macroconidia tend to be numerous, ellipsoid, have thinner cell walls and four to six cells. Some microscopic characteristics of *T. mentagrophytes* that can be seen are spiral hyphae and grape-like clusters of one-celled microconidia.



Microsporum canis macroconidia



Microsporum gypseum

Integrating the results of any color change, the macroscopic colony appearance, and the microscopic findings yields the most accurate interpretation. The fungal culture is one of the most important laboratory procedures in dermatology. It should be used to rule out ringworm, as much as to confirm it.

Sabouraud's Dextrose Agar (SDA):

Dextrose	40 g
Peptone	10 g
Agar	20 g

Mix the ingredients, boil to melt the agar, adjust pH to 5.6 and sterilize. Autoclave for 10 min.

A selective isolation media for dermatophytes can be made by supplementing SDA with chloramphenicol (50 mg/ L) and cycloheximide (0.5 g/L). To add chloramphenicol add 50 mg in 2 ml of 95 % ethanol, add this to boiling medium and quickly cool. To add cycloheximide add 0.5 g in 2 ml acetone; add it to hot medium while stirring.

Modified Sabouraud's Dextrose Agar (SDA):

Dextrose	40 g
Peptone	10 g
Agar	20 g
Yeast extract	05g

Prepare as above except: addition of yeast extract (5 g /L), and gentamicin sulfate dissolved in 2ml sterile distilled water to give a final concentration in the medium of 100 ug/ml of active drug.

Potato Dextrose Agar (PDA)

Potato (peeled and diced)	200 g
Dextrose (glucose)	20 g
Water	1000 ml

Rinse potato under running water, and then add to water. Boil for 1 hour. Filter through a cloth, squeezing through as much pulp as possible. Autoclave at 15 psi for 30 minutes. Useful for the growth of many fungi, especially phytopathogens, and some bacteria.

Culturing skin scales, nail scrapings, and hairs:

a. Place skin scales, nail scrapings, or hairs on agar culture media (SDA or modified SDA supplemented with chloramphenicol 0.005 %, gentamicine sulfate 0.01 %, and cycloheximide 0.05 %) and submerge portions beneath the surface of the agar with an inoculating needle.
b. Incubate cultures at 25 – 30 ºC for 1-4 weeks.
c. Examine growing fungal colonies

Culturing nails: bacteria and other factors limit the chance of isolating a dermatophyte from large pieces of nail. There are two main types nail infection. 1. the type invading beneath the nail plate and 2. the white spot type invading the top surface of the nail plate. In the deep type KOH positive material may be obtained from beneath the nail plate, and spread out on the agar. In the superficial white spot type of onychomycosis the white chalky spots on the nail plate are easily scraped off and spread on agar. Non-dermatophyte fungi may be involved in nail infections, and a percentage of clinically suggestive nails seem to harbor no filamentous fungi at all.

Culturing highly inflammatory lesions of the body, beard, and scalp (kerion): These are sometimes difficult to culture because the fungus and parasitized hairs may be scarce and may be in pus in the depths of the follicles or small abcesses in a granulomatous tissue reaction. Plucking and culturing hairs and pus on DTM is recommended. Sparse chains of spores may be found in the hairs by KOH examination. The Gram stain often reveals bits of hyphae in pus more successfully than a KOH preparation. Tinea corporis and tinea barbae of this type is frequently associated with *T. mentagrophytes, T. verrucosum*, and sometimes *T. rubrum*.

Culturing siblings and contacts of infected children and suspected pets in the household: Hair brush (massage brush) is used to sample scalps of infected children and also suspected pets fur. The brush is then pressed into a Petri dish containing medium after a vigorous brushing of the scalp or a pit's fur. Clinically infected subjects produce fungal colonies from many points of the brush.

Culturing animals for ringworm infections: Before making the culture, the hair on the lesions should be first clipped and the site thoroughly washed with soap and water. The culture should consist of clean scrapings of epidermal scales and selective culture of broken or fluorescent hairs, rather than inoculation of the agar with matted and crusted tufts of hair from the lesions without prior washing. Multiple cultures are recommended whenever a contamination problem is anticipated. The pH indicator in DTM is particularly advantageous in detecting the early outgrowth of dermatophytes among contaminant fungi. Isolation of T. verrucosum (cattle ringworm) is favored at 37 °C.

Record your observations.

Experiment 5: Identification of isolates

Fungi are usually identified by the recognition of morphological features and, to a lesser degree, by their biochemical properties.

Macroscopic features such as the texture of the colony, the surface colour and production of pigments seen on the reverse side of the plate may be diagnostic.

To examine under the microscope, a small portion of the culture is teased out in a drop of lactophenol cotton blue.

Lactophenol (Aman's medium):

Lactic acid	20 ml
Phenol crystals	20 g
Glycerol 40 ml	
Water	20 ml
Aniline blue	0.05 g

Add in order: 1. lactic acid and glycerin, and distilled water, 2. add phenol and dissolve with even heat, 3. add the stain (0.05-g aniline blue (cotton blue), or trypan blue per 100 ml) and dissolve, 4. filter through paper. (Alternative method: Lactophenol can be prepared by warming the phenol with the water until dissolved, and then adding lactic acid and glycerol.).

If sporulation is adequate, a strip of sellotape pressed on to the culture surface and subsequently placed on a drop of stain and pressed onto the slide will reveal the spore arrangement satisfactorily.

A slide culture prepared by inoculating each side of a block of agar on a slide is more time consuming but may be necessary to encourage sporulation. After sufficient growth has developed, the agar block is discarded and stained preparations made of the growth remaining on the slide and coverslip.

In some cultures (e.g. *T. schoenleinii*), the diagnostic features can be seen by placing the culture plate directly on the stage of the microscope and examining the reverse side directly with a low power objective.

For species identification see species descriptions in Rebell & Taplin (1978) and any other available references.

Illustrate and record your observations.

Identification of common dermatophytes

- The identification of dermatophytes (Table 2) is primarily based on the microscopic morphology of the fungus.
  - A good slide preparation is required and in some strains sporulation stimulation may be required.
  - Culture characteristics, such as surface texture, topography and pigmentation, are variable and are therefore the least reliable criteria for identification.
- Clinical information such as
  - the site,
  - appearance of the lesion,
  - geographic location,
  - travel history,
  - animal contacts and
  - race is also important, especially in identifying rare non-sporulating species such as *M. audouinii, T. concentricum, T. schoenleinii* and many others.

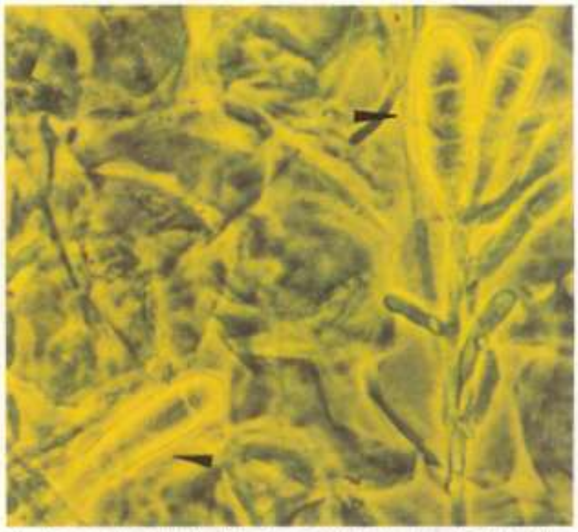
Table 2 Practical identification of common dermatophytes

Key features	**Group**	**Common species**
1) Smooth thin-walled macroconidia only, no microconidia; colonies are green-brown to khaki colour	*Epidermophyton*	*E. floccosum*
2) Macroconidia with rough walls present; microconidia may also be present	*Microsporum*	*M. canis* *M. gypseum* *M. Nanum*
3) Microconidia present, smooth-walled macroconidia may or may not be present	*Trichophyton*	*T. rubrum* *T. mentagrophytes* *T. tonsurans* *T. equinum*
4) No conidia present, colonies sterile. Note: chlamydoconidia are non- diagnostic.	Non-sporulating *Microsorum* and *Trichophyton* species	*M. audouinii* *M. ferrugrineum* *T. verrucosum* *T. violaceum* *T. schoenleinii* *T. soudanense* *T. concentricum*

Epidermophyton group

- *Epidermophyton floccosum* is an anthropophilic dermatophyte with a worldwide distribution that often causes tinea pedis, tinea cruris and tinea corporis.
- Key features include
  - characteristic greenish brown or khaki coloured cultures,
  - the production of smooth, thin-walled, club-shaped macroconidia and the absence of microconidia (Figure)

10. Culture of E. floccosum, partly pleomorphic



11. Microscopic examination of a fragment of an E. floccosum culture: club-shaped spindles in typical banana-like clusters. Phase-contrast x 400

***Microsporum* group**

- In this group it is essential to observe macroconidia to make the identification.

Difficulties may occur with non-sporulating strains of *M. canis and with the differentiation between M. canis* and *M. audouinii*.

Microsporum canis

- is a zoophilic dermatophyte of worldwide distribution that is a frequent cause of ringworm in humans, especially children.
  - It invades hair, skin and, rarely, nails.
  - Cats and dogs are the main sources of infection.
  - Invaded hairs show an ectothrix infection and fluoresce a bright greenish- yellow under Wood's ultraviolet light.

- Key features include
  - distinctive spindle-shaped macroconidia,
  - culture characteristics (Figure), and
  - abundant growth and sporulation on polished rice grains and
  - *in vitro* perforation of hair.

8. Culture of M. canis

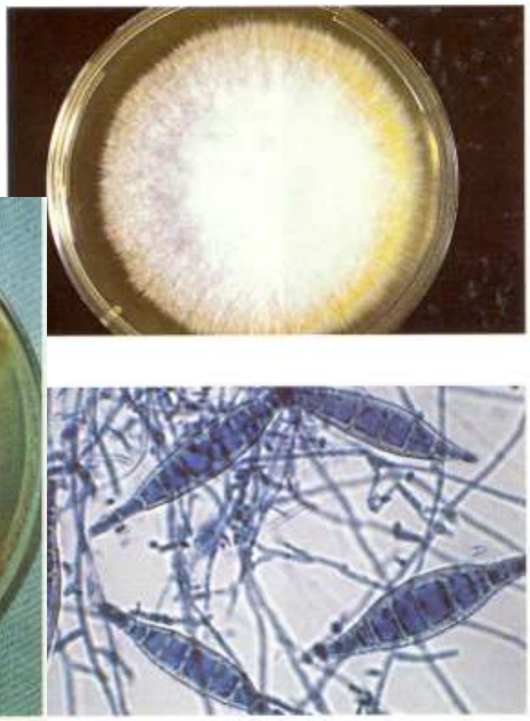


Figure 1.2
(a) Cultures of M. canis *are flat, spreading white to cream-coloured, with a dense cottony surface, and they usually have a bright golden-yellow to brownish-yellow reverse pigment.*
(b) Macroconidia of M. canis *are typically spindle shaped with 5–15 cells, verrucose, thick walled, and they often have a terminal knob.*

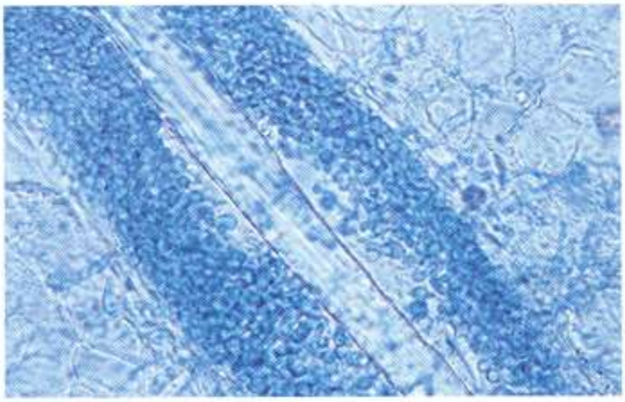


Figure 1.17
Ectothrix hair invasion showing the formation of arthroconidia on the outside of the hair shaft. The cuticle of the hair is destroyed.

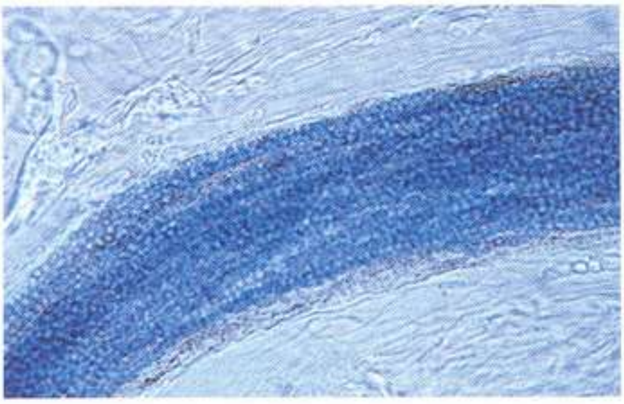


Figure 1.18
Endothrix hair invasion showing the development of arthroconidia within the hair shaft only. The cuticle of the hair remains intact.

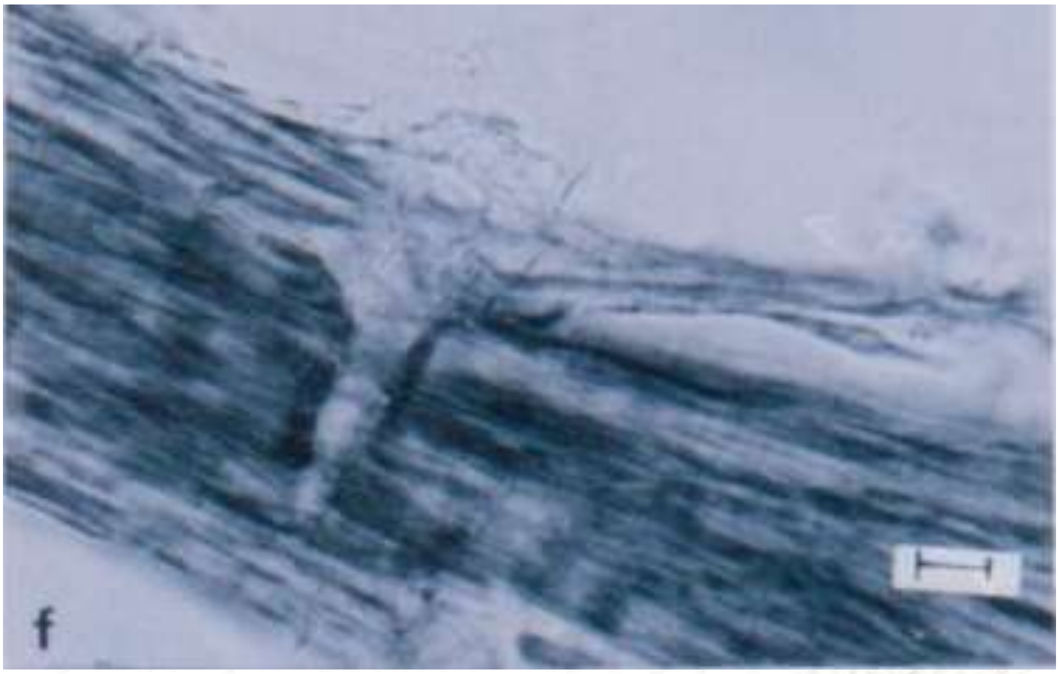
Wood's lamp

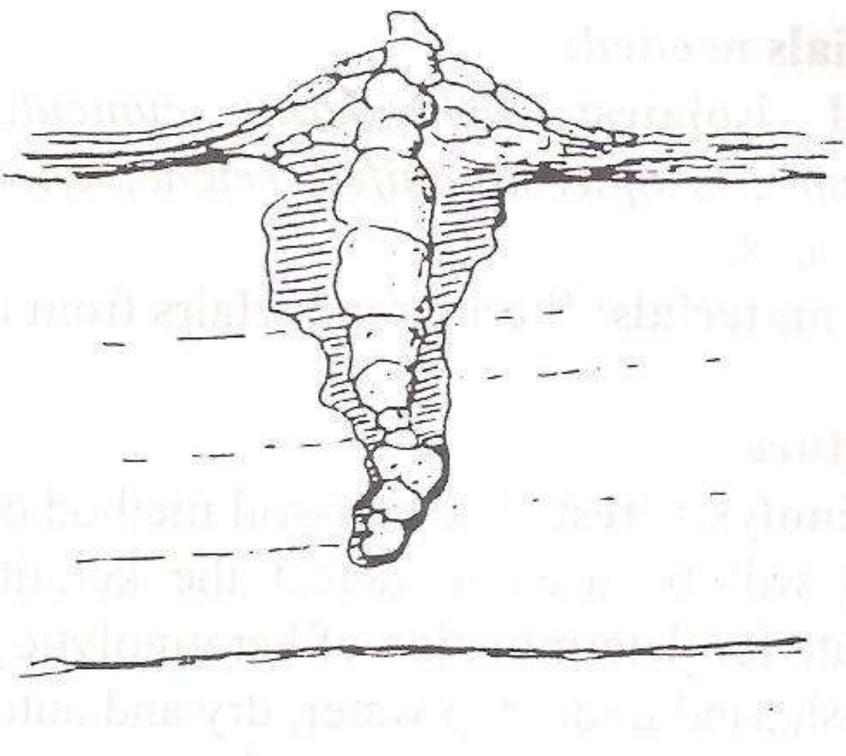
Etymology: Robert W. Wood, American physicist, 1868-1955

- an illuminating device with a nickel oxide filter that holds back all light except for a few violet rays of the visible spectrum and ultraviolet wavelengths of about 365 nm.
- It is used extensively to help diagnose fungus infections of the scalp and erythrasma.
- The light causes hairs infected with a fungus such as *Microsporum canis* to become brilliantly fluorescent.

(Principle: The rays from a quartz lamp pass through Wood's filter, which screens out visible UV rays and transmits longer invisible UV rays which upon contact with some chemicals cause a luminescence.)

Hair perforation





Microsporum gypseum

- is a *geophilic* fungus with a worldwide distribution that may cause infections in animals and humans, particularly children and rural workers during warm humid weather.
- It usually produces a single inflammatory skin or scalp lesion.
- Invaded hairs show an *ectothrix* infection but do not fluoresce under Wood's ultraviolet light.

- Key features include
  - distinctive macroconidia and
  - culture characteristics (Figure)

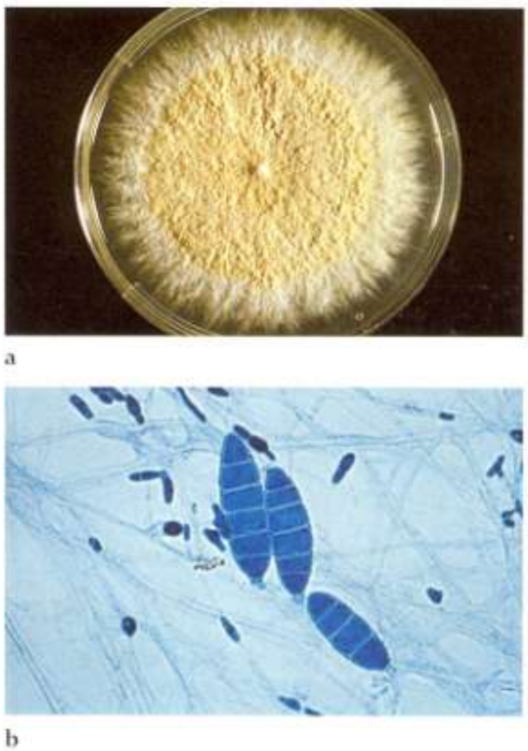
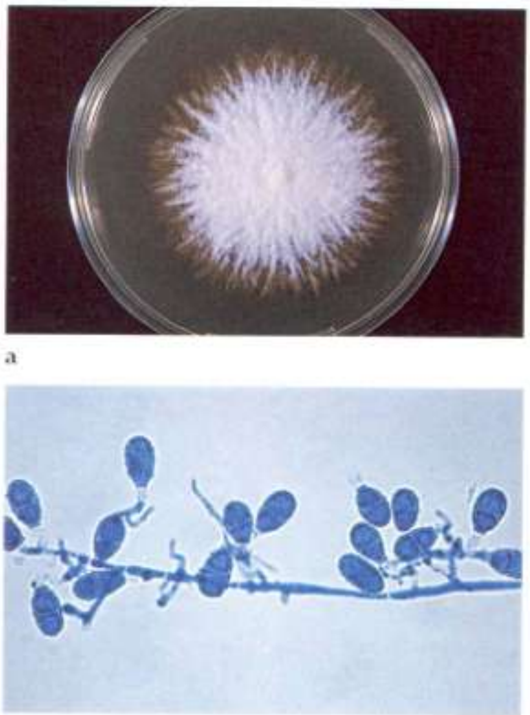


Figure 1.3
(a) Cultures of M. gypseum *are usually flat, suede-like to granular, with a deep cream to tawny-buff to pale cinnamon-coloured surface and a yellow-brown reverse pigment. (b) Macroconidia of* M. gypseum *are ellipsoidal, thin walled, verrucose and 4–6-celled.*

Microsporum nanum

- is a *zoophilic* fungus frequently causing chronic non-inflammatory lesions in pigs and, rarely, causing tinea in humans; it is also present in the soil of pig-yards.
- Human infections are usually contracted directly from pigs or fomites.
- Invaded hairs typically show a sparse *ectothrix* or *endothrix* infection but *do not fluoresce* under Wood's ultraviolet light.
- The geographical distribution is worldwide.

- Key features include
  - distinctive macroconidia and
  - culture characteristics (Figure)



a

b

Figure 1.4
(a) Colonies of M. nanum *are flat, cream to buff in colour, with a suede-like to powdery surface texture and a dark reddish-brown reverse. (b) Macroconidia of* M. nanum *are small, ovoid to pyriform, mostly 2-celled with relatively thin, finely echinulate (rough) walls, and broad truncate bases.*

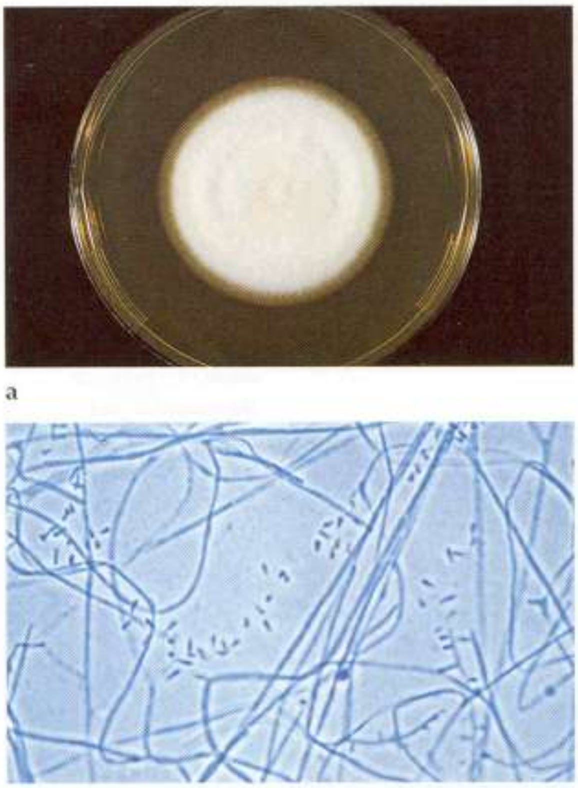
Trichophyton group

- In this group macroconidia are less distinctive and are often absent.
- Microconidia are more important and their shape, size and arrangement should be noted. Culture characteristics are also useful.
- Common species include:
    - *T. rubrum,*
    - *T. mentagrophytes* and varieties,
    - *T. tonsurans* and
    - *T. equinum.*

Trichophyton verrucosum may occasionally produce conidia on some media.

Trichophyton rubrum

- is an anthropophilic fungus that has become the most widely distributed dermatophyte of humans (Figure 1.5(a) and (b)).
- It frequently causes chronic infections of skin, nails and, rarely, scalp.
- Granulomatous lesions may sometimes occur.
- Key features include
    - culture characteristics,
    - microscopic morphology and
    - failure to perforate hair in *vitro*.



a

b

Figure 1.5
(a) Cultures of T. rubrum *are usually flat to slightly raised, white to cream, suede-like to downy, with a yellow-brown to wine-red reverse. (b) The microscopic morphology of* T. rubrum *showing the production of scanty to moderate numbers of slender clavate to pyriform microconidia. Macroconidia are usually absent.*

Trichophyton rubrum (granular strain)

- a frequent cause of tinea corporis in many countries.
- represents the parent strain of *T. rubrum* (downy type); the latter evolved by establishing a niche in feet (tinea pedis).
- Invaded hairs show ectothrix or endothrix infection but do not fluoresce under Wood's ultraviolet light.
- Key features include
  - the presence of cigar-shaped macroconidia with terminal appendages (Figure 1.6(a) and (b)).

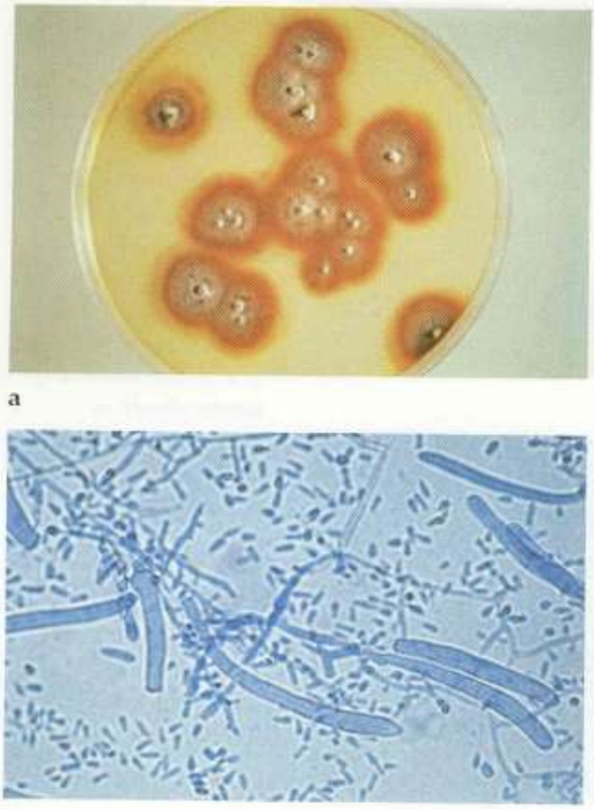

a
b

Figure 1.6
(a) Cultures of T. rubrum *(granular strain) are usually flat to slightly raised, white to cream, suede-like to downy, with a wine-red reverse. (b) The microscopic morphology of* T. rubrum *showing numerous numbers of clavate to pyriform microconidia and moderate numbers of smooth, thin-walled, cylindrical-shaped macroconidia.*

Identification of *T. rubrum*

Invasion of hair: reported as ectothrix chains of rather large spores and as typical endothrix. not fluorescent. Usually does not form perforating organs in vitro and does not invade the nail plate.

Clinical disease: Primarily a cause of tinea corporis, tinea pedis, tinea manum, and onchomycosis.

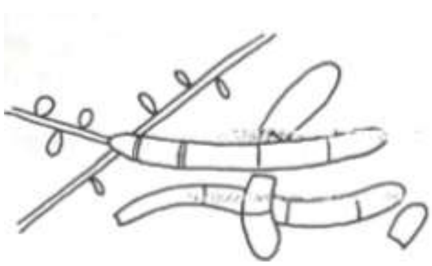
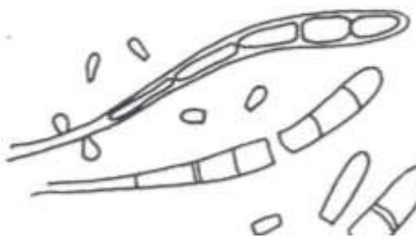
Epidemiology: A widespread anthropophilc species which appears to be increasing everywhere and displacing *T. mentagrophytes* as the principal cause of tinea pedis.

Geographical distribution: World-wide.

Appearance of thallus: Typically a downy to fluffy white thallus which slowly develops deep venous-blood color on the underside as the culture matures. *T. rubrum* is distinguished from other red pigmented dermatophytes by its usually white fluffy or fuzzy surface and dark-red non-diffusing pigment under the thallus and by its microscopic morphology.

Microscopic morphology: The usual thallus of *T. rubrum* consists of long strands of hyphae with small, lateral characteristically tear-shaped or peg-shaped microaleuriospores. Macroaleuriospores are absent or rare.

In sporulating strains macroaleuriospores may be abundant and characteristically narrow, long, and pencil-shaped, often developing directly at the ends of thick hyphae or attached together in a fashion resembling the macroaleuriospores of *E. floccosum*. Microaleuriospores tend to be larger, clavate to round, and in small open clusters as well as along the hyphae.


The production of microaleuriospores directly on macroaleuriospores is a rather common and characteristic feature in *T. rubrum*. Both hyphae and macroaleuriospores tend to break up into numerous arthrospores.

Variants. A very variable species. Typical cultures of *T. rubrum* may vary in macroscopic appearance from fluffy through suede-like and folded (suggestive of *T. tonsurans*) to glabrous and heaped up (suggestive of *T. violaceum*) and in microscopic appearance from few to numerous peg-shaped microaleuriospores with or without macroaleuriospores to abundant macroaleuriospores with microaleuriospores tend to be egg-shaped and plump. An additional common variable feature is the tendency to produce arthrospores. Occasional strains produce little or no color and may be difficult to distinguish from pleomorphic *T. mentagrophytes*. In some strains and under alkaline conditions or poor aeration the under color of the thallus may be yellow rather than red. Rare strains produce a dark brown, diffusing melanoid pigment.

Special media.

Potato dextrose agar is useful to separate tinea pedis and tinea cruris strains of *T. rubrum* an *T. mentagrophytes*. Pigment formation is usually very intense on this medium and fluffy surface growth is reduced.

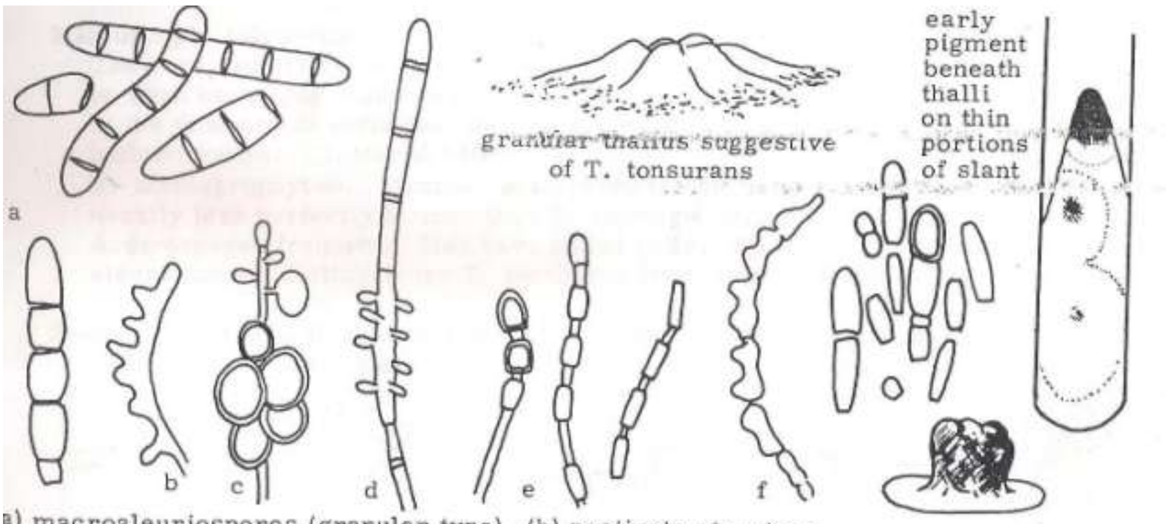
Meat infusion agar will stimulate production of a few macroaleuriospores in many fluffy strains.

T. rubrum grows well in absence of thiamine, serving to distinguish it from *T. tonsurans* and *T. violaceum*, and unlike *T. megninii* it does not require l-histidine.

T. rubrum may be distinguished from *T. mentagrophytes* by its failure to perforate hair in vitro and by the production of red pigment on corn meal dextrose agar.

Urease activity weak except in granular variants.

Clinical characteristics. (1) Characteristics and pigment of thallus, (2) Tear-shaped microaleuriospores, (3) Characteristic macroaleuriospores, (4) Independent of thiamine and histidine, (5) Failure to perforate hair in vitro.



a) macroaleuriospores (granular type), (b) pectinate structure,
c) enlarged microaleuriospores and chlamydospores,
d) macroaleuriospores bearing lateral microaleuriospores,
e) arthroaleuriospores, (f) peridial hypha from sterile ascocarp.

microscopic features and thallus of glabrous (rodhainii) variant

Trichophyton mentagrophytes var. interdigitale

- is an anthropopilic fungus of worldwide distribution that is a common cause of tinea pedis (particularly the vesicular type), tinea corporis and sometimes superficial nail-plate invasion.
- It is not known to invade hair in vivo.
  - Key features include culture characteristics, microscopic morphology and in vitro perforation of human hair.
- *Trichophyton mentagrophytes* var. *interdigitale* can be distinguished from *T. rubrum* and from other varieties of *T. mentagrophytes by*
  - *its culture* characteristics and microscopic morphology on Sabouraud's dextrose agar and / or lactritmel agar, and
  - by its growth and colony morphology on Sabouraud's salt agar (Figure 1.7).

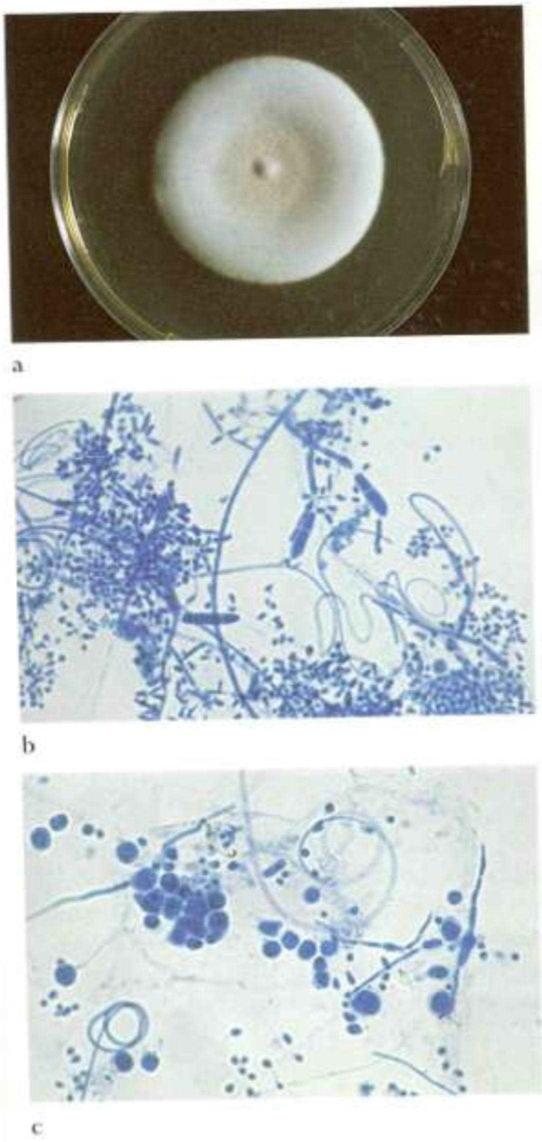


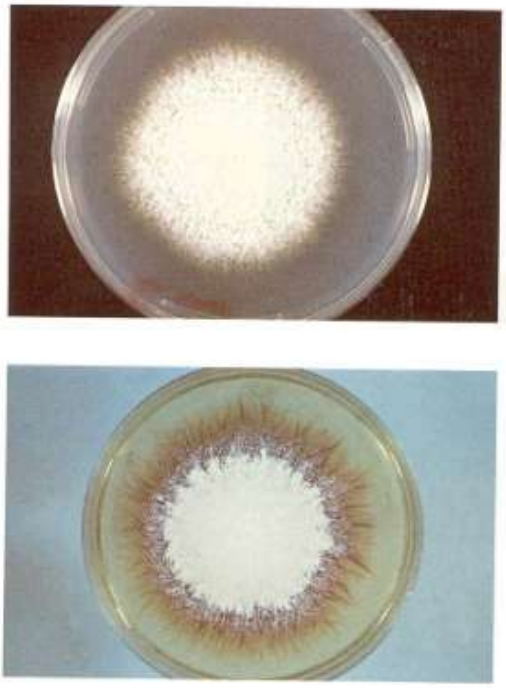
Figure 1.7
(a) Cultures of T. mentagrophytes *var.* interdigitale *are usually flat, white to cream in colour, with a powdery to suede-like surface and a yellowish to pinkish-brown reverse pigment, often becoming a darker red-brown with age.*
(b and c) The microscopic morphology of T. mentagrophytes *var.* interdigitale *showing numerous subspherical to pyriform microconidia, occasional spiral hyphae and spherical chlamydoconidia. There are occasional slender, clavate, smooth-walled macroconidia.*

T. mentagrophytes **var.** ***mentagrophytes*** **is**

- the zoophilic form of *T. mentagrophytes* with a
- wideworld distribution and
- a wide range of animal hosts including mice, guinea-pigs, kangaroos, cats, horses, sheep and rabbits.
- It produces inflammatory skin or scalp lesions in humans, particularly in rural workers.
- Kerion of the scalp and beard may *occur*.
- Invaded hairs show an ectothrix infection but do not fluoresce under Wood's ultraviolet light.

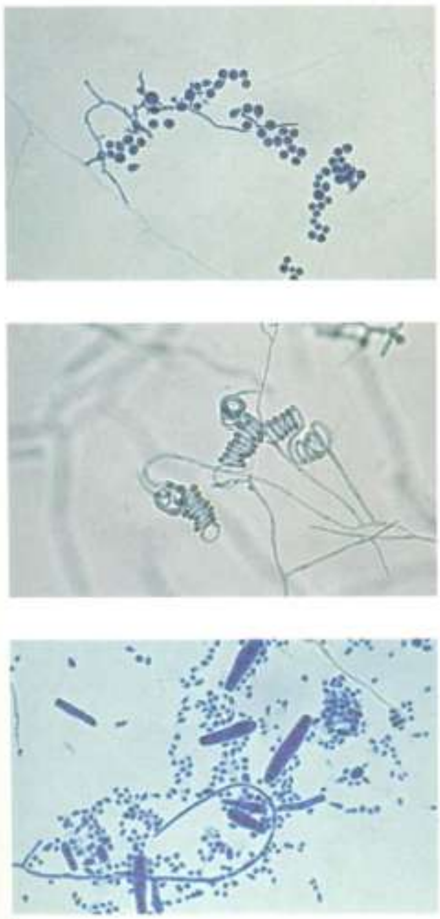
Kerion: Ringworm infection of the hair follicles of the scalp and beard that is usually results in a pustule-covered swelling that oozes fluid.

- **Key features include:**
  - culture characteristics, microscopic morphology and clinical disease with known animal contacts (Figure 1.8(a) and (b)).



a

Figure 1.8
(a) Cultures of T. mentagrophytes *var.* mentagrophytes *are generally flat, white to cream in colour, with a powdery to granular surface and a yellow-brown to reddish-brown reverse.*

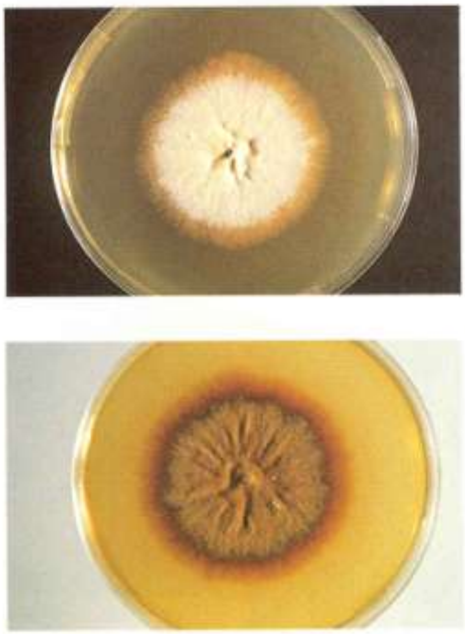


b

(b) The microscopic morphology of T. mentagrophytes *var.* mentagrophytes *showing numerous single-celled, spherical to subspherical microconidia, often in dense clusters. Varying numbers of spherical chlamydoconidia, spiral hyphae and smooth, thin-walled, clavate-shaped, multicelled macroconidia may also be present.*

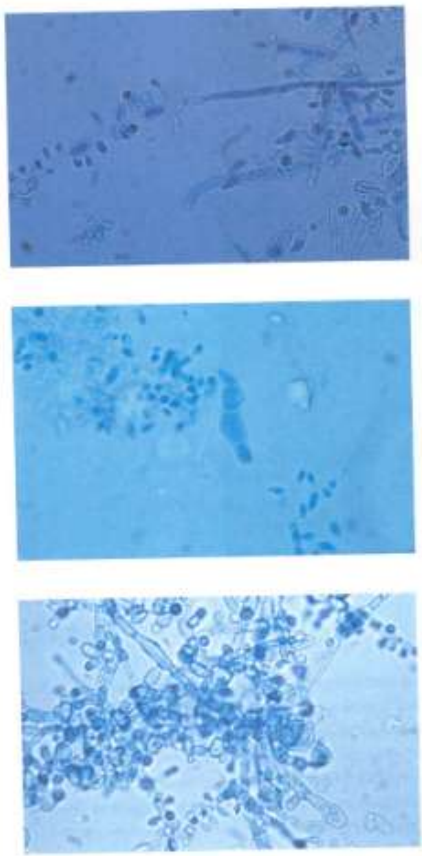
T. tonsurans

- is an anthropophilic fungus with a worldwide distribution that causes inflammatory or chronic non-inflammatory finely scaling lesions of skin, nails and scalp.
- Invaded hairs show an endothrix infection and do not fluoresce under Wood's ultraviolet light.
- Key features include:
  - microscopic morphology, culture characteristics, endothrix invasion of hairs and partial thiamine requirement (Figure 1.9(a) and (b)).



a

Figure 1.9
(a) Cultures of T. tonsurans *show considerable variation in texture and colour. They may be suede-like to powdery and flat with a raised centre or folded, often with radial grooves. The colour may vary from pale buff to yellow—the so-called sulfureum form which resembles* Epidermophyton floccosum*—to dark brown. The reverse colour varies from yellow-brown to reddish-brown to deep mahogany.*



b

(b) The microscopic morphology of T. tonsurans *showing relatively broad, irregular, much branched hyphae with numerous septa. Numerous characteristic microconidia (varying in size and shape from long clavate to broad pyriform) are borne at right angles to the hyphae, which often remain unstained by lactophenol cotton blue. Very occasional smooth, thin-walled, irregular, clavate macroconidia may be present on some cultures. Numerous swollen giant forms of microconidia and chlamydoconidia are produced in older cultures.*

Non-sporulating Microsporum/Trichophyton species

- Cultures of these species are usually sterile with no conidia present.
- Chlamydoconidia or other hyphal structures may be present but are non-diagnostic.
- In practice sporulation may need stimulation, e.g. to decide between non-sporing strains of *M. canis* or *T. rubrum*.
- Common species in this group include *M. audouinii, T. verrucosum* and *T. violaceum*.
- Less common ones are *T. concentricum, T. schoenleinii, T. soudanense* and *M. ferrusineum*.

T. verrucosum

- is a zoophilic fungus causing ringworm in cattle (Figure).
- Infections in humans result from direct contact with cattle or infected fomites and are usually highly inflammatory involving the scalp, beard or exposed mainly hairy areas of the body.
- Invaded hairs show an ectothrix infection, and fluorescence under Wood's ultraviolet light has been noted in cattle but not in humans.
- Geographic distribution is worldwide.

Key features include: culture characteristics and requirements for thiamine and inositol, large ectothrix invasion of hair, clinical lesions and history.

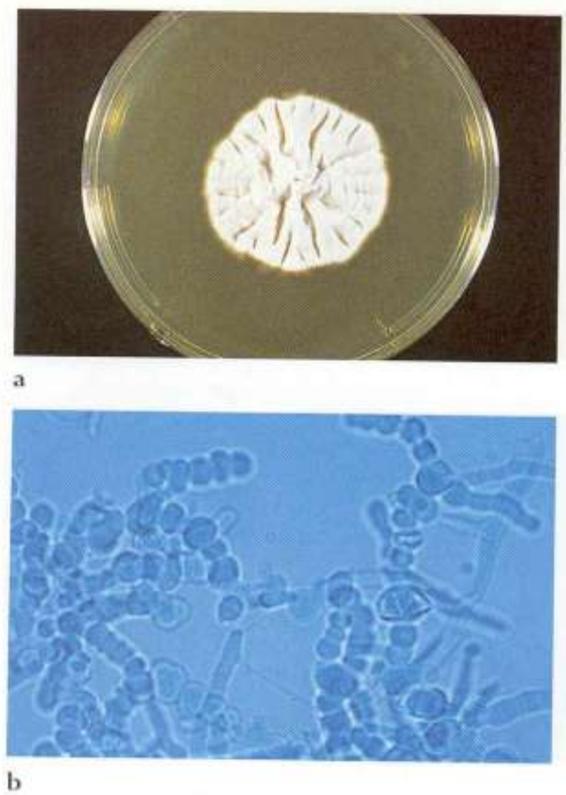
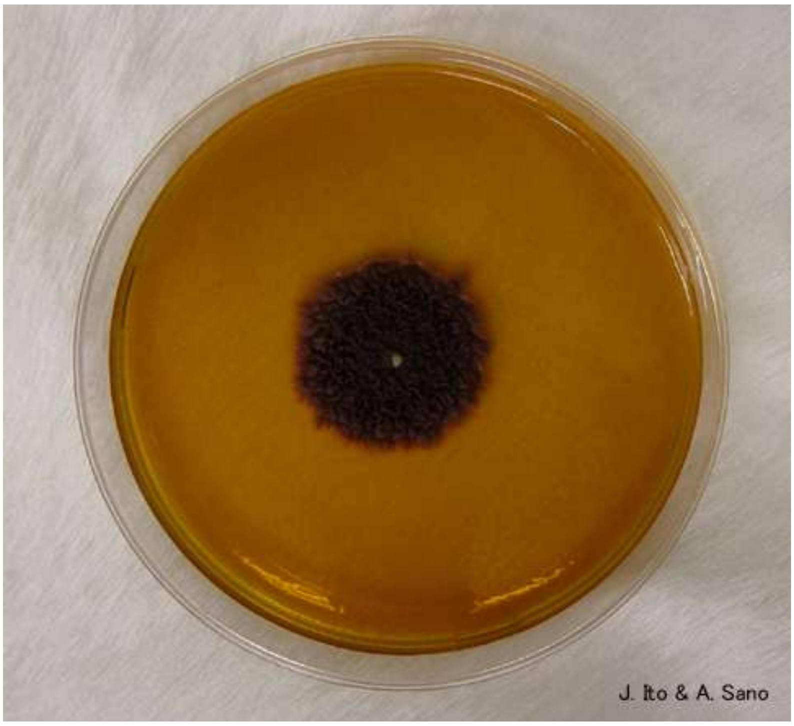


Figure 1.10
(a) Cultures of T. verrucosum *are slow growing, white to cream coloured, with a suede-like to velvety surface, a raised centre and a flat periphery with some submerged growth. Reverse pigment may vary from non-pigmented to yellow. (b) The microscopic morphology of* T. verrucosum *showing typical chains of chlamydoconidia when grown in brain heart infusion broth at 37°C.*

T. violaceum

- is an anthropophilic fungus causing inflammatory or chronic non-inflammatory finely scaling lesions of skin, nails, beard and scalp, producing the so-called 'black dot' tinea capitis.
- Distribution is worldwide, particularly in the Near East, Eastern Europe, the former USSR and north Africa.
- Key features include:
    - culture characteristics,
    - endothrix hair invasion and
    - a partial requirement for thiamine, which separates this organism from *T. gourvillii, T. rubrum* and other species that may produce purple-pigmented colonies.



T. schoenleinii

- Is an anthropophilic fungus causing favus in humans.
- Favus is a chronic, scarring form of tinea capitis characterized by saucershaped crusted lesions or scutula and permanent hair loss. Favus is common in Eurasia and Africa.
- Invaded hairs remain intact and flouresce a pale greenish-yellow under Wood's ultraviolet light.
- Key features include: clinical history, culture characteristics and microscopic morphology showing favic chandeliers (Figure)

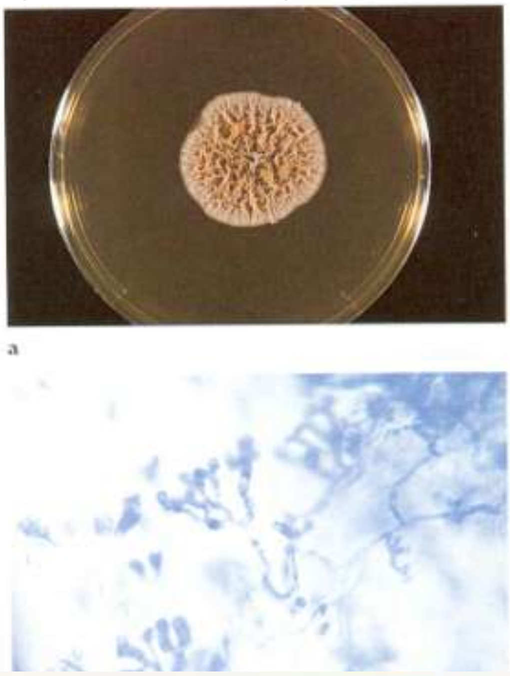


Figure 1.11
(a) Cultures of T. schoenleinii *are slow growing, waxy or suede-like, with a deeply folded honey-comb-like thallus and some subsurface growth. The thallus is cream coloured to yellow to orange-brown. Cultures are difficult to maintain in their typical convoluted form, and rapidly become flat and downy. (b) The microscopic morphology of* T. schoenleinii *showing characteristic antler 'nail head' hyphae also known as 'favic chandeliers'.*

Figure 2.54
Favus of the scalp showing extensive hair loss and numerous small scutula. T. schoenleinii *was isolated.*

Identification of non-dermatophyte moulds

- The identification of non-dermatophyte moulds is primarily based on:
- Microscopic morphology.
- Culture characteristics, although less reliable, may also be useful. These include:
  - surface texture,
  - topography, and
  - pigmentation,
  - reverse pigmentation and
  - growth at 37 °C.
- Non-dermatophyte moulds reported as causative agents of skin infection and/ or onychomycosis include the following: *Acremonium, Aspergillus, Chrysosporium, Chrysosporium, Fusarium, Geotrichum candidum, Nattrassia manaiferae (Nattrassia manaiferae (*synonym *Hendersonula toruloidea,* anamorph of *Scytalidium), Scopulariopsis brevicaulis*.

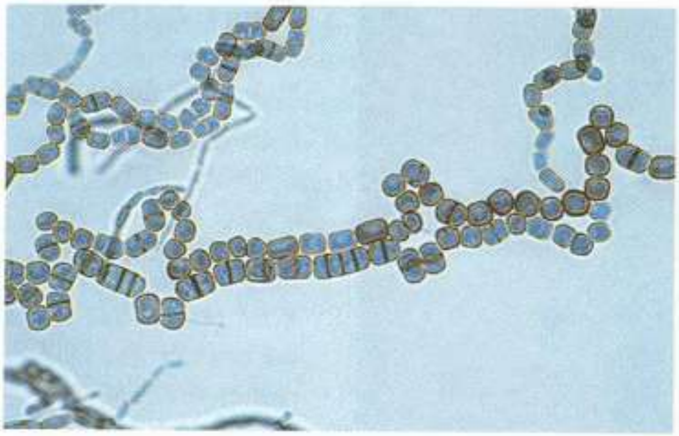


Figure 1.14 Scytalidium *anamorph of* Nattrassia mangiferae *showing chains of 1–2-celled, darkly pigmented arthroconidia.*

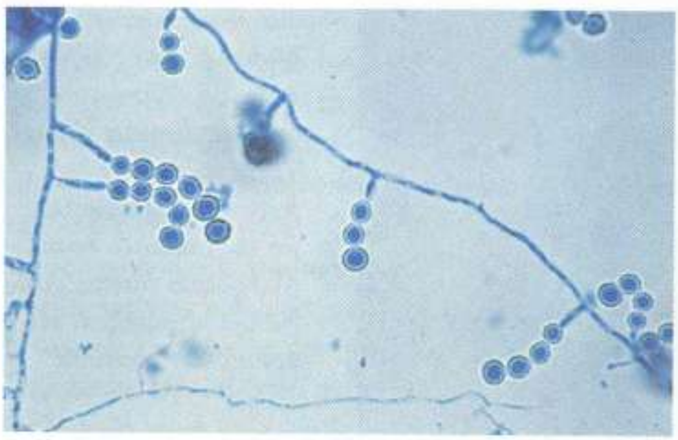


Figure 1.15 Scopulariopsis brevicaulis *showing chains of single-celled conidia produced by a specialised condiogenous cell called an annellide.*

Experiment 6: Additional tests used in identification of dermatophytes.

1. Urease Test

Urea Agar Base (Difco) is prepared in solid form and in tubes.

Urease Test Medium:

Peptone	1 g
NaCl	5 g
KH_2PO_4	2 g
Glucose	5 g
Agar Agar	20 g
Distilled water	1000 ml

Dissolve by heat, add 5 ml of phenol red solution (0.2% in 50% ethanol). Autoclave at 121 °C for 15 minutes. Cool, and add 100 ml of filter sterilized 20% aqueous solution of urea. Slant in tubes or pour plates. Place a small amount of colony in the medium and incubate at 24-26 °C. Read result in seven days. A deep red color through the medium is a positive reaction. Urease activity causes an alkaline (yellow → red).

The ability of fungi to attack urea and change the colour of the medium from straw to red within 7 days will distinguish floccose forms of *T. rubrum* from *T. mentagrophytes* and *T. megninii*, and also *T. erinacei* from *T. mentagrophytes*.

A positive reaction is shown by *T. mentagrophytes* and *T. megninii* while *T. rubrum* and *T. erinacei* are both urease negative. (since all dermatophytes produce an alkaline pH change with growth, only early pH changes in urea-rich medium are indicative of urease activity).

2. Penetration of hair in vitro

The ability to produce transverse perforations in sterile human hair will distinguish *T. mentagrophytes* from *T. rubrum*.

Short lengths of human hair are sterilized and added to distilled water in a Petri dish with 2 to 3 drops of 10% yeast extract.

Inoculate plates with small fragments of test fungus culture grown on SDA.

After inoculation the hairs are incubated for up to 4 weeks and examined at intervals (by scanning them under the microscope in a drop of lacto-phenol cotton blue, slightly warmed, under a coverslip; examination with KOH permits visualization of the perforating organs) for the formation of wedge shaped perforations which are formed by *T. mentagrophytes*. No perforations are formed by *T. rubrum*.

3. Growth on rice grains

Rice grain medium

A few grains of rice are placed in a small flask or bottle, covered with distilled water and sterilized. The test organism is inoculated onto the surface of the rice and incubated for 10 to 14 days.

This test differentiates *M. audouinii*, which will not grow on the rice grains, from *M. canis* or *M. gypseum*, both of which produce thick growth. This medium also encourages the production of characteristic macroconidia in atypical isolates of *M. canis*.

4. Pigment production on 1 % peptone agar

Microsporum persicolor will develop a pink colour on the surface when grown on agar containing 1 % peptone after 7 to 14 days. *Trichophyton mentagrophytes* will not produce any surface pigment.

5. Nutritional requirements (Media to test for vitamin, amino acid, and nitrogen requirements):

The series of *Trichophyton* Agars No. 1 - 7 can be used to differentiate some *Trichophyton* species by demonstrating the requirement of growth factors. Examples are T. equinum which requires nicotinic acid, *T. violaceum* requires thiamine and *T. megninii* requires histidine.

Illustrate and record your observations.

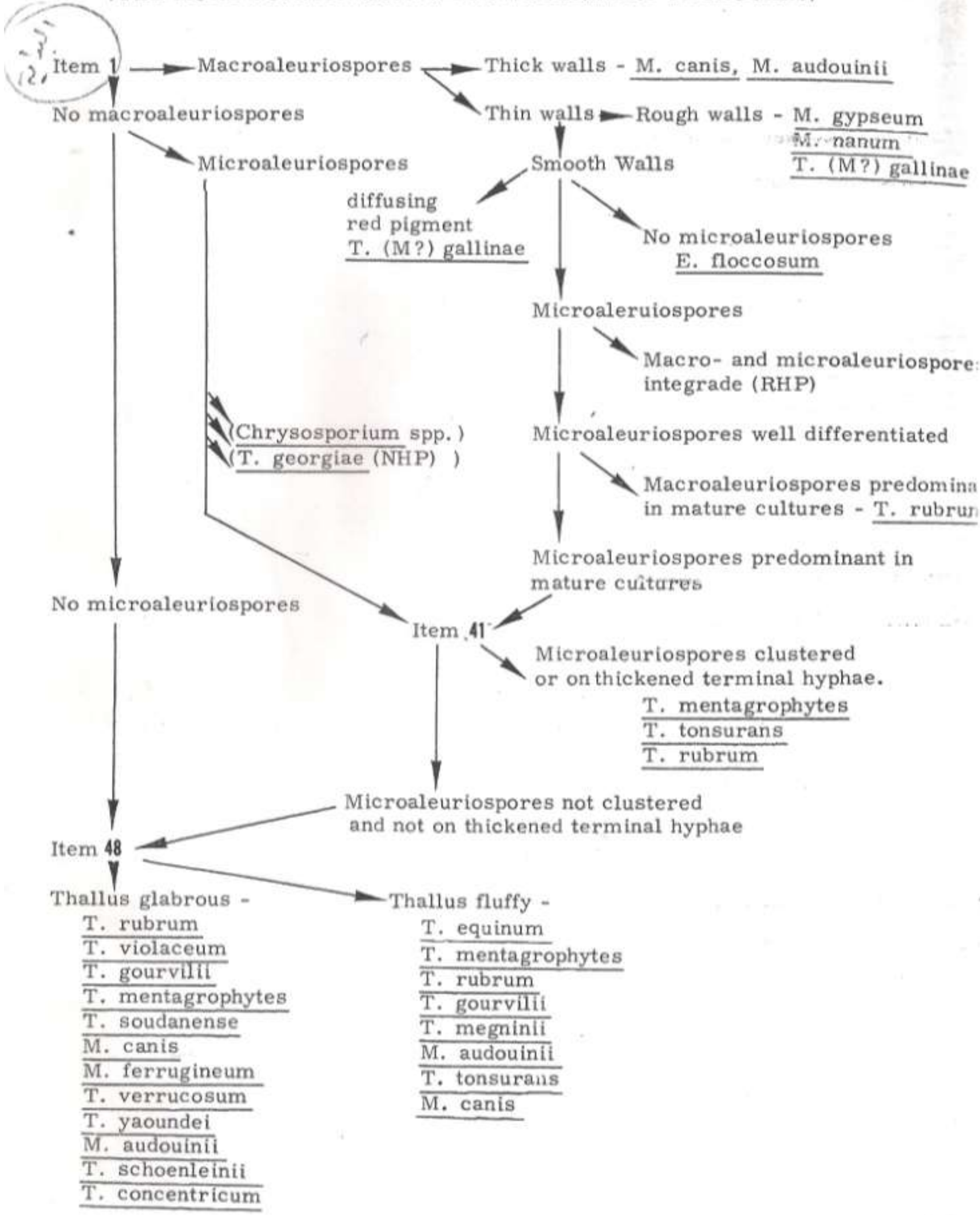
Experiment 7: Isolation of dermatophytes from soil – hair bait technique.

Moisten the soil samples in Petri dishes with sterile distilled water, and with chopped autoclaved horse or pre pubertal human hair, which serves as a bait and becomes colonized by dermatophytes and other keratinophilic fungi present in the soil. It is advisable to add cycloheximide (500 μg/ ml) and bacterial antibiotics. A peptone dextrose agar medium designated SAB-PSA (contains 10,000 units penicillin, 1 mg streptomycin, and 2 mg cycloheximide /ml).

Illustrate and record your observations.

Taxonomic Key of the Most Common Human and Veterinary Fungal Pathogens

SKELETON OUTLINE OF THE KEY
(THE MORE COMMON HUMAN AND VETERINARY PATHOGENS)



1 Macroaleuriospores (predominantly with 2 or more cells) present 2

Macroaleuriospores absent 34

2 Walls of some or most macroaleuriospores noticibly thicker than the septa (photomicrographs 1 - 6) 3

Walls of macroaleuriospores not thicker than septa (photomicrographs 7 - 21, 23, 27, 29, 32, 34, 35) 10

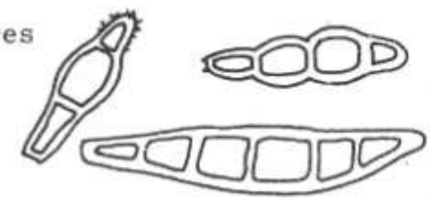
3 Macroaleuriospores frequently with a distinct beak at tip and spindle-shaped, frequently asymmetric; or else distorted or aborted in shape (photomicrographs 1 - 3). Frequently associated with fluorescent scalp ringworm 4

Macroaleuriospores usually without a terminal beak and symmetrical, spindle-shaped, or long ellipsoidal (photomicrographs 4 - 9) 7

4 Macroaleuriospores spindle-shaped, frequently 75 to 150μ long and with up to 15 cells, usually with terminal beak which is echinulate and often deflected to one side. Microaleuriospores usually not conspicuously abundant. Thallus colorless or more frequently deep yellow beneath (if yellow pigment is absent check 16 M. audouinii). . . 13 M. canis (CHP), cat and other animals (also check 19 M. ferrugineum).

Macroaleuriospores somewhat as above, but aborted, misshapen; or substantially longer than 150μ but with less than 8 cells 5

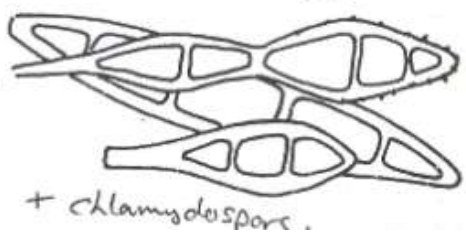
5 As in M. canis (see item 4), but macroaleuriospores small, blunted (often without terminal beak), aborted, or beaded (frequently with constrictions at the septa). . . 13 M. canis var. obesum (CHP), frequently primates and horses.



Macroaleuriospores variously misshapen or frequently constricted in mid-length (hour-glass or tadpole shape), or longer than 150μ 6

6 Macroaleuriospores variously misshapen. Microaleuriospores sometimes very numerous. Undersurface of thallus white or yellow. . . 15 M. distortum (RHP), man and animals.

Macroaleuriospores frequently constricted in mid-length (hour-glass or tadpole shape) and smooth to densely echinulate, or in excess of 150μ long, fusiform, but with less than 8 cells. Undersurface of thallus colorless or with salmon or peach-tan tints. . . 16 M. audouinii (CHP), rare in animals.

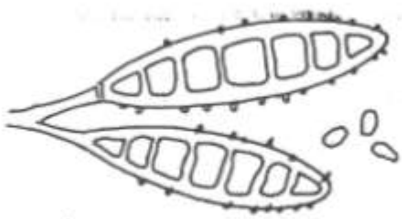


+ chlamydospore.

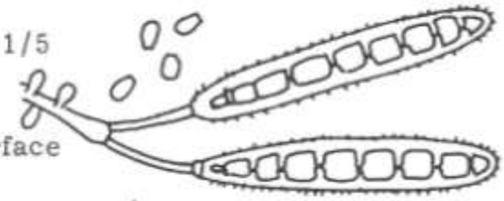
7 Surface of macroaleuriospores more or less echinulate or roughened. Microaleuriospores numerous.. 8

Surface of macroaleuriospores entirely smooth. Microaleuriospores usually rare (see photomicrograph 6)................................ 9

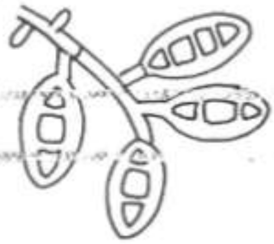
8 Macroaleuriospores more or less plump, spindle-shaped, or club-shaped (width usually more than 1/5 the length) echinulate, most containing 7 or less cells. Thallus more or less powdery buff, usually with deep red, non-diffusing pigment beneath the thallus. . .29 M. cookei (RHP), soil (also check 65 M. amazonicum, and 19 M. ferrugineum).



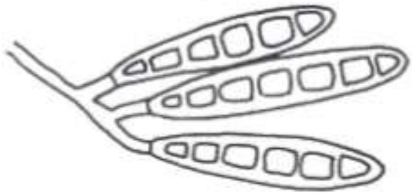
Macroaleuriospores long (width often less than 1/5 the length), cylindrical, or cigar-shaped, frequently containing 8 or more cells and finely echinulate. Microaleuriospores abundant. Surface of thallus powdery cream to pink. . .30
9 M. vanbreuseghemii (RHP), soil.



Macroaleuriospores short spindle-shaped, most with 2 - 5 cells. Thallus more or less powdery orange buff. . .31 T. ajelloi var nanum (NHP), soil.



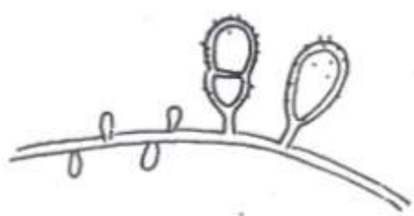
Macroaleuriospores long cylindrical, club-shaped to spindle-shaped, most with 6 or more cells. Microaleuriospores usually rare. Thallus with a more or less rich orange-buff powdery surface, frequently producing purple-black pigment beneath. . .31 T. ajelloi (NHP), common in soil (also check 30 M. vanbreuseghemii).



10 Macroaleuriospores spindle-shaped, egg-shaped, club-shaped, or cylindrical, with surface more or less roughened or echinulate, generally more than 7μ in diameter (photomicrographs 7-15) 11

Macroaleuriospores principally club-shaped or cylindrical. Surface not roughened or echinulate, in most cases less than 7μ in diameter if microaleuriospores also present (photomicrographs 16 - 21, 23, 27, 29, 32, 34, 35).. 19

11 Macroaleuriospores egg-shaped or short ellipsoidal most containing 2 cells. Microaleuriospores present (if microaleuriospores are not present, check 71 Chrysosporium). . .26 M. nanum (RHP), usual in pigs and soil in pigyards.



Macroaleuriospores spindle-shaped or elongate, most with more than 3 cells.. 12

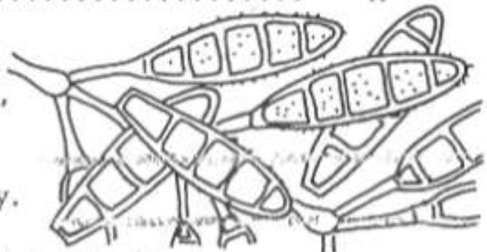
12 Macroaleuriospores predominantly spindle-shaped, clavate, club-shaped, bullet-shaped, or cigar-shaped, symmetrical and uncurved, most with 5 or more cells. Attachment scar frequently more than 1/3 the diameter of the macroaleuriospore at its greatest width (photomicrographs 8 - 12) 13

Macroaleuriospores with attachment scar less than 1/3 the diameter of the spore at its greatest width. Macroaleuriospores either (a) spindle-shaped and most containing 4 cells, or (b) fusiform, club-shaped, and tadpole-shaped (frequently curved), with rounded tips, and containing up to 10 cells (photomicrographs 13 and 14)........................... 18

13 Macroaleuriospores spindle-shaped, club-shaped, clavate, bullet-shaped, or cigar-shaped, less than 60μ long, most with 5 to 8 cells or less. Surface of thallus powdery buff to ochre-pink.......................... 14

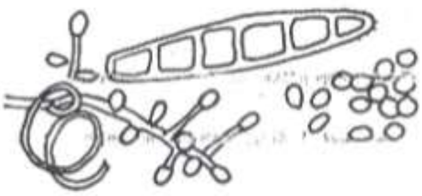
Macroaleuriospores spindle-shaped (not bullet-shaped), frequently more than 60μ long and with more than 6 cells. Surface of thallus buff or powdery cream to grape red...................................... 17

14 Macroaleuriospores mostly spindle-shaped or ellipsoidal with diameter often 1/3 to 1/4 the length, most containing 6 or less cells, sometimes with a slight terminal beak and produced usually in large numbers in clusters along the hyphae and terminally. Entire surface of thallus usually powdery, olive-buff to cinnamon-tan. A nearly glabrous (silky) isolate with few macroaleuriospores has been reported. Also isolates with smooth macroaleuriospores. . . 23 M. gypseum (CHP), common in soil (also check 25 M. fulvum).



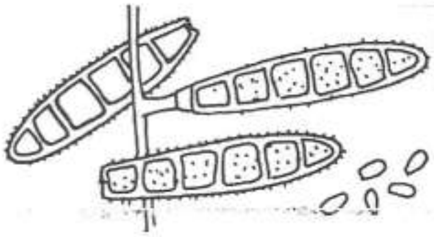
Macroaleuriospores club-shaped, bullet-shaped, cigar-shaped, or spindle-shaped with diameter less than 1/4 the length, most with 6 or more cells, abundant or few, and scattered rather than clustered................ 15

15 Thallus powdery or fluffy, cream color to buff, at times with violet or pink tinted margin. Macroaleuriospores few. Microaleuriospores spherical or club-shaped, in large clusters. Coils frequent. Macroaleuriospores club-shaped and tapered, nearly smooth or else echinulate near the tip. 26 M. persicolor (RHP), geographically limited to Europe and carried by the Bank Vole (also check 25 M. fulvum, 40 T. mentagrophytes, and 64 M. racemosum).

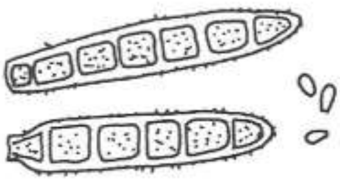


Thallus usually heavily powdery, buff to reddish buff, frequently with a white fluffy border. Macroaleuriospores conspicuously echinulate, usually abundant. Microaleuriospores not in conspicuous clusters, and not the predominant feature... 16

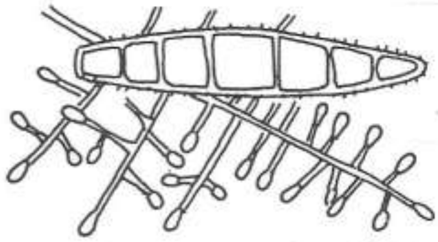
16 Macroaleuriospores thin-walled (photomicrograph 10), mostly less than 50μ in length, club-shaped, spindle-shaped, cigar-shaped, or bullet-shaped, and with 7 or less cells. Thallus buff to reddish buff. . . 25 M. fulvum (RHP), soil (also check 23 M. gypseum and 63 M. boullardii).



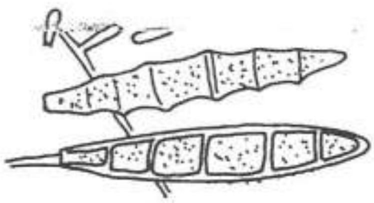
Macroaleuriospores somewhat thick-walled (photomicrograph 11), spindle-shaped, cigar-shaped, or bullet-shaped, frequently with 7 or more cells and frequently achieving length of 50μ or more. Thallus reddish buff. . . 63 M. boullardii (NHP), soil, Africa (also check 25 M. fulvum, 64 M. racemosum, and 64 M. praecox).



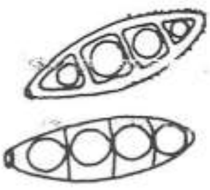
17 Thallus flat, powdery white to cream or stained with grape-red and with dark grape-red under-color (see Plate IV). Club-shaped microaleurio-spores in conspicuous wand-like open terminal clusters (which may be the predominant feature). Macroaleuriospores few to numerous, spindle-shaped, varying in length to over 60μ, and with 5 to 10 cells. . . 64 M. racemosum (NHP), soil.



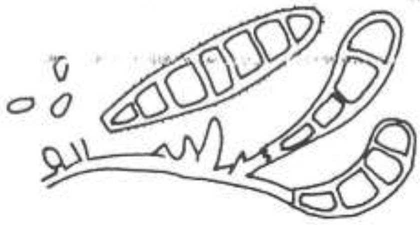
Surface of thallus powdery buff. Undersurface yellow-orange. Microaleuriospores not in con-spicuous clusters. Macroaleuriospores slender spindle-shaped, many over 60μ long, and with up to 9 cells. . . 64 M. praecox (RHP) (also check 23 M. gypseum, 25 M. fulvum, and 62 M. boullardii).



18 Thallus olive-buff to olive-grey, fluffy to heavily powdery. Macroaleuriospores containing up to 7 or more cells, but most with only 4 cells and short, symmetrical, spindle-shaped, with both cell walls and septa frequently thickened, and frequently containing oil drop-like inclusion in each cell (photomicrograph 13). Attachment scar small, usually less than 1/5 diameter of the aleuriospore at its greatest width. . . 65 M. amazonicum (NHP), soil, Brazil (also check 23 M. gypseum).



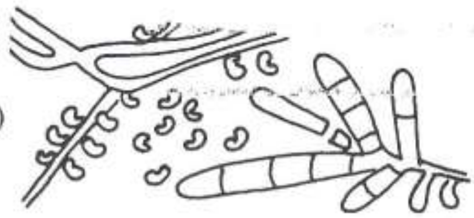
Thallus usually white to pink white or brown, satiny to fluffy, rarely powdery buff. Macroaleuriospores slightly echinulate or smooth, blunt-tipped, frequently tadpole shaped and curved, containing up to 10 cells, and frequently on comb-like hyphal branches (photomicrograph 14). Attachment scar usually less than 1/3 diameter of the spore at its greatest width. A diffusing yellow to strawberry red pigment usually produced beneath the thallus. . . 21 T. (M?) gallinae (RHP), chicken (also check 29 M. cookei).



19 Macroaleuriospores and attachment scar usually less than 1/3 the diameter of the spore at its greatest width. Macroaleuriospores blunt-tipped, spindle-shaped, club-shaped, or tadpole-shaped, frequently curved (photomicrograph 14). Microaleuriospores usually present. Mature thallus usually developing a yellow to strawberry red diffusing pigment. . . 21 T. (M?) gallinae (RHP), chicken.

Macroaleuriospores with attachment scar in most cases more than 1/3 the diameter of the spore at its greatest width, if not then microaleuriospores absent. Macroaleuriospores generally thin-walled, club-shaped, or cylindrical. Strawberry red diffusing pigment rarely produced..... 20

20 Microaleuriospores present and predominantly cashew nut-shaped (photomicrograph 24), and produced laterally or abundantly in ascocarp-like structures composed of thickened hyphae (pycnidia) . . . 66 T. phaseoliforme (NHP), soil (also check 27 M. persicolor).

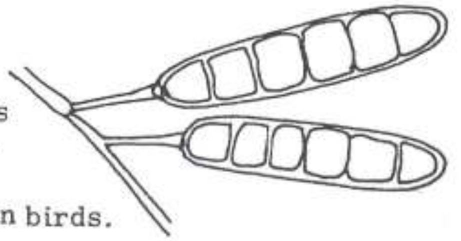


Microaleuriospores absent, or if present not predominantly cashew nut-shaped or curved, and rarely in ascocarp-like structures........... 21

21 Microaleuriospores (small, one-celled aleuriospores) absent; or macroaleuriospores usually large, frequently more than 9μ in diameter or more than 100μ long. 22

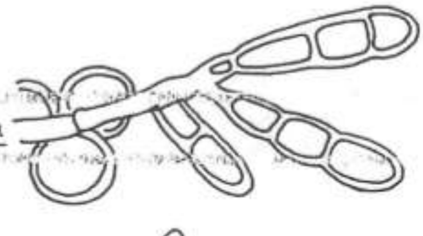
Microaleuriospores (small, one-celled aleuriospores) present. Macroaleuriospores usually less than 9μ in diameter and less than 100μ long. 24

22 Macroaleuriospores large, cylindrical (usually more than 9μ in diameter) and produced on slender stalks. Attachment scar may be less than 1/3 the diameter of the spore at its greatest width. Thallus flat, white to buff, frequently with concentric rings of growth, and yellow underneath. . . 70 A. flavescens (NHP), from feathers of Australian birds.

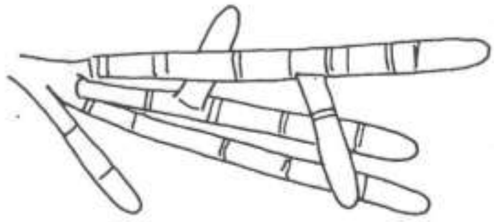


Macroaleuriospores club-shaped or cylindrical and attached together and joined in groups (photomicrographs 16 and 21)................... **23**

23 Macroaleuriospores blunt, club-shaped, frequently more than 7μ in diameter, and attached together in groups. Thallus usually mustard or khaki color, with rough or folded surface. . . 62 <u>E. floccosum</u> (CHP), principally tinea cruris and pedis (also check 33 T. terrestre, 31 T. ajelloi, and 50 T. rubrum).



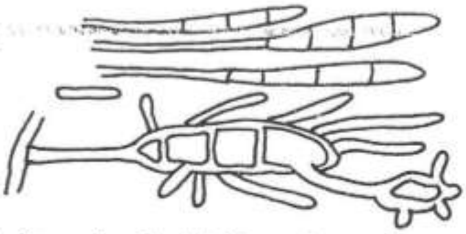
Macroaleuriospores elongate, cylindrical, and frequently attached together. Thallus flat and powdery, yellowish or cream-white to tan. . 67 <u>T. longifusus</u> (NHP), soil.



24 Macroaleuriospores narrow (usually less than 7μ in diameter) principally born laterally or terminally, not markedly differentiated from lateral clavate, one-celled microaleuriospores (photomicrographs 23, 29, 32). **25**

Macroaleuriospores narrow as above, or thick (more than 7μ in diameter) and born singly or in groups, sometimes with "microaleuriospore-like", one-celled elements, but clearly differentiated from the small, more or less spherical, club-shaped, or teardrop-shaped microaleuriospores... **29**

25 Macroaleuriospores principally terminal or lateral on slender stalks, and when attached tapering toward the base; pencil-shaped, spindle-shaped, or club-shaped when detached, and sometimes appearing slightly roughened (photomicrograph 24). "Propagules" present in the depths of the thallus . . . 67 <u>T. proliferans</u> (RHP), ecology unknown (also check 44 T. erinacei, 27 M. persicolor, and 50 T. rubrum).

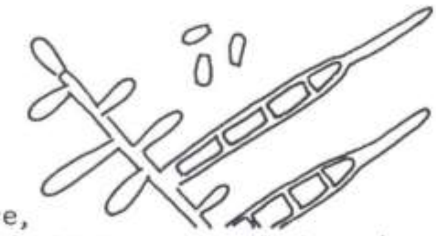


Macroaleuriospores predominantly lateral, club-shaped, or cylindrical, scattered or in terminal clusters, and not tapering toward the base when attached (photomicrographs 23 and 29)............................... **26**

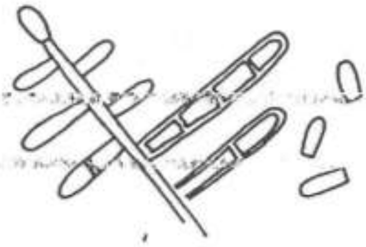
26 Thallus reddish or reddish-brown.................................. **27**

Thallus white, yellowish, greenish, or cinnamon buff................ **28**

27 Macroaleuriospores frequently long cylindrical or tapered (exceeding 40μ) and frequently forming terminal filaments while still attached to the hyphae. Mature thallus frequently developing a cracked surface. . . 27 <u>T. fluviomuniense</u> (RHP), ecology unknown (also check 33 T. terrestre, 52 T. tonsurans, 49 T. megninii, 50 T. rubrum, and 40 T. mentagrophytes).

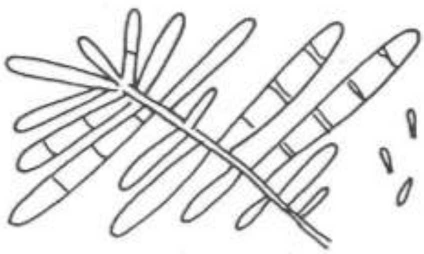


Macroaleuriospores predominantly small and cylindrical, usually less than 6 X 40μ, and not forming terminal germ tubes before detachment . . . 33 T. terrestre, 68 T. thuringiense, etc. (NHP), soil. Separations largely depend on demonstrating the perfect states. T. terrestre may produce diffusing red pigment (also check 69 T. fluviomuniense, 35 T. georgiae, and 44 T. erinacei).



28 Thallus white, yellowish, or greenish. Smallest mature, detached, one-celled elements (microaleuriospores) predominantly large and clavate (most more than 1.5 X 3μ) (photomicrographs 29). Lateral macroaleuriospores not conspicuously clustered terminally, but scattered more or less evenly throughout the aerial hyphae. . . 33 T. terrestre, 70 A. cuniculi, etc. (NHP), soil. Separations largely depend on demonstrating the perfect states.

Thallus white, yellowish, or cinnamon buff. Smallest mature, detached microaleuriospores usually small and tear-drop shaped or elongate (less than 1.5 X 3μ). Lateral macroaleuriospores usually conspicuously clustered terminally in groups of up to 50 macroaleuriospores (photomicrographs 23 and 34). . . 36 T. gloriae (NHP), soil (also check 49 T. rubrum and 38 T. simii).

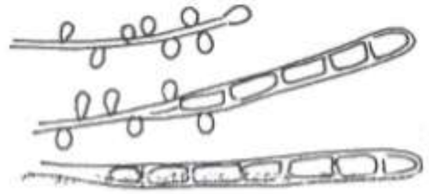


29 Macroaleuriospores remaining a conspicuous and abundant feature in maturing thallus. Microaleuriospores predominantly or exclusively lateral on non-terminal hyphae or on terminal hyphal branches.............. **30**

Microaleuriospores a more conspicuous feature in maturing thallus than macroaleuriospores and either lateral on non-terminal hyphae or terminal hyphal branches; or in conspicuous branched and unbranched terminal clusters; or on thickened terminal or subterminal hyphae (photomicrographs 25, 26, 27, 33, 36); or both macroaleuriospores and microaleuriospores rare... **41**

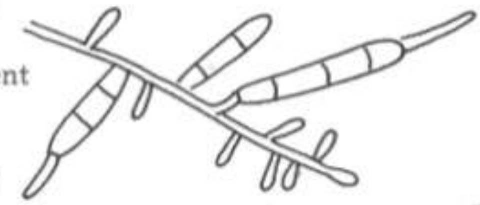
30 Macroaleuriospores narrow (most less than 7μ in diameter), the largest usually appearing long, cylindrical, or fragmented, length frequently more than 7 times diameter (photomicrographs 23, 34, 35)................ **31**

Macroaleuriospores frequently 7μ in diameter or larger, the largest usually appearing well-formed, and club-shaped, narrow fusiform or short, cylindrical, length usually less than 7 times the diameter (photomicrographs 17, 18, 19).. **33**

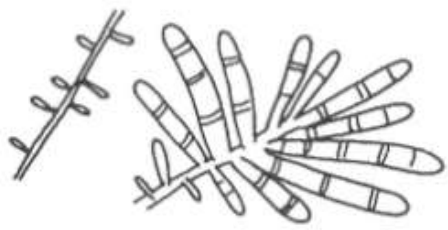


31 Macroaleuriospores narrow, long, cylindrical, mainly terminal, sometimes poorly differentiated from thickened hyphae tips or arthrospores, but well differentiated from egg-shaped to peg-shaped or short club-shaped lateral microaleuriospores. Microaleuriospores sometimes produced laterally on the macroaleuriospores. Thallus usually purplish-red beneath and with a fluffy or more or less heaped and glabrous surface. . . 50 T. rubrum (CHP), principally tinea corporis, tinea pedis and tinea manus (also check 38 T. simii and 23 M. gypseum).

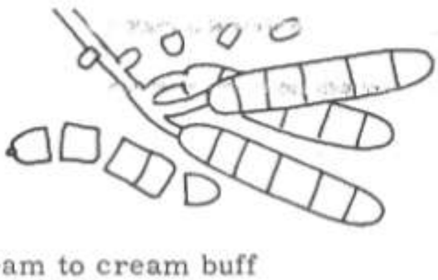
Macroaleuriospores narrow, varying in length from short one-celled and microaleuriospore-like to long cylindrical, with up to 10 cells, and produced mostly laterally along the hyphae or in terminal clusters............. **32**



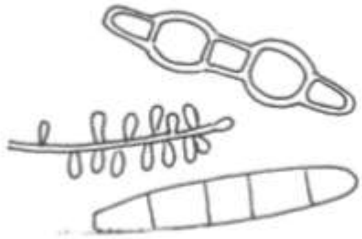
32 Macroaleuriospores, frequently with terminal filaments produced laterally, but not in conspicuous clusters. Smallest microaleuriospore elements short clavate (about 3 X 5.5) with broad attachment scars. Surface of thallus granular, folded, and cracked, and red-brown in color. . . 69 T. fluviomuniense (RHP), ecology unknown (also check 33 T. terrestre, 52 T. tonsurans, 49 T. megninii, 50 T. rubrum, 40 T. mentagrophytes, and 23 M. gypseum).



Macroaleuriospores without frequent terminal germ tubes and produced laterally in terminal clusters of up to 50 (photomicrograph 23). Small tear-drop shaped microaleuriospores (about 1.5 X 3μ) also present (photomicrograph 34). Surface of thallus more or less powdery, flat, and yellowish to cinnamon; yellow beneath. Arthroaleuriospores may be frequent. . . 36 T. gloriae (NHP), soil (also check 27 M. persicolor, 33 T. terrestre, and 50 T. rubrum).



33 Macroaleuriospores blunt, cylindrical, and thick (7 to 10μ in diameter), most of relatively uniform length or varying in length from one-celled to larger elements, and produced terminally and laterally in small clusters or in masses. Cells of macroaleuriospores frequently disarticulating when mature (photomicrograph 17). Surface of thallus cream to cream buff . . . 37 T. vanbreuseghemii (RHP), soil (also check 38 T. simii, 36 T. gloriae, 40 T. mentagrophytes, and 23 M. gypseum).



Macroaleuriospores tapered and club-shaped to long cylindrical, frequently developing distinctive swollen cells when mature (photomicrograph 18). Macroaleuriospores frequently clustered or massed in large numbers. Microaleuriospores clavate to teardrop-shaped, sometimes with slender bases or stalks, and produced laterally on non-terminal hyphal branches. Surface of thallus powdery white to cream or reddish. Undersurface usually yellow to reddish. . . 38 T. simii (RHP), monkey, chicken, and soil, restricted to penisular India (also check 40 T. mentagrophytes, 36 T. vanbreuseghemii, 44 T. erinacei, and 50 T. rubrum).

34 Microaleuriospores (predominantly one celled) present. 35

Neither macroaleuriospores nor microaleuriospores present. 48

35 Microaleuriospores frequently or predominantly cashew nut-shaped (photomicrograph 24) and frequently produced in ascocarp-like structures (pycnidia). . . 66 T. phaseoliforme (NHP), soil (also check 27 M. persicolor).

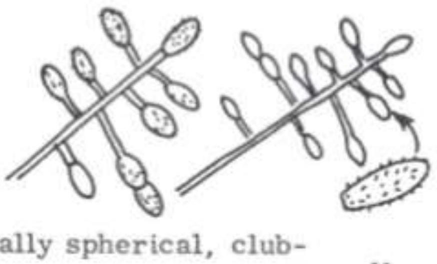
Microaleuriospores not predominantly cashew nut-shaped and rarely produced in ascocarp-like structures. 36

36 Microaleuriospores mostly large (more than 7μ long) egg-shaped, spherical, or ellipsoidal, predominantly with one cell, but approaching in size and shape the macroaleuriospores of M. nanum (photomicrograph 30). 37

Microaleuriospores mostly small (less than 7μ long) spherical, pear-shaped, egg-shaped, club-shaped, or oat-shaped. 38

37 Microaleuriospores predominantly echinulate, 70 A. tuberculatum, 71 C. asperatum, and 71 C. serratus (NHP), soil.

Microaleuriospores predominantly smooth, 70 A. multifidum, 70 A. curreyi, and 71 C. keratinophilum (NHP), soil.

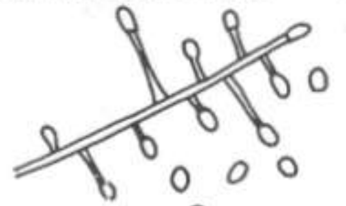


38 Microaleuriospores more or less oat-shaped, surface finely echinulate. No macroaleuriospores produced (photomicrograph 22). Surface of thallus powdery white to cream. . . 71 C. indicum and 71 C. evolceanui (NHP), soil (differentiate by size and perfect state).

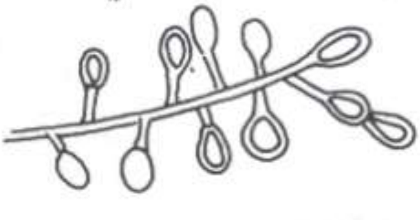
Microaleuriospores with smooth surface and generally spherical, club-shaped, egg-shaped, teardrop-shaped, etc. 39

39 Microaleuriospores frequently on long lateral stalks (more than 2X the length of microaleuriospores) or frequently in chains of two or more (photomicrographs 28 and 31), and scattered more or less abundantly throughout the surface branches in the thallus, but not conspicuously or predominantly in branched terminal clusters or on thickened terminal or subterminal hyphae (photomicrographs 28 and 31). No macroaleuriospores produced... 40

Microaleuriospores not conspicuously on long stalks or in chains; or if on stalks, stalks less than 2X the length of the microaleuriospores; or microaleuriospores conspicuously in more or less branched terminal clusters or cluster-like masses, or on thickened terminal or subterminal hyphae (photomicrographs 25, 26, 27, 33, 36)...................... 41

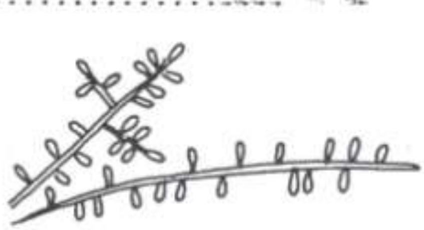


40 Microaleuriospores rarely in chains of two or more (catenulate). Microaleuriospores club-shaped, small, less than 2.5 X 4.5μ. Thallus rosy buff, loosely powdery, and slightly domed (see Plate IV). . . 35 T. georgiae (NHP), soil (also check 71 C. pannorum).



Microaleuriospores club-shaped, oat-shaped, and egg-shaped, sometimes in chains of 2 or more (catenulate) and large (3.5 X 8μ or more). Thallus white to cream-color usually flat and powdery . . . 71 C. tropicum (NHP), soil (also check 71 C. evolceanui, 71 C. indicum, 71 C. pannorum, 72 C. pruinosum, and 33 T. terrestre).

41 Microaleuriospores usually numerous in more or less conspicuous, variously branched, terminal clusters, or on thickened terminal hyphae, and in some cases differing in size and shape from those produced laterally on non-terminal hyphae. Microaleuriospores in some cases predominantly spherical (photomicrographs 25, 26, 27, 33). 42

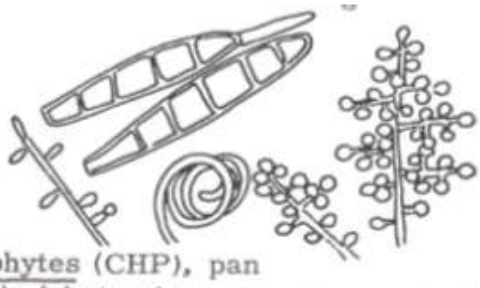


Microaleuriospores few to numerous, mainly lateral on non-terminal hyphae, or clustered on undifferentiated terminal hyphal branches. Microaleuriospores clustered terminally not differing conspicuously in size and shape from those produced laterally on non-terminal hyphae, and not predominantly spherical.. 48

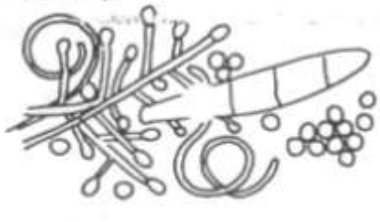
42 Microaleuriospores in conspicuous branched clusters in the mature thallus and predominantly spherical when mature and detached. Macroaleuriospores and spiral hyphae often present..................................... 43

Microaleuriospores in branched clusters or not, but predominantly club-shaped, teardrop-shaped, or peg-shaped when mature and detached... 44

43 Immature microaleuriospores in clusters in primary culture or subculture mostly not stalked and frequently spherical (photomicrograph 25). Macroaleuriospores few to numerous, club-shaped usually with 4 or more cells. Lateral microaleuriospores usually more teardrop-shaped. Spiral hyphae often present. . . 40 T. mentagrophytes (CHP), pan zoophilic, principally tinea pedis, and tinea corporis (photomicrograph 19) (also check 52 T. tonsurans and 27 M. persicolor).



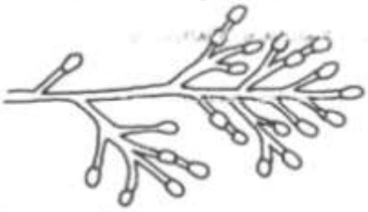
Immature microaleuriospores in clusters frequently on long stalks (stalks more than 3X of the length of microaleuriospores) and club-shaped (photomicrograph 33). Macroaleuriospores usually scattered and club-shaped or somewhat spindle shaped (photomicrograph 9). Spiral hyphae often present. . . 27 M. persicolor (RHP), geographically limited to Europe and carried by the Bank Vole (also check 40 T. mentagrophytes, 25 M. fulvum, and 52 T. tonsurans).



44 Microaleuriospores in clusters, or on thickened hyphae, frequently on g stalks (stalks more than 3X length of microaleuriospores)....... 45

Microaleuriospores in clusters, or on thickened hyphae, mostly not on long stalks (stalks, if present less than 3X length of microaleuriospores). 47

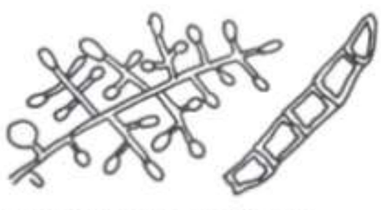
45 Microaleuriospores in clusters conspicuously on forward-directed branches and stalks and produced singly or in chains of two or more. No macroaleuriospores produced. . . 71 C. pannorum (NHP), soil (also check 71 C. tropicum).



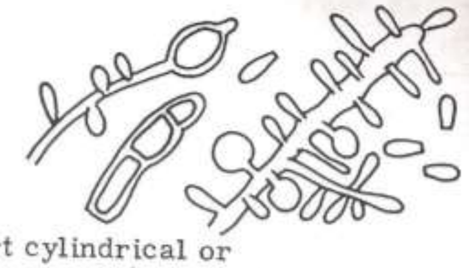
Microaleuriospores in clusters not conspicuously on forward directed branches and stalks. Chains of aleuriospores rare or absent......... 46

46 Microaleuriospores usually in large twiggy clusters or masses and usually or relatively uniform size and shape (photomicrograph 33). Macroaleuriospores usually present and well formed and tapered or club-shaped (photomicrograph 9). Thallus fluffy to densely powdery, cream-buff, frequently developing pink tints. . . 27 M. persicolor (RHP), geographically limited to Europe and carried by the Bank Vole (also check 40 T. mentagrophytes).

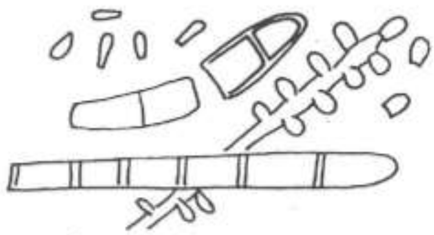
Microaleuriospores of variable size and shape, predominantly club-shaped, long club-shaped, or filiform, frequently lateral on thickened terminal hyphae (photomicrograph 26), and in clusters usually of twiggy aspect, and frequently with spherical balloon forms. Macroaleuriospores usually rare and short cylindrical or poorly formed. Young thallus slightly powdery, mature thallus suede-like and flat or folded. Undersurface of thallus mahogany brown, yellow, or colorless. . . 52 T. tonsurans (CHP) primarily endothrix scalp ringworm (also check 40 T. mentagrophytes).



47 Microaleuriospores of variable size and shape, frequently stalked, predominantly long, club-shaped, or filiform, and frequently lateral on thickened terminal hyphae (photomicrograph 26) and in clusters usually of twiggy aspect, and frequently developing into spherical balloon forms. Macroaleuriospores usually rare and short cylindrical or poorly formed (length less than 6 times diameter). Undersurface of thallus mahogany brown, yellow, or colorless. . . 52 T. tonsurans (CHP), primarily endothrix scalp ringworm (also check 50 T. rubrum).



Microaleuriospores in clusters or on thickened terminal hyphae, and often short club-shaped, or plump, frequently with attachment scars more than 1/2 the length of the spore. Lateral microaleuriospores on non-terminal hyphae frequently small and peg-shaped with attachment scar less than 1/3 the length of the spore. Filiform microaleuriospores not a predominant feature. Spherical balloon forms sometimes present. Thickened terminal hyphae bearing lateral microaleuriospores may be present, or microaleuriospores may be born directly on the basal portion of macroaleuriospores. Macroaleuriospores usually present and frequently long, cylindrical, length more than 7 times diameter, or if thicker frequently fragmented into segments. Microaleuriospores on stalks infrequent (photomicrograph 27). Undersurface of thallus frequently becoming venous blood color with maturity, rarely yellow or brown. . . 50 T. rubrum (CHP), mostly tinea corporis and tinea pedis (also check 49 T. megninii and 52 T. tonsurans).



48 Thallus glabrous and heaped up, waxy, verrucose, or convoluted - See Color Plates I(16, 19); II(47); III(50, 54, 56, 58, 59, 61); IV(40). 49

Thallus downy to fluffy, and flat or folded; or powdery - See Color Plates I(13, 16); II(40, 44, 45, 49); III(50, 52); IV(13, 16, 40, 52). 64

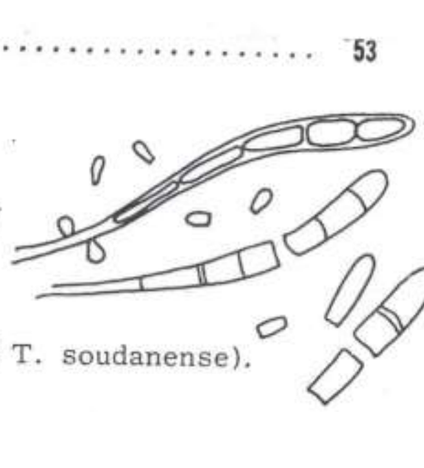
49 Thallus red, purple, or yellow. 50

Thallus white or brown. 59

50 Thallus red or purple. 51

Thallus yellow to orange. 53

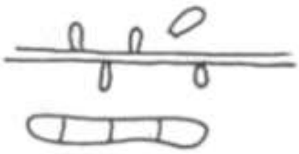
51 Mycelia usually with abundant disconnected hyphal segments and elongate macroaleuriospores or fragments of macroaleuriospores. More or less fluffy mutants with abundant microaleuriospores commonly appear. Only slight enhancement of growth with the thiamine. . . 50 T. rubrum (CHP), usually from tinea corporis, tinea pedis, and tinea manus (also check 54 T. violaceum, 55 T. gourvilii, and 56 T. soudanense).



Mycelia without abundant arthrospores. Macroaleuriospores few or absent. Commonly from endothrix tinea capitis.................... 52

52 Thallus waxy or verrucose, and usually dark purplish red. Mycelia much branched and entangled, usually without abundant arthrospores and without aleuriospores. Microaleuriospores rarely or never produced. Not commonly mutating to aleuriospore producing types. Requiring thiamine for growth . . . 54 T. violaceum (CHP), principally endothrix tinea capitis (also check 55 T. gourvilii, 49 T. rubrum, and 56 T. soudanense).

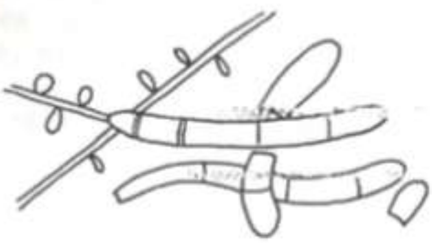
Thallus purplish red or garnet red, glabrous to slightly fluffy, frequently centrally accuminate with a flat radiating border. Microaleuriospores often present. Macroaleuriospores short, club-shaped, and infrequent. Not requiring thiamine . . . 55 T. gourvilii (CHP), principally endothrix tinea capitis. Geographically localized in equatorial and west Africa (also check 56 T. soundanense, 54 T. violaceum, 50 T. rubrum, and 49 T. megninii).



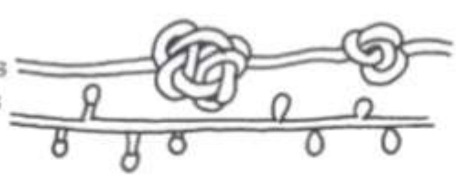
53 Culture producing frequent nodular organs or regularly developing a fluffy perimeter - Plate I(40), with microaleuriospores suggestive of T. mentagrophytes; or mutating to fuzzy violet type - Plate III(50), with macro- and microaleuriospores typical of T. rubrum................ 54

Macro- and microaleuriospores usually rare or absent. Culture not producing frequent nodular organs or regularly developing a fluffy perimeter and microaleuriospores suggestive of T. mentagrophytes; or not mutating to fuzzy violet type of T. rubrum......................... 55

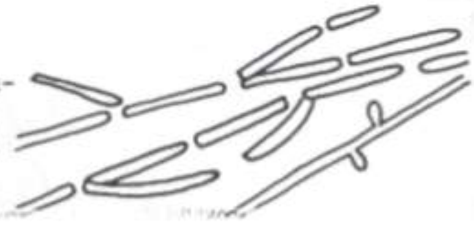
54 Culture usually mutating to fuzzy violet type with microscopic features of T. rubrum (elongate macroaleuriospores and tear-shaped microaleuriospores, see item 50). . . 50 T. rubrum (CHP), principally tinea corporis and tinea pedis (also check 56 T. soudanense and 55 T. gourvilii).



Mycelia with frequent nodular organs. Arthrospores may be present. Thallus usually developing a fluffy pleomorphic perimeter in about one week, and with spherical or tear-shaped microaleuriospores and other structures suggestive of T. mentagrophytes . . . 40 T. mentagrophytes var. nodular (CHP), principally tinea pedis (also check 62 E. floccosum, 13 M. canis and 52 T. tonsurans).



55 Thallus glabrous to slightly fluffy, heaped or centrally accuminate, frequently with a conspicuously fringed perimeter - Plate III(56). Hyphae and abundant arthrospores in brush-like bundles and with frequent reflexive branching. Microaleuriospores sometimes present. . . .



56 T. soudanense (CHP), principally tinea capitis, rare outside of equatorial Africa (also check 19 M. ferrugineum, 50 T. rubrum, 13 M. canis, and 46 T. verrucosum).

Reflexive branching not present. Thallus glabrous to slightly fluffy, and heaped, centrally accuminate, cerebriform, submerged, or spreading and with radial grooves. Perimeter of thallus irregular, submerged, sometimes more or less fringed - Plates I(19); II(47); III(61). Arthrospores or disarticulated, clubbed hyphae may be present, but not in brush-like bundles; or hyphae mainly branched and clubbed with frequent chalmydospores. 56

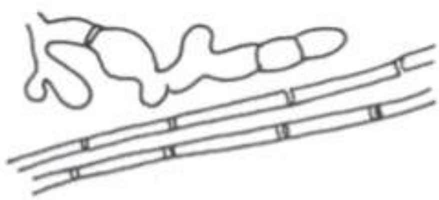
56 Thallus yellow-orange to rust-colored, heaped and slow growing, or spreading. Mycelium irregular, swollen and fragmented, or straight with or without terminal branching, but not highly branched throughout. Subcultured thallus retaining these features, or becoming coarse, fluffy, with hyphae straight and relatively unbranched. Frequently associated with ectothrix, fluorescent tinea capitis.......................... 57

Initial or subcultured thallus yellow or orange, without fragmented hyphae as a predominant feature. Mycelia consisting predominantly of a mass of irregular, antler-like, frequently clubbed branches and chlamydospores. Mainly associated with inflammatory tinea capitis, tinea corporis, tinea barbae, or favus, or tokelau.................... 58

57 Often converting in a week or so from fragmented, swollen mycelia to typical M. canis, with spreading fluffy thallus and fusiform macroaleuriospores (see item 4), or else growth poor and limited, with production of abortive terminal branching. . .

13 M. canis disgonic forms (CHP), zoophilic (also check 19 M. ferrugineum and 52 T. tonsurans).

Thallus yellow to rust colored, heaped up or cerebriforme; or white and somewhat flat, with yellow to rust colored, glabrous concentric zones. Hyphae irregular, fragmented, and swollen, or else thick and straight with prominent double septa (suggesting bamboo). May mutate to peach tan forms suggestive of M. audouinii, 19 M. ferrugineum (CHP), fluorescent tinea capitis, principally East Asia, North and tropical Africa, Eastern Europe (also check 13 M. canis, 56 T. soudanense, and 16 M. audouinii).

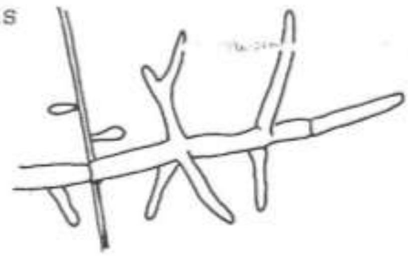


58 Thallus usually heaped or spreading with radial "alluvial" folds. Color of thallus lemon yellow to ochraceous - Plate II(47). Stimulated by thiamine to produce a flatter more downy thallus, sometimes with tear-shaped microaleuriospores. Usually connected with inflammatory ringworm and large spore ectothrix hair parasitism. . . 47 T. verrucosum (ochraceum form) (RHP to CHP), cattle, principally tinea corporis, tinea capitis, and tinea barbae (also check 59 T. schoenleinii, 40 T. mentagrophytes, 19 M. ferrugineum, and 13 M. canis).

Thallus usually heaped and much convoluted or warty. Color of thallus orange to honey-colored or brown, not bright yellow - Plate III(61, 59). Growth may be stimulated by thiamine but aleuriospores not produced. Associated with tokelau, a tropical form of tinea corporis restricted to South Asia, South Pacific Islands, and endemic areas in central and south America; or with favus, a form of tinea capitis, world-wide, but principally in East Europe, USSR, North Africa, Middle East, Iceland. Locally endemic elsewhere.. **63**

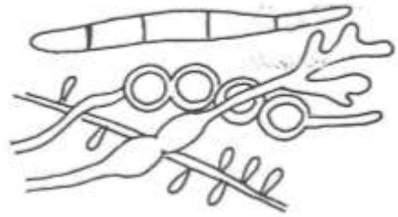
59 Thallus brown or honey colored................................. **60**

Thallus white or near white.. **61**



60 Thallus initially cream white, with a heaped glabrous surface, usually becoming more or less chocolate brown with maturity and producing brown diffusing pigment - Plate III(58). Microaleuriospores may be present. . . 58 T. yaoundei (CHP), principally tinea capitis, endemic equatorial Africa (also check 55 T. gourvilii and 54 T. violaceum).

Thallus honey colored to brown with a cerebriform or verrucose, glabrous surface, not becoming chocolate brown with maturity and not producing diffusing brown pigment - Plate III(47). Mycelia usually much branched with clubbed and antler-like tips......................... **61**

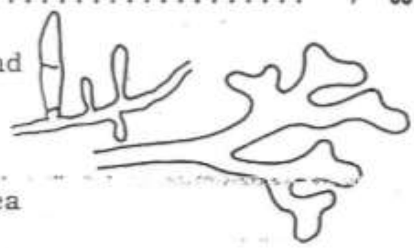


61 Thallus heaped up or flat, white or grey, glabrous button-like and tough, or somewhat fuzzy or downy. With addition of thiamine becoming more or less flat and downy, sometimes with production of aleuriospores. Mycelia irregular, branched, antler-like, and clubbed. Growth and chlamydospore production enhanced at 37°C. (If antler-like, clubbed hyphae and chlamydospores are not a conspicuous feature check T. violaceum var. glabrum). Associated with inflammatory ringworm and large spore ectothrix hair parasitism. . . 47 T. verrucosum (RHP to CHP), cattle (also check 59 T. schoenleinii, 61 T. concentricum, 54 T. violaceum, and 50 T. rubrum).

Thallus more or less cerebriform or folded and spreading, glabrous or somewhat fuzzy or downy, white to grey white or cream colored. Not producing aleuriospores with addition of thiamine. Hyphal elements frequently antler-like, or pectinate, or enlarged terminally. Associated with tinea imbricata, tinea corporis, favus type of tinea capitis, or fluorescent ectothrix tinea capitis.................................. 62

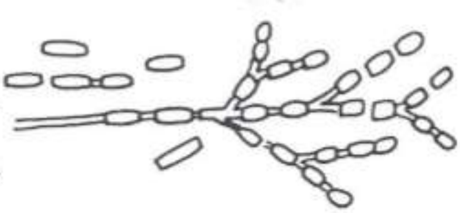
62 Thallus grey to white, glabrous to fluffy, rapidly growing, and relatively friable. With addition of yeast extract, becoming more fluffy and radially folded. Mycelia with terminal inflated and bulbous hyphal elements and pectinate hyphae. Macro-aleuriospores of the M. audouinii type sometimes present. . . 16 M. audouinii var. rivalieri (CHP), principally fluorescent ectothrix tinea capitis (also check 59 T. schoenleinii).

Thallus usually tough and leathery, not frankly fluffy, on first isolation entirely glabrous, and not exhibiting a marked change of morphology with addition of yeast extract.. 63



63 Thallus in subculture often becoming grey white and somewhat spreading with radial folds and tending to crack the agar. Branched antler and nail head hyphae present. Not stimulated by thiamine.
59 T. schoenleinii (CHP), favus, principally tinea capitis. World-wide but principally North Africa, Eastern Europe, Russia, Middle East, and Iceland (also check 47 T. verrucosum, 61 T. concentricum, 19 M. ferrugineum and 54 T. violaceum).

Thallus not normally becoming grey white and spreading with radial folds if subcultured regularly. Thallus predominantly heaped and cerebriforme. Branched hyphae frequent, nail head hyphae not well developed. . . 61 T. concentricum (CHP), tokelau, tinea corporis only, tropical and restricted to South Pacific area, South Asia, and limited foci in Central and South America (also check 47 T. verrucosum, 59 T. schoenleinii, and 50 T. rubrum).



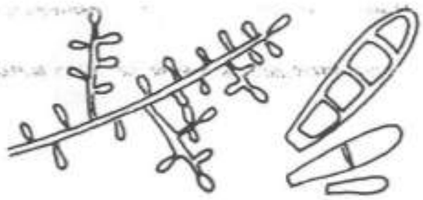
64 Culture producing abundant rectangular arthrospores (Arthroaleuriospores) and few or no micro- or macroaleuriospores. . . 50 T. rubrum (CHP), 36 T. gloriae (NHP), 40 T. mentagrophytes (CHP), 13 M. canis (CHP), 73 Malbranchea sp. (NHP), etc. Differential separation based upon evidence of microscopic and macroscopic morphology otherwise typical of these species.

Culture not producing abundant rectangular arthrospores or aleuriospores as a predominant or only microscopic feature....................... 65

65 Margin of growing thallus yellow beneath or thallus predominantly yellow beneath. 66

Undersurface of growing thallus not yellow, predominantly or at the growing margin. 69

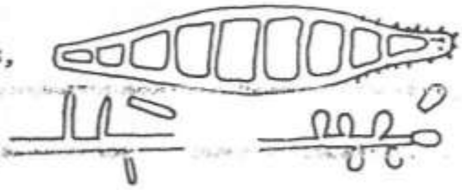
66 Surface of thallus powdery, white to cream, flat, remaining friable and powdery when mature - Plate II(44). Undersurface with clear yellow diffusing pigment. Microaleuriospores usually uniform, narrow, club-shaped, abundant, and in effect clustered on terminal hyphal branches. Macroaleuriospores frequently present and club-shaped, usually variable in size, the smallest intergrading with microaleuriospores.



44 T. erinacei (RHP), carried by hedgehog (also check 40 T. mentagrophytes and 45 T. equinum).

Mature thallus fluffy, or folded and suede-like, and forming skin-like pellicle on the agar rather than powdery and friable - Plates I(13), II(45), and IV(40). 67

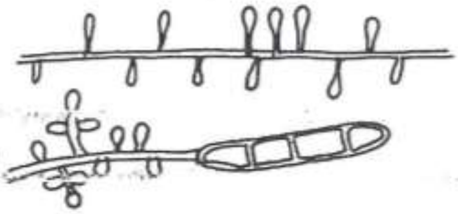
67 Surface of mature thallus frequently with loose cottony tufts and sometimes sparse glabrous areas, and flat and somewhat coarse and spreading, usually with 10 or more radial grooves. Micro-aleuriospores usually few and slender club-shaped or teardrop-shaped. Further study of culture usually reveals large, spindle-shaped macroaleuriospores (see item 4).



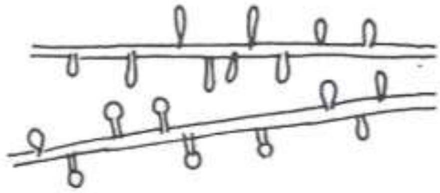
. . .13 M. canis (CHP) cat (also check 19 M. ferrugineum and 16 M. audouinii).

Surface of mature thallus densely and usually uniformly downy, velvety, or suede-like, flat or folded, and white, grey, tan, or pink. 68

68 Microaleuriospores usually predominantly lateral on non-terminal hyphae and variously spherical, teardrop-shaped, or club-shaped, sometimes with slender attenuated bases or stalks. Surface of thallus white to brownish or pink, fluffy or velvety, usually forming a compact folded pellicle on the agar. Undersurface of thallus usually yellow or yellow at the margin, becoming reddish or brownish centrally. Macro-aleuriospores club-shaped, but usually rare on Sabouraud Agar and associated with microaleuriospores clustered on terminal hyphal branches. . .45 T. equinum (RHP), horse. Some isolates may be confirmed by demonstrating a requirement for nicotinic acid (also check 40 T. mentagrophytes var. quinkeanum, 44 T. erinacei, 13 M. canis, 52 T. tonsurans var sulfureum, 50 T. rubrum, and 38 T. simii).



Lateral microaleuriospores spherical, egg-shaped, teardrop-shaped, peg-shaped, or club-shaped, but usually not conspicuously on slender stalks, or stalks if present consisting of a short supporting pillar of about the same diameter as the microaleuriospores. . .

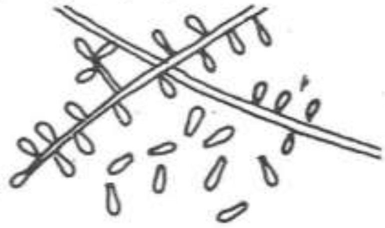


40 T. mentagrophytes (var. quinkeanum) (RHP) rodents, T. mentagrophytes var nodulare (CHP) principally tinea pedis (also check 50 T. rubrum, 36 T. gloriae, and 52 T. tonsurans).

69 Undersurface of thallus developing a dark red or garnet red pigment, surface glabrous to fluffy, pink to white, or greenish - Plate II(49); III(50, 55): IV(50). 70

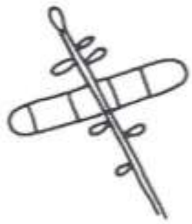
Undersurface of thallus not becoming distinctly red (may be brown, pinkish tan, or colorless) - Plates I(16); II(40); III(50, 52); IV(40). 72

70 Surface of thallus more or less fluffy and white, pink or greenish. Mature thallus usually developing a venous blood undercolor as it matures (or on potato dextrose agar). Microaleuriospores usually small and peg-shaped and rare to exceedingly abundant. Macroaleuriospores, if present narrow, appearing long cylindrical. . . 50 T. rubrum (CHP) principally tinea corporis, tinea pedis, and tinea manus (also check 49 T. megninii, 55 T. gourvilii, and 40 T. mentagrophytes var quinkeanum).

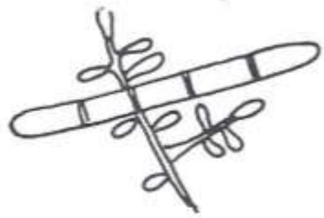


Surface and undersurface of thallus pink to garnet red, surface waxy, glabrous, fluffy, or suede-like. Microaleuriospores club-shaped or teardrop-shaped, rare to numerous but not exceedingly abundant. 71

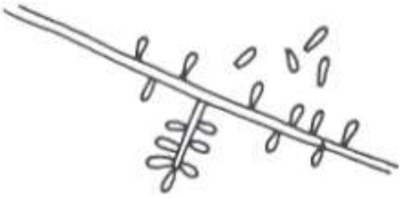
71 Thallus usually glabrous or waxy initially, and heaped and purplish pink to garnet red. Dark brown pigment may be produced beneath the thallus. Microaleuriospores usually few. . . 55 T. gourvilii (CHP) principally endothrix tinea capitis. Tropical West Africa (also check 54 T. violaceum, 56 T. soudanense, 49 T. megninii, and T. yaoundei).



Thallus pink and downy or suede-like with radial furrows. Undersurface garnet red. Microaleuriospores club-shaped to teardrop-shaped. Macroaleuriospores narrow, appearing long cylindrical. Histidine requiring . . 49 T. megninii (CHP) geographically restricted to Europe and Africa (need to distinguish from 40 T. rubrum by requirement for histidine) (also check 69 T. fluviomuniense and 55 T. gourvilii).



72 Thallus essentially white and fluffy, but producing a diffusing dark brown pigment- Plate III(50). aleuriospores lateral and peg-shaped . . . 50 T. rubrum (CHP), principally tinea corporis, tinea pedis, and tinea manus (also check 45 T. equinum).



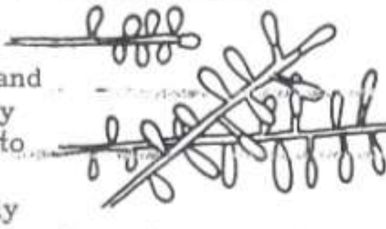
Thallus not producing a diffusing brown pigment or brown surface. 73

73 Undersurface of thallus tinted with salmon or peach-tan or brown. 74

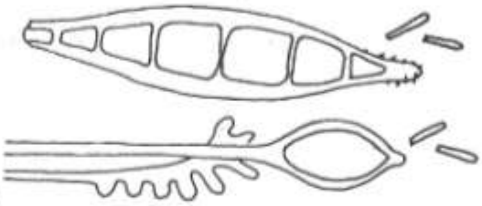
Undersurface of thallus colorless. 75

74 Surface of thallus flat and spreading, sparsely to densely and fluffy, developing radial furrows, and white, tan, or light pinkish brown. Microaleuriospores usually rare and pyriform to slender clavate. Macroaleuriospores usually absent. Terminal chlamydospores often present. Pectinate hyphae frequently present. . . 16 M. audouinii (CHP), principally tinea capitis (also check 52 T. tonsurans and 40 T. mentagrophytes).

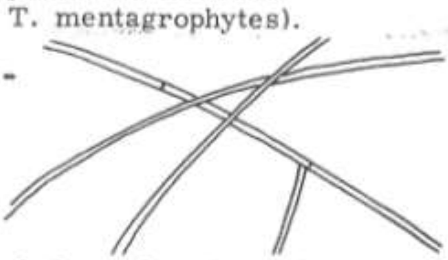
Surface of thallus somewhat powdery, white, yellow, or greyish, flat or becoming suede-like and folded with maturity. Microaleuriospores usually abundant and variable in size and shape, clavate to filiform. Terminal chlamydospores frequently present. . . 52 T. tonsurans (CHP), principally tinea capitis (also check 16 M. audouinii, 40 T. mentagrophytes and 50 T. rubrum).



75 Thallus essentially colorless on surface and below, flat spreading usually developing radial grooves Advancing mycelium usually relatively coarse, straight and succulent. Microaleuriospores pear-shaped to slender clavate and usually rare or absent. . . 13 M. canis (CHP), or 16 M. audouinii (CHP). Principally fluorescent tinea capits. Differentiate on rice media and by search for macroaleuriospores, pectinate hyphae, and terminal chlamydospores (also check 50 T. rubrum and 40 T. mentagrophytes).



Thallus white, flat and more or less fluffy. Advancing mycelium usually thin and flexuous. Lateral, pear-shaped microaleuriospores rare or more or less common throughout the thallus. . . 50 T. rubrum (CHP), 40 T. mentagrophytes (CHP), and pleomorphic forms of other dermatophytes. Differentiate T. rubrum from T. mentagrophytes by hair perforation and urease tests. These are difficult and often insolvable problems of identification. If the thallus is fluffy and no macro- or microaleuriospores or other special microscopic features are present, the culture is probably unidentifiable, constituting a completely pleomorphic dermatophyte or other fungus classed taxonomically as "Mycelia Sterilia".



Identification of yeast-like fungi

Experiment 8. Yeasts.

Yeasts are considered normal flora of oropharynx and gastrointestinal tract and may therefore be recovered from sputum, throat swabs, bronchial washings, gastric washings, and stool specimens. However, the repeated isolation of yeasts from a series of clinical specimens of urine, nail scrapings, and vaginal washings, from the same patient usually indicates infection with the organism recovered and identification of the isolates is necessary. Furthermore, the presence of yeast in normally sterile body fluids such as blood, cerebrospinal fluid (CSF), or fluids aspirated from the pleural cavity, or the pericardial sac, is also considered as a clinical situation in which species identification is justified. *Candida albicans* is the species of yeast most frequently cultured from clinical specimens.

The principal tests for the identification of yeasts involve the investigation of isolates for their ability to ferment sugars and also to assimilate various sources of carbohydrates and nitrates. However, commercial identification kits which give sufficient information to identify most isolates encountered in a medical laboratory are available.

The Urease test as previously described is also useful in yeast identification. *Cryptococcus* species can be confirmed by a positive urease reaction, whereas most *Candida and Saccharomyces* species are urease negative.

1. Collection of clinical specimens:

1. Swab the infected area with 70% alcohol to remove bacterial contaminants.
2. Collect infected material (in sterile containers, as smears on slides or culture directly) as follows: scraping from skin or nail; mucus patches from the mouth, vagina, or anus; sputum, blood, or cerebrospinal fluid.

2. Culturing of specimens:

1. Culture infected material on media (e.g., SDA) supplemented with penicillin and streptomycin or chloramphenicol.
2. Incubate at room temperature or 37 ºC for 3-4 days.

By the end of incubation period colonies of *C. albicans* appear as cream colored, smooth and pasty, and have a yeasty odor.

3. Identification of *Candida albicans*:

Microscopically a slide mount of *C. albicans* will show oval, budding cells 2.5-4 by 6 µm in size and some pseudomycelium if taken from submerged growth.

Illustrate and record your observations.

Experiment 9. Germ tube test.

a. Suspend a very small portion of an isolated colony of the yeast to be tested in a test tube containing 0.5 ml of rabbit or human serum.
b. Incubate the test tube at 37 ºC for no longer than 3 hours.
c. Place a drop of the yeast-serum suspension on a microscope slide, and cover with a coverslip.
d. Examine under the microscope for the presence of germ tube (**Figure**).

Note that the germ tube of *Candida albicans* has no constrictions at the point of origin, in contrast to that of *C. tropicalis*.

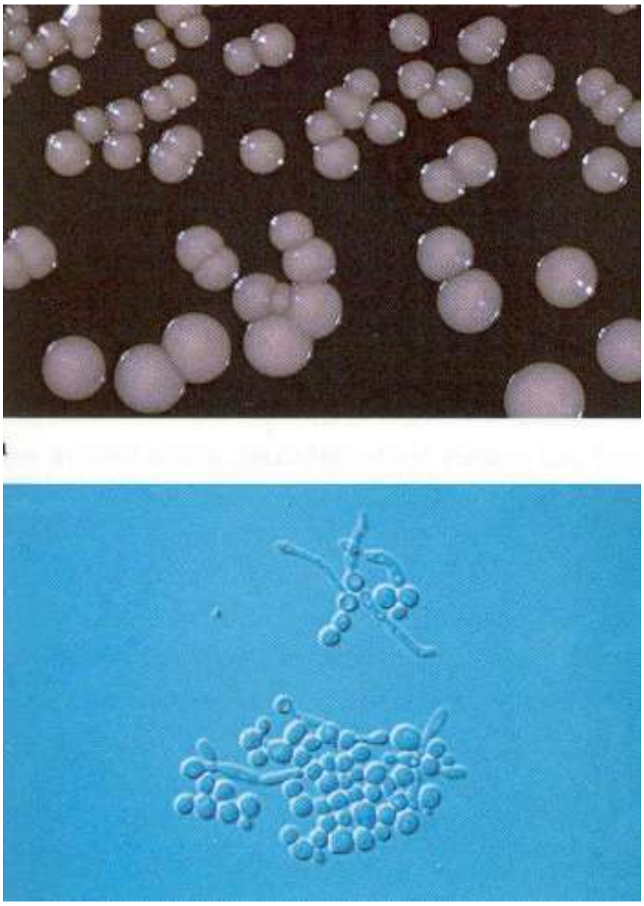


Figure. (a) A culture of Candida albicans on Sabouraud Dextrose Agar showing typical cream-colored, smooth-surfaced waxy colonies. (b) The microscopic morphology of *Candida albicans* showing budding spherical to ovoid blastoconidia.

Illustrate and record your observations.

Experiment 10. Production of chlamydospores on CMA-Tween 80 (1% tween 80 CMA).

a. Inoculate a portion of the yeast colony on CMA plates by making three parallel cuts about 1 cm apart into the agar, holding the inoculating wire at about 45- degree angle.

b. Place a coverslip on the surface of the agar covering the inoculating streaks.

c. Incubate at 30 ºC for 24-48 h.

d. Examine under the microscope for the presence of chlamydospores.

Illustrate and record your observations.

Experiment 11. Carbohydrate fermentation test for yeast identification:

Physiological tests for yeast identification

The principal tests for the identification of yeasts involve the investigation of isolates for their ability to ferment sugars and also to assimilate various sources of carbohydrates and nitrates. However, commercial identification kits which give sufficient information to identify most isolates encountered in a medical laboratory are available.

The Urease test as previously described is also useful in yeast identification. *Cryptococcus* species can be confirmed by a positive urease reaction, whereas most Candida and *Saccharomyces* species are urease negative.

Yeast fermentation in appropriate culture media containing a single carbohydrate source is detected by gas and acid production.

a. To each of the yeast fermentation tubes (glucose, maltose, sucrose, and lactose) add 0.2 ml of a yeast suspension in saline equivalent to a McFarland No. 4 standard (see Appendix A).
b. Incubate at 37 ºC for 48 hours.
c. Leave all negative test in incubator for 6-10 days before discarding.

Note the presence of bubbles or a drop in the liquid level, in the inverted Durham's tube, which indicates the fermentation. The development of a yellow color is not a reliable indicator of fermentation and should be ignored (Table B.1 & B.2 in Appendix B)

McFarland Nephelometer standards:

Determination of the number of microorganisms in a liquid medium by using the turbidity method:

The number of microorganisms in a liquid medium can be determined by visually comparing the turbidity of the liquid medium to a standard that represents a known number of microorganisms in suspension.

Turbidity standards can be prepared by mixing chemicals that precipitate to form a solution of reproducible turbidity. Such solutions, using barium sulfate, were developed by McFarland to approximate numbers of bacteria in solutions of equal turbidity, as determined by colony counts (Table B.2).

Preparation of McFarland Nephelometer standards:

Principle: A chemically induced precipitation reaction can be used to approximate the turbidity of a bacterial suspension.

Method:

1. Set up 10 test tubes or ampoules of equal size and of good quality. Use new tubes that have been thoroughly cleaned and rinsed.
2. Prepare 1 % chemically pure sulfuric acid.
3. Prepare a 1.175 % aqueous solution of barium chloride ($BaCl_2 . 2 H_2O$).
4. Slowly, and with constant agitation, add the designated amounts of the two solutions to the tubes as shown in Table 1 to make a total of 10 ml per tube.

5. Seal the tubes or ampoules. The suspended barium sulfate precipitate corresponds approximately to homogenous E. coli cell densities per millimeter throughout the range of standards as shown in Table B.2.
6. Store the McFarland standard tubes in the dark at room temperature. They should be stable for 6 months.

Illustrate and record your observations.

Malassezia furfur (Pityrosporum)

- Is the causative agent of pityriasis versicolor and
- is also implicated as a causative agent of seborrhoeic dermatitis and dandruff.
- It has also been recovered in blood cultures from neonate and adult patients undergoing lipid replacement therapy.

• Diagnosis requires

- special culture media, and
- blood drawn back through the catheter is the preferred specimen.
- Culture of the catheter tip is also recommended.
- *M. furfur is characterised by*
  - globose, oblong-ellipsoidal to cylindrical yeast cells (Figure).
  - Reproduction is by budding on a broad base and from the same site at one pole (unipolar).
  - It is a lipophilic yeast; therefore in vitro growth must be stimulated by natural oils or other fatty substances.

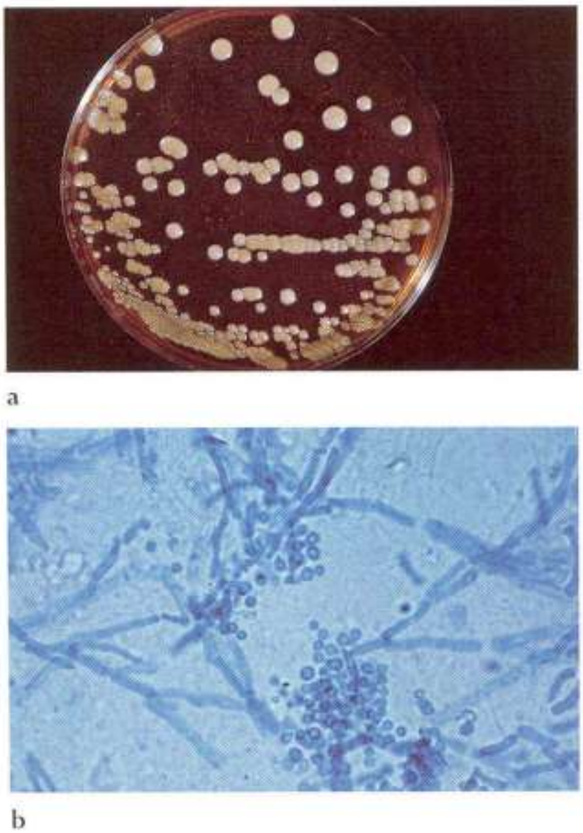


Figure 1.13
(a) Colonies of Malassezia furfur *on Dixon's agar. (b) Skin scrapings taken from patients with pityriasis versicolor stain rapidly when mounted in 10% KOH, glycerol and Parker ink solution, and show characteristic clusters of thick-walled, round, budding yeast-like cells and short angular hyphal forms up to 8 µm in diameter (average diameter 4 µm). These microscopic features are diagnostic for the causative agent* Malassezia furfur, *and culture preparations are usually not necessary.*

Exercise 12. Keratinolytic activity of fungi

Materials needed:

Fungal isolates: *Arthroderma cuniculi; Chrysosporium keratinophilum; Microsporum gypseum; Rhizopus stolonifer; Trichophyton ajelloi*. All fungal isolates are maintained on SDA slants.

Other materials: Sterile sand; Hairs from a blond-child, 1 cm long; Lactophenol cotton blue.

Procedure:

Keratinolysis test. The hair-soil method will be used to detect the keratinolytic activity. Culture the isolates on a sand-hair substrate for determination of keratinolytic activity as follows:

1. Wash sand under tap water, dry and autoclave.
2. Place 25-ml sand in Petri dishes (9-cm diameter), and moisten with 10-ml sterile distilled water.
3. Scatter hairs from a blond-child, 1 cm long, on the surface of the sand.
4. Inoculate the Petri dishes with aqueous spore suspension prepared by scraping off the surface of 14-day old fungal colonies cultured on SDA, and then wash with 10 ml sterile distilled water.
5. Incubate the cultures at room temperature for 20 days, and moisten with sterile distilled water when necessary. Employ two replicate plates for each isolate.
6. Mount the inoculated hairs in lactophenol cotton blue for microscopic examination (10 X, 40 X, and 100 X).
7. Explain results (based on the examination of 10 hairs, 5 from each replicate plate for each isolate) in light of the model shown in the following Figure. The term surface erosion (SE) is used to indicate progressive destruction of the hair from the exterior or inwards; this may occur either uniformly (U) along the length of the hair, or in localized areas forming more or less extensive pockets (P). Random attack on the hair by more or less specialized hyphae that penetrate the hair at right angles to the surface is termed radial penetration (Rp). A boring hypha is used to describe fine hypha about 1 μm diam. which penetrates the substratum at right angle to the layer of keratinized cells, while a perforating organ is used to describe single column of up to 10 short hyphal cells, usually 3-4 times wider than normal mycelial cells, that penetrates radially into the hair cortex, passing straight through its keratinized cells irrespective of their boundaries. Wider boring hyphae (wbh) indicate structure intermediate in diameter between boring hyphae and perforating organs. Swollen boring hyphae (sbh) are structures that are similar to boring hyphae when penetrating the outer cortex, but dilated in a series of balloons on reaching what are probably less compact regions of the hair.
8. Estimate the intensity of keratinolytic activity (IKA) as follows. The activity is based on a scale of 0 to 100, and is estimated by giving different weights to the presence of various features of hair keratinolysis (i.e., invasive structures, hair degradation, etc.) as follows: IKA= G + F + U + P + bh + sbh + wbh + po. Where G, fungal growth 0-5%; F, fruiting 0-

5%; U, uniform surface erosion 10%; P, pocket-like surface erosion 20%; bh, boring hyphae 10%; sbh, swollen boring hyphae 10%; wbh, wider boring hyphae 20%; and Po, perforating organ 20%. In case of complete degradation of hair, IKA is considered 100%.

Illustrate and record your observations.

Exercise 13. Antifungal susceptibility testing

In this exercise, nystatin (anticandidal drug), griseofulvin, and extracts of some medicinal plants will be compared for their antifungal activity against some selected human-pathogenic fungi.

1. Collection of plant material

Some plant species, commonly used in folk medicine in Palestine for the treatment of fungal skin infections, will be selected in this exercise. Mature plants can be collected, dried in the shade, and ground into a powdered material using an appropriate seed mill.

2. Plant extracts (ethanolic extract) and discs preparation:

a) Soak a 20-g portion of the powdered plant material in 100 ml of 95% ethanol for 5 days at room temperature, and stir the mixture daily for regular infusion.

b) After a five-day period filter the extract in muslin or Whatman filter paper no.1.

c) Dry the extracts using a rotary evaporator at 60 ºC, and store the final dried extract in labeled sterile glass bottles and keep at –20 ºC

d) Reconstitute one gram of the dried extract using 5 ml of the solvent.

e) Sterilize paper discs and impregnate each with 50 µl of the extract to give a final concentration of 10 mg of dried extract per disc.

3. Preparation of inocula

a. Inoculate part of an isolated *C. albicans* colony into a 5ml Muller-Hinton broth tube and incubate for 4-18 hrs at 37 ºC.

b. Adjust the growth turbidity in Muller-Hinton broth by further incubation or dilution with sterile physiological saline, after comparison with that of a McFarland nephelometer tube no. 0.5 (10^8 CFU/ml) using a spectrophotometer at 625 nm (optical density 0.08-0.1).

I. Anticandidal susceptibility test:

A. Disk diffusion method

a. With a sterile cotton applicator swab 10^8 CFU/ml of *C. albicans* culture on the surface of SDA agar by dipping the cotton applicator into the candidal suspension, rotate several times and press against the inside wall of the tube to remove excess inoculum.

b. Streak the agar plate in three different directions and around the agar margin to ensure even distribution of the inoculum.

c. Leave the plates to dry for 3-5 minutes.

d. Using sterile forceps, distribute the selected extract disks evenly on the surface of the agar plates.

e. Use a positive control (nystatin, 10 mg / disc), and a negative control (solvent). Use three replicate plates for each test.

f. Incubate the plates upside-down at 37 ºC for 48 hrs.

g. Measure the inhibition zone around each disk using a transparent ruler.

B. Determination of minimum inhibitory concentration (MIC) - broth dilution method

1. Preparation of media

For each isolate, 15 tubes, each with 9.9 ml Muller Hinton broth were prepared and autoclaved.

2. Preparation of the active ingredient dilutions

1. Reconstitute one gram of the dried plant extract using 5 ml of the solvent to give a final concentration of 200 mg/ml (stock solution).
2. Prepare several dilutions of the stock solution as shown in (Table B.3).

3. Incorporation of active ingredients into media and reading the results:

1. Into broth tube 1, add 0.1 ml of stock solution. Into broth tube 2, add 0.1 ml of dilution 2 (Table B.3). Repeat the procedure for the remaining dilutions. The final concentrations of the active ingredients in broth are shown in Table B.4.
2. For positive control, add 0.1 ml of reference antibiotic, while for negative control prepare two tubes by adding 0.1 ml of solvent to the first tube and 0.1 ml of sterile water to the second.
3. Add 10 µl of *Candida albicans* suspension of (10^8 CFU/ml) concentration to each of the 15 tubes of extract serial dilutions.
4. Incubate the tubes at 37 ºC for 24 hrs.
5. Prepare a positive control by adding 0.1 ml of nystatin to a 9.9-ml broth tube.
6. Prepare a negative control by adding 0.1 ml of sterile water to a 9.9-ml broth tube.
7. Examine turbidity after incubation. The lowest concentration of the extract that inhibits the growth of the organism (visually clear) is designated MIC.

Record and discuss your results.

C. Minimum fungicidal concentration (MFC)
Prepare subcultures from the visually clear tubes on SDA plates. The tube that has subcultured and shows no growth on agar plate indicates the MFC.

II. Screening for antifungal activities against mycelial fungi - agar dilution method:
Antimycotic activity is carried out by the poisoned-food technique method, which involves the cultivation of the test organism on a medium containing the test chemical, and then measuring its growth. The use of poisoned agar is most common, but use of a shake culture in a liquid media provides more precise results. The growth of fungi in poisoned liquid medium is measured using dry weight. The following is an outline of the poisoned agar medium method (Dikshit& Husain, 1984; see also Abu-Ghdaib, 1998; Ali-Shtayeh *et al*., 1998a).

A. Poisoned-food technique method:

1. Inoculate test isolates (see fungal isolates in **Materials needed**) onto SDA plates and incubate at 25 ºC for 7-10 days to obtain young, actively growing cultures consisting of mycelia and conidia.
2. Dissolve the required amount of the dried plant extract or reference antimycotic drug in 2 ml ethyl alcohol or 10 % aqueous dimethylsulfoxide (DMSO), and sterilize by filtration through a 0.45 um membrane), and then mix in requisite amount of pre-sterilized SDA medium to give a final concentration of 15 µg/ml.
3. Cut out a mycelial disc of 5-mm diameter, from the periphery of the 7-10 day old cultures, and aseptically inoculate onto the medium.
4. In controls, use sterile DMSO or distilled water in place of plant extract.
5. Incubate the inoculated plates at 25 ºC.
6. After 7 days measure and record colony diameter. Percentage of mycelial inhibition is calculated as follows. % Mycelial inhibition = [(dc-dt)/dc] x 100; dc = colony diameter in control, dt = colony diameter in treatment. Use three replicate plates for each treatment.

B. Minimum inhibitory concentration determination is performed by a serial agar dilution plate technique as follows:

1. Reconstitute extracts solutions (0.01mg - 3 mg/ml concentrations).
2. Incorporate into SDA sterilized pre-poured medium.
3. Pour the medium, and allow the agar in the plates to set.
4. Inoculate the plates with the test fungi and incubate as mentioned above. Control plates, which contain no plant extracts, are also made with the test.

5. Determine the MIC of each plant after 7 days. This is the lowest concentration at which no visible growth is observed.

C. Minimum Fungicidal Concentration (MFC) determination is performed as follows:

1. Re-inoculate the inhibited discs of each fungal isolate on SDA medium separately.
2. Incubate as above.
3. Examine the plates after 7 days. Absence of mycelial growth on the seventh day indicates fungicidal nature. MFC is regarded as the lowest concentration of the test compound that prevented growth of the fungus, indicating > 99.5 % killing of the original inoculum.

Illustrate and record your observations.

APPENDIX A

MEDIA AND REAGENTS

Corn Meal Agar (CMA): (Satisfactory for the growth and sporulation of many fungi)

Ground maize kernels ………….…………………….60 g
Agar……………………………………………..……15 g
Water ……………………………………………1000 ml
Boil 60 g freshly ground maize kernels in 1-liter water for 1 hour. Strain the suspension through two layers of muslin. Add the agar and heat until dissolved. Autoclave at 15 psi for 15 minuets.

Czapek-Dox Agar (CzA): (suitable for growing *Aspergillus*, *Penicillium* and *Nocardia* spp.)

$NaNO_3$ ……..……………..…………………………2 g
K_2HPO_4 ……………………………………………....1g
KCl ………………....……………..…..………….....0.5 g
$MgSO_4 . 7H_2O$ …………..……..……..………......0.5 g
$FeSO_4 . 7H_2O$ ……………………………..……....0.01 g
Sucrose …………………………..…………...…..30 g
Agar ………………………………….……….……15 g
Distilled water …………………………..……...1000 ml
Sucrose is added just before sterilization in order to reduce contamination.

Hoyer's medium:

Gum Arabic ………………………………….. …30g
Chloral hydrate ……………………………….200g
Glycerin ………………………………….. ……..20 g
Water ………………………………….. ……...50 ml.

Soak gum Arabic in the water and chloral hydrate. Leave the mixture to stand and occasionally agitate over several days until the material is dissolved. Then add glycerin. Wetting with absolute ethanol for 1 min followed by a 1-min treatment in 2% KOH and washing in 70% ethanol may be necessary for certain specimens before mounting in this medium.

Malt Extract Agar (MEA):

Useful for the growth of wood destroying and many other fungi
Malt extract ……………………...………………...25 g
Agar ………..………………………………………..15 g
Water ……………………………………………1000 ml
Heat the malt extract in water until dissolved. Add agar and dissolve. Fill up the liquid with distilled water to 1 liter. Adjust the medium to a final pH of 6.5 by NaOH.

M2 Agar:

Malt extract …………………………….……...…5%
Yeast extract ……………………………….……0.2%
Agar …………………………………………….……1.8%

Potato Dextrose Agar (PDA):

Useful for the growth of many fungi, especially phytopathogens, some bacteria
Potato (peeled and diced) …………….………….. 200 g
Dextrose (glucose) ……………………..……….. 20 g
Water …………..……………………………..…1000 ml
Rinse potato under running water, and then add to water. Boil for 1 hour. Filter through a cloth, squeezing through as much pulp as possible. Autoclave at 15 psi for 30 minutes.

Sabouraud's Dextrose Agar (SDA):

Dextrose …..………………………………..………. 40 g
Peptone ………..………………..………………….. 10 g
Agar ……………………………………………….20 g
Mix the ingredients, boil to melt the agar, adjust pH to 5.6 and sterilize. Autoclave for 10 min.

Sterile Soil Extract:

Soil ……………………...……..………………… 50 g
Distilled water………………………..……… 1000 ml
Add soil to water and shake well and allow to settle for 48 hours. Filter through Whatman no. 1 filter paper and autoclave for 20 min.

3P Medium:

Cornmeal agar ……………………………..……17 g
Pimaricin ……………………………………….5 mg
Polymixin-B ……………………………………..50 mg
Penicillin……..……….………………………… 50 mg
Distilled water …………………………………1000 ml

Tween 80:

Tween 80 ……………………….………………200 ml
Distilled water …………………..………………800 ml

2P Medium:

Cornmeal agar …………………….…….……….17 g
Polymixin-B …………………….…….….………50 mg
Penicillin …………………………..…….….…50 mg
Distilled water …………………………..……....1000 ml

APPENDIX B

Table B.1. Characteristics of *Candida* species most often isolated from clinical specimens.

	Assimilations												Fermentations						Other reactions					
Species of candida	**Glucose**	**Maltose**	**Sucrose**	**Lactose**	**Galactose**	**Melibiose**	**Cellobiose**	**Inositol**	**Xylose**	**Raffinose**	**Trehalose**	**Dulcitol**	**Glucose**	**Maltose**	**Sucrose**	**Lactose**	**Galactose**	**Trehalose**	**Urease**	**KNO3**	**Pseudohyphae**	**Growth at 37 oC**	**Germ tubes**	**India ink capsule**
C. albicans	+*	+	+	-	+	-	-	-	+	-	+	-	AG	AG	A†	-	AG or A	AG or A†	-	-	+	+	+	-
C. stellatoidea‡	+	+	-	-	+	-	-	-	+	-	+†	-	AG	AG	-	-	-	-	-	-	+	+	+	-
C. parapsilosis	+	+	+	-	+	-	-	-	+	-	+	-	AG or A	-	-	-	AG or A	AG or A†	-	-	+	+	-	-
C. tropicalis	+	+	+	-	+	-	+†	-	+	-	+	-	AG	AG	AG	-	AG	AG	-	-	+	+	-	-
C. pseudotropi-calis	+	-	+	+	+	-	+	-	+†	+	-	-	AG	-	AG	AG	AG	AG or A†	-	-	+	+	-	-
C. krusei	+	-	-	-	-	-	-	-	-	-	-	-	AG	-	-	-	-	-	+†	-	+	+	-	-
C. guillermindii	+	+	+	-	+	+	+	-	+	+	+	+	AG	-	AG	-	AG or A†	AG or A†	-	-	+	+	-	-
C. rugosa	+	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-

* += Assimilation, growth density greater than 1+ turbidity by the Wickerham card; AG= acid and gas; A= acid only produced in fermintation broth; -= negative.
† Strain variation. ‡ Report as *C. albicans* when *C. stellatoidea* is identified.

Table B.2. McFarland Nephelometer standards

	Tube number										
	0.5	1	2	3	4	5	6	7	8	9	10
Barium chloride (ml)	0.05	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
Sulfuric acid (ml)	9.95	9.9	9.8	9.7	9.6	9.5	9.4	9.3	9.2	9.1	9
Approximate bacterial cell density (x 10^8/ml)	1.5	3	6	9	12	15	18	21	24	27	30
Approximate candidal cell density (x 10^6/ml)	1-5										