# B.Sc. MEDICAL MICROBIOLOGY LAB MANUAL

3rd Semester

Prepared By
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BMLT

# MIDNAPORE CITY COLLEGE

# B.Sc. Medical Microbiology Lab Manual (3rd Sem)

## Paper: Mycology & Virology

## Code: BMM 395

### COLLECTION AND TRANSPORT OF MYCOLOGICAL SAMPLES:

All efforts should be made to collect specimens for fungal culture as free from bacterial contamination as possible.

### HAIR, SKIN AND NAILS

- The scalps of patients with suspected tinea capitis may be examined with a Wood's lamp. Fluorescent distorted or fractured hairs should be removed with forceps. Infected hairs can easily be removed, but normal hairs are more difficult to dislodge. A comb or brush may be used to collect loose hair and skin squames.
- Skin, when involved, should be cleansed with an alcohol wipe before a specimen is collected. Epidermal scales at the active border of a lesion should be removed with a scalpel. Nails should be cleansed with alcohol wipe, and the outermost layer should then be removed by scraping with a scalpel. Deeper scrapings, debris from under the edges of the infected nails, and nail clippings from infected areas are also suitable for culture.
- Samples of hair, skin and nails should be collected and placed in a sterile culture dish for transport to the laboratory. Storage of 4°C is not recommended since at least one dermatophyte is susceptible to cold temperatures. In addition, storage in closed containers is unsuitable due to overgrowth of contaminating bacteria and saprobic fungi in a moist environment.
- Nail clippings may be ground in a mortar before being inoculated onto culture media. Skin scrapings and hair may be inoculated directly onto the surface of appropriate culture media.

### BODY FLUIDS INCLUDING CEREBROSPINAL FLUID

- All body fluids are collected aseptically by needle aspiration and should be sent to the laboratory in a sterile container as quickly as possible. However, if a delay is unavoidable, CSF should not be refrigerated, since it is an excellent culture medium and fungi will continue to replicate at 25-30°C.
- Other body fluid specimens may be stored at 4°C overnight, if necessary before culturing. All body fluids should be concentrated by centrifugation for 15 minutes at 1000 x g, and a minimum of 0.5 ml of sediment should be inoculated onto the surface of media. Small volumes of CSF not suitable for centrifugation may be dropped directly on media surface.

### BLOOD AND BONE MARROW

- As with the collection of other sterile body fluids, good skin antisepsis should be practiced for blood sample collection. Ten millilitres of blood should be collected at periodic intervals as determined by the physician. Blood is inoculated to blood culture bottles at bedside. Bottles should be returned immediately to laboratory for incubation at 35°C for 28 days.

- Bone marrow aspirates and biopsies are collected after good skin antisepsis and are commonly submitted to the laboratory in a sterile syringe or tube containing EDTA. The collected specimen should be transported to the laboratory as soon as possible; however, it may be refrigerated for no longer than 12 hours, if immediate culturing is impossible. Direct plating on mycology media can be done at bedside. Media may be obtained from the microbiology department.

**URINE**

- The urinary meatus must be adequately cleansed if a clean-catch or catheterized specimen is to be submitted for culture. Suprapubic aspirates are obtained after good skin antisepsis is used in the area of aspiration. Specimens should be cultured promptly since bacteria and yeasts replicate rapidly in specimens kept at room temperature. If specimens cannot be cultured soon after receipt, they should be refrigerated at 4°C for no longer than 12 to 15 hours. Twenty-four-hour specimens or those collected from indwelling catheter collection bags are not suitable for culture.

**VAGINAL SECRETIONS**

- Vaginal and cervical specimens collected by a physician are usually submitted on a swab. Transport to the laboratory should be rapid; however overnight refrigeration before culturing is satisfactory. Specimens should not be stored at room temperature.

**RESPIRATORY SPECIMENS**

Specimens from the ear, nose, nasopharynx, and mouth are usually submitted on a sterile swab.

- All specimens from the lower respiratory tract should be collected in a sterile wide-mouth bottle or sputum cup. A first morning expectorated sputum specimen is optimal. Before a sputum specimen is collected the patient's, teeth must be extensively brushed or his or her dentures be removed. The mouth should be cleansed by a mouthwash or several rinses of sterile water of saline. Only a specimen expectorated from deep within the lungs if satisfactory. For those patient's incapable of expectoration, sputum induction is necessary.
- Specimens should be transported to the laboratory as soon as possible to ensure maximum recovery of fungi. If culturing is delayed, specimens may be refrigerated at 4°C.

**TISSUES/BIOPSIES**

- Tissue should be divided aseptically by the surgeon in the operating room, and material representative of the infectious process should be submitted to the surgical pathology lab for culture and direct microscopic examination. All biopsy tissues should be placed in a sterile container containing a small amount of saline without a preservative. When an abscess is drained, a portion of the abscess wall should be submitted for culture. The surgical pathologist will visually examine specimens prior to submission to the microbiology lab.
- Surgical specimens should be transported to the surgical pathology laboratory as soon as possible after collection. If immediate culturing is impossible, specimens should be stored at 4°C for no longer than 8-10 hours.

**VIRAL SPECIMEN COLLECTION:**

| SPECIMEN TYPE | GUIDELINES | DEVICE AND MINIMUM VOLUME | TRANSPORT TIME AND TEMP | COMMENTS |
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| **Blood** | 1.Cleanse venipuncture site with 70% isopropyl alcohol.<br>2. Starting at the site, swab concentrically with 2% iodine tincture.<br>3. Allow the iodine to dry (≈ 1 min).<br>4. Do not palpate the vein at this point.<br>5. Collect 8-10 ml in an anticoagulant tube (viral transport is not required).<br>6. After venipuncture, remove iodine from the skin with alcohol. | Except for body fluids (BAL, CSF, urine, blood), place all viral specimens in UTM[a]. | Most viruses remain stable at 4°C for 2-3 days and almost indefinitely at –70°C.<br>Do not freeze at –20°C. | Commonly ordered for: CMV. Collect blood during the early, acute phase of infection.<br>Maintain at RT[a].<br>Do not refrigerate. |
| **CSF** | 1. Disinfect site with 2% iodine tincture.<br>2. Insert a needle with stylet at L3-L4, L4-L5, or L5-S1 interspace.<br>3.On reaching the subarachnoid space, remove the stylet and collect 2-5 ml in a sterile leakproof tube. [UTM[a] (Universal Testing Medium) not required]. | Heparin tube, 8-10ml/tube. You may need to draw 2 or more tubes from patients who are leukopenic. | Submit at RT | Frequently isolated: coxsackievirus (some), echovirus, enterovirus, mumps virus.<br>Less frequently isolated: arboviruses, HSV, LCMV, rabies virus. |
| **Cervical or vaginal swab** | 1. If lesions are present, swab vigorously. Place swab in UTM[a].<br>2. If lesions are not | Sterile screw-cap tube, 1.0 ml. | Submit immediately at 4°C. | Frequently isolated: HSV, CMV<br>Noncultivable: papillomavirus, |

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| | present, remove mucus from the cervix with a swab and discard the swab.<br>3. Firmly sample the endocervix (≈ 1 cm). into the cervical canal) with a fresh swab by rotating the swab for 5 seconds.<br>4. Place swab in UTM[a].<br>5. Carry out a vulvar sweep using a second swab; place both swabs in the same transport tube. | | | molluscum contagiosum virus. Although a cervical swab sample is the specimen of choice in the monitoring of pregnant women with a history of genital HSV infection, recovery of HSV may be increased by also sampling the vulva. |
| **Conjunctiva swab** | 1. Collect material from the lower conjunctiva with a flexible, fi ne-shafted swab moistened with sterile saline.<br>2. Place swab in UTM[a]. | Swab | Immediately place swab in UTM[a].<br>Submit at 4°C. | Frequently isolated: adenovirus; coxsackievirus A (some), CMV, HSV, enterovirus (including type 70), Newcastle disease virus. |
| **Feces** | 1. Pass directly into a clean, dry container.<br>2. 2-4 g of stool to sterile, leak proof container and transport immediately to lab. | Swab | Immediately place swab in UTM[a].<br>Submit at 4°C. | Frequently isolated: adenoviruses; enteroviruses.<br>Less frequently isolated: rotavirus Rotavirus antigen is detected by EIA. |
| **Nasal swab** | 1. Pass a flexible, fi ne shafted swab into the nostril.<br>2. Rotate slowly for 5 seconds to absorb secretions.<br>3. Remove swab and place in UTM[a].<br>4. Repeat for other nostril using a fresh swab. Place both swabs in the | Sterile, leakproof, wide-mouth container, at least 2 g or more. | Submit at 4°C. | Frequently isolated; influenza virus, parainfluenza virus, rhinovirus (limited), RSV (nasopharyngeal preferred)<br>Influenza A virus and RSV are detected by antigen assay |

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| | same transport tube. | | | (EIA). |
| **Nasopharynx aspirate or wash** | 1. Pass appropriate size tubing or catheter into the nasopharynx.<br>2. Aspirate material with a small syringe.<br>3. If material cannot be aspirated, tilt patient's head back about 70°<br>and in still 4ml. of sterile PBS until it occludes the nostril.<br>4. Reaspirate. Place in sterile container.<br>5. Place specimen at 4°C immediately. | Swab | Immediately place UTM. Submit at 4°C. | Frequently isolated: influenza virus, parainfluenza virus, rhinovirus (limited), RSV. Influenza A virus and RSV can be detected by antigen assay. |
| **Nasopharynx swab** | 1. Pass a flexible, fi ne shafted swab into the nasopharynx.<br>2. Allow secretions to absorb for 5 seconds; then carefully remove swab and place it in UTM[a].<br>3. Repeat for other nostril using a fresh swab. Place both swabs in the same transport tube. | Sterile container | Submit at 4°C. | Frequently isolated: influenza virus, parainfluenza virus, rhinovirus (limited), RSV. |
| **Oral swab** | 1. Firmly sample base of lesion(s) with a swab.<br>2. Place swab in UTM. | Swab | Immediately place swab in UTM[a]. Submit at 4°C. | Frequently isolated: enterovirus (some), HSV. |
| **Rash Maculopapular** | 1. Gently cleanse area with sterile saline.<br>2. Disrupt the surface of the lesion and firmly | Swab | Immediately place swab in UTM[a]. Submit at 4°C. | Frequently isolated: adenovirus, enterovirus, rubella virus, measles virus |

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| | sample its base with a swab moistened with sterile saline.<br>3. Place swab in UTM[a]. | | | (rubeola virus)<br>Less frequently isolated: pox viruses<br>Noncultivable: parvovirusB19 |
| **Vesicular** | 1. Sample only fresh vesicles because older crusted vesicles may not contain viable virus.<br>2. Cleanse area with sterile saline.<br>3. Carefully open the vesicle with needle or scalpel blade.<br>4. Using a swab, collect fluid and cellular material by vigorously sampling the base of the lesion.<br>5. Place in UTM[a]. | Swab | Immediately place swab in UTM[a].<br>Submit at 4°C. | Frequently isolated: enterovirus (some), echovirus, HSV, VZV<br>Less frequently isolated: poxviruses<br>The preferred specimen for VZV is a vesicle aspirate placed in 1 ml. UTM[a]. |
| **Throat swab** | 1. Using a tongue depressor, depress the tongue to prevent contamination with saliva.<br>2. Firmly sample the posterior pharynx, tonsils, and inflamed areas with a sterile swab.<br>3. Place swab in UTM[a]. | Swab | Immediately place swab in UTM[a].<br>Submit at 4°C. | Frequently isolated: adenovirus, CMV, enterovirus, HSV, influenza A and B viruses, measles virus, mumps virus, parainfluenza virus.<br>Less frequently isolated: RSV. |
| **Tissue** | 1. Obtain samples from areas directly adjacent to affected tissue.<br>2. Place specimen in a sterile vial containing UTM[a]. | UTM[a] | Submit at 4°C. | Always submit as much tissue as possible. Never submit a swab that has simply been rubbed over the surface. |
| **Urethral swab** | Patient should not have urinated within 1h prior to collection.<br>1. Express and | Swab | Immediately place swab in UTM[a].<br>Submit at 4°C. | Frequently isolated: CMV, HSV |

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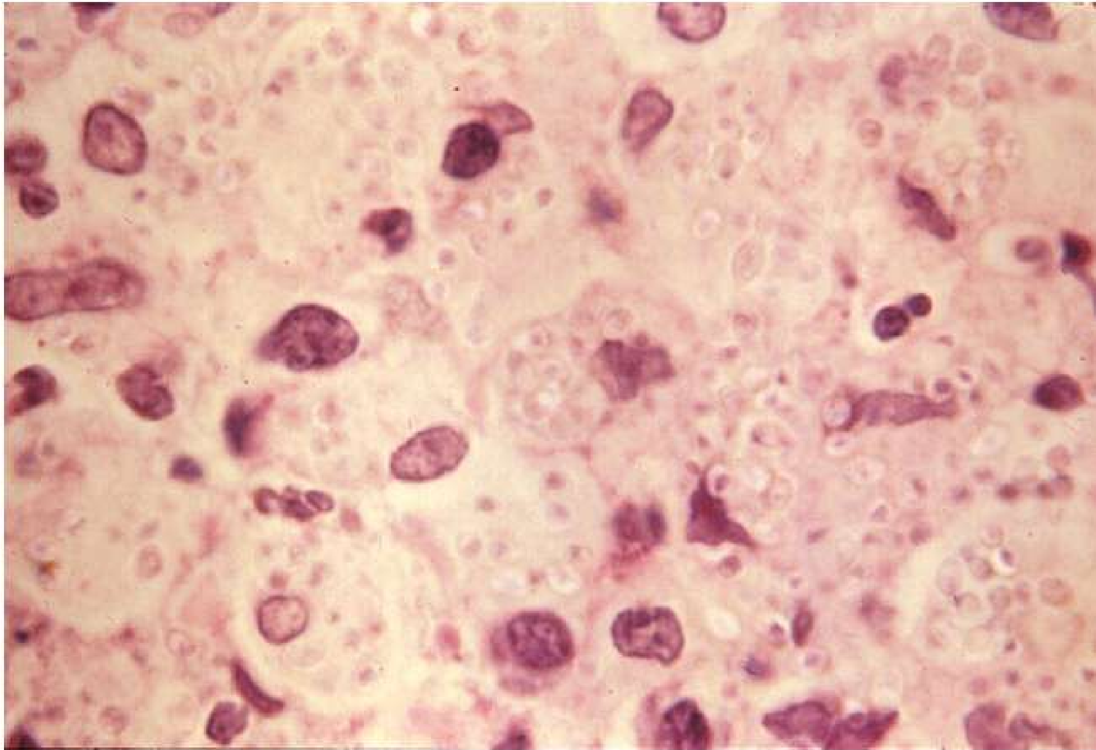
| | discard any exudate.<br>2. Carefully insert flexible, fine-shafted swab 4 cm. into urethra.<br>3. Rotate swab 2-3 times to obtain an adequate number of cells.<br>4. Remove swab and place in UTM[a]. | | | |
| **Urine** | Refer to specific guidelines for urine collection.<br>Collect 5 ml. of midstream clean, voided urine in a sterile container (UTM[a] not required). | Sterile container, 5 ml | Submit at 4°C. | Frequently isolated: adenovirus, CMV, HSV, mumps virus<br>Less frequently isolated: polyomavirus (JC virus), rubella virus<br>Two or three specimens on successive days maximize recovery of CMV. |

**Short forms:** BAL, bronchoalveolar lavage; CSF, cerebral spinal fluid; UTM, virus, PBS, phosphate buffered saline, RT, room temperature; CMV, cytomegalovirus; HSV, herpes simplex virus; EBV, Epstein-Barr virus; HIV-1, human immunodeficiency virus type 1: LCMV, lymphocytic choriomeningitis virus: EIA, enzyme immunoassay; RSV, respiratory syncytial virus; ELISA, enzyme-linked immunosorbent assay; VZV, varicella-zoster virus.

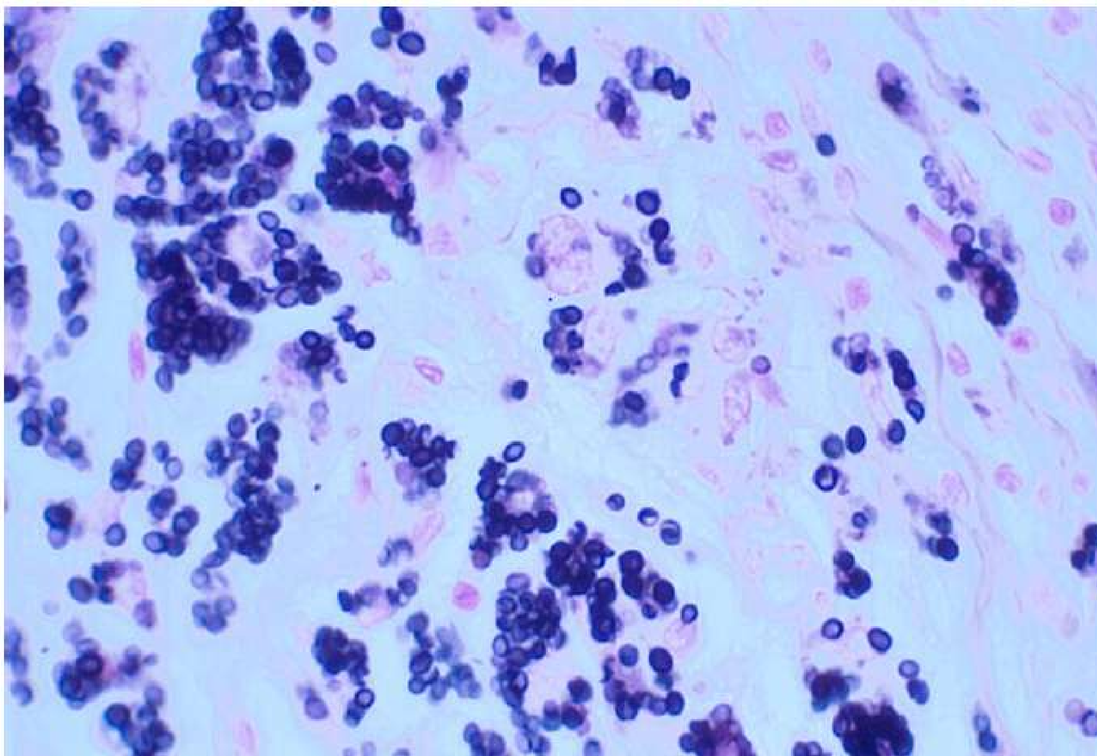
**UTM:** UTM Universal Transport Medium is an FDA-cleared collection and transport system suitable for the collection, transport, preservation, and long-term freeze storage of clinical specimens containing viruses for viral molecular diagnostic testing, including COVID-19, chlamydia, mycoplasma or ureaplasma organisms. The transport medium comes in a plastic, screw cap tube and maintains organism viability for 48 hours at room or refrigerated temperature.

## HISTOPATHOLOGY SLIDES OF FUNGAL DISEASES

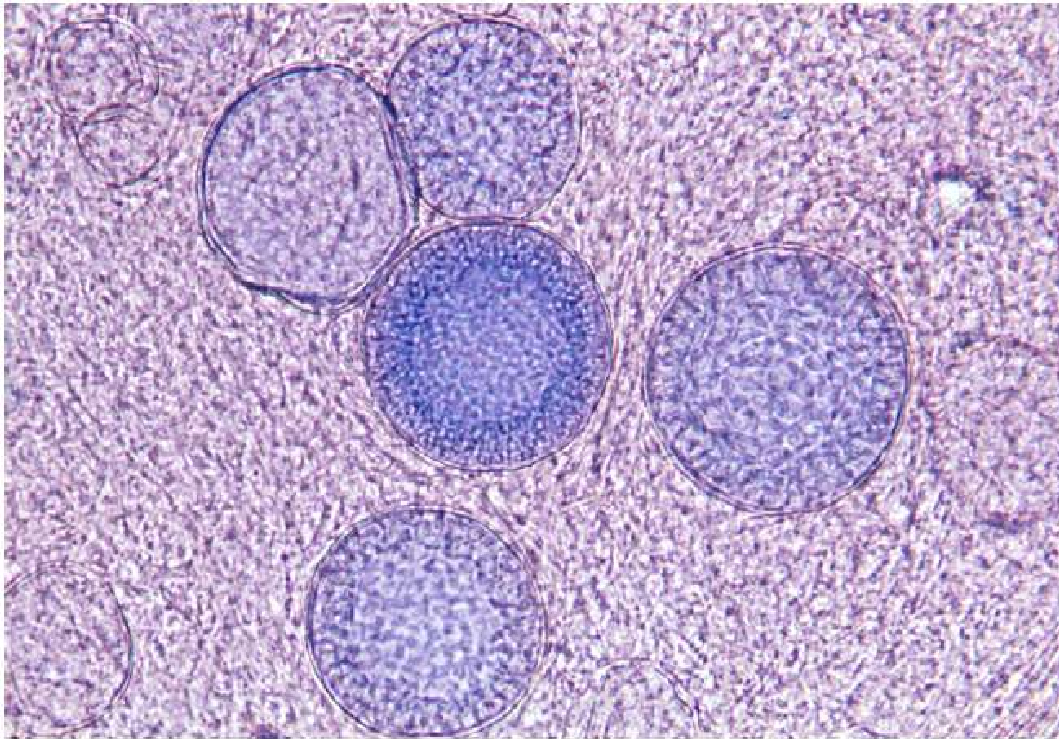
1. H&E showing macrophages containing numerous yeast cells of *Histoplasma capsulatum*. The basophilic cytoplasm of the fungal cells is retracted from the poorly stained cell wall, giving the false impression of a capsule.



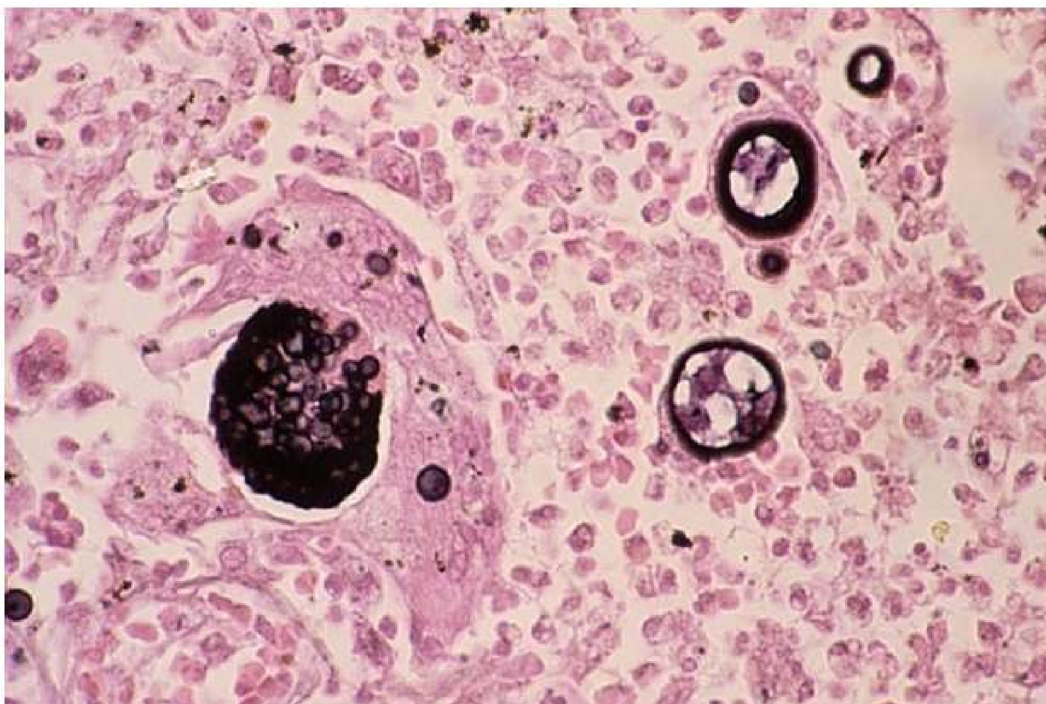
2. Grocott's methenamine silver (GMS) from a lung biopsy showing numerous yeast cells of Histoplasma capsulatum inside macrophages.



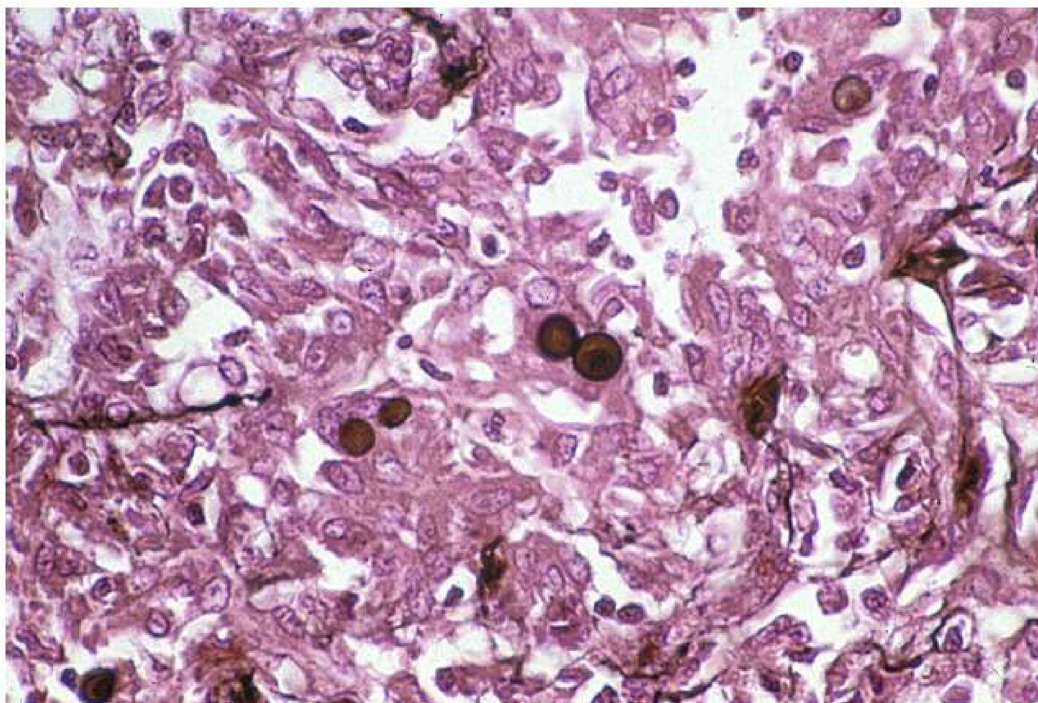
3. Direct microscopy of skin scrapings from a cutaneous lesion mounted in 10% KOH and Parker ink solution showing characteristic endosporulating spherules (sporangia) of *Coccidioides immitis*. The presence of spherules with endospores is diagnostic.



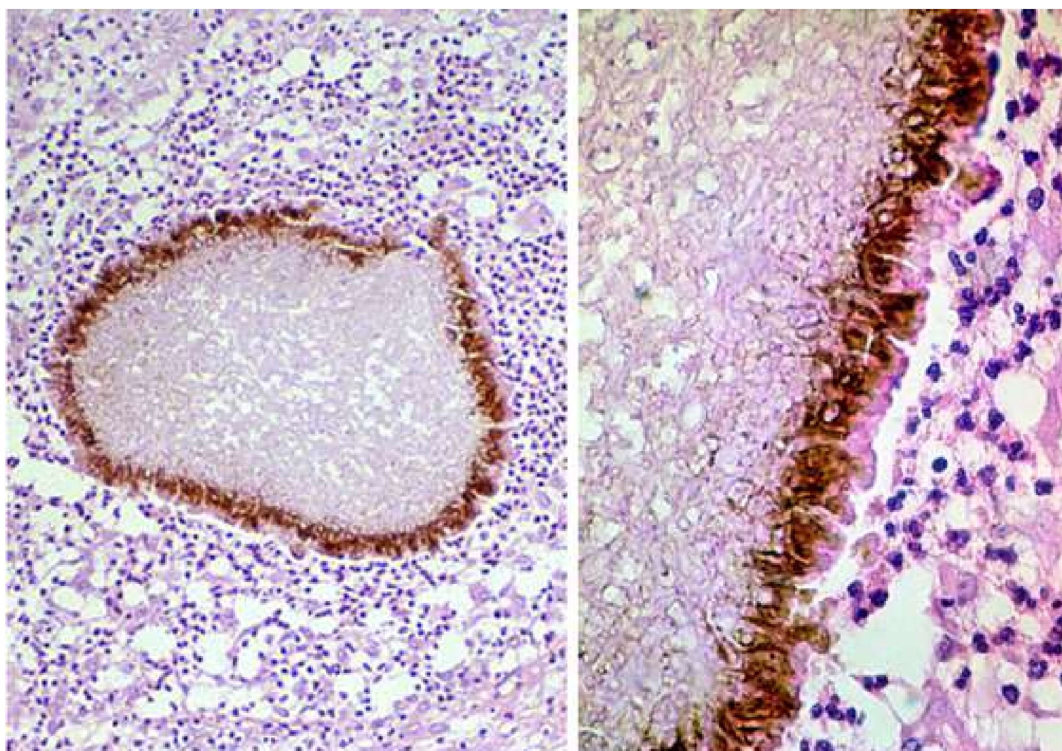
4. Periodic Acid-Schiff (PAS) stained tissue section showing typical endosporulating spherules of *Coccidioides immitis*. Young spherules have a clear centre with peripheral cytoplasm and a prominent thick wall. Endospores (sporangiospores) are later formed within the spherule by repeated cytoplasmic cleavage. Rupture of the spherule releases endospores into the surrounding tissue where they re-initiate the cycle of spherule development.



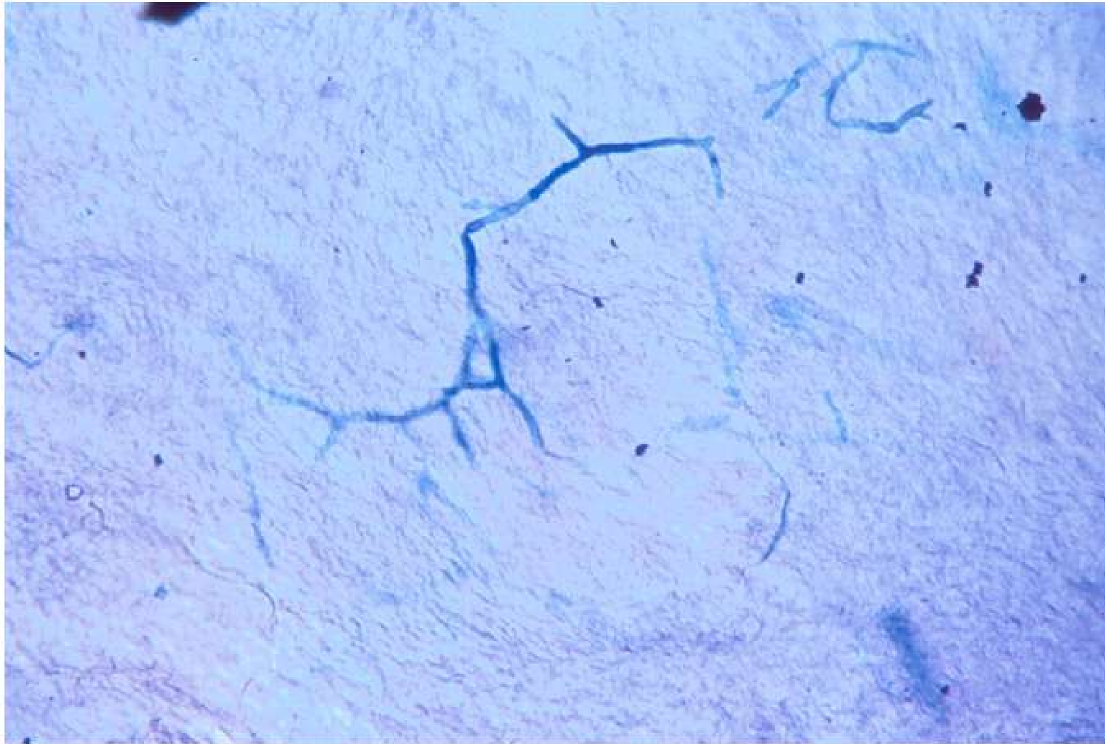
5. Blastomycosis: Tissue sections showing large, broad-base, unipolar budding yeast like cells, 8-15m in diameter. Note: tissue sections need to be stained by Grocott's methenamine silver method to clearly see the yeast-like cells, which are often difficult to observe in Haematoxylin and eosin (H&E) stained preparations.



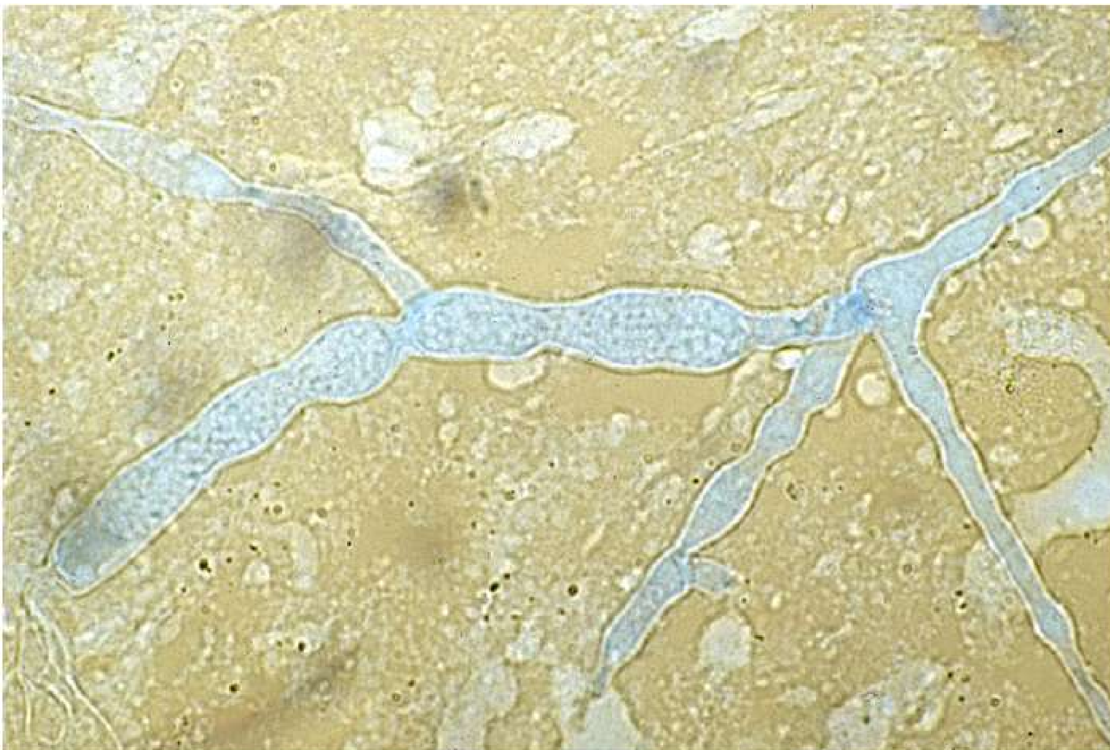
6. Haematoxylin and eosin (H&E) stained tissue section showing black grained eumycotic mycetoma caused by *Madurella mycetomatis*.



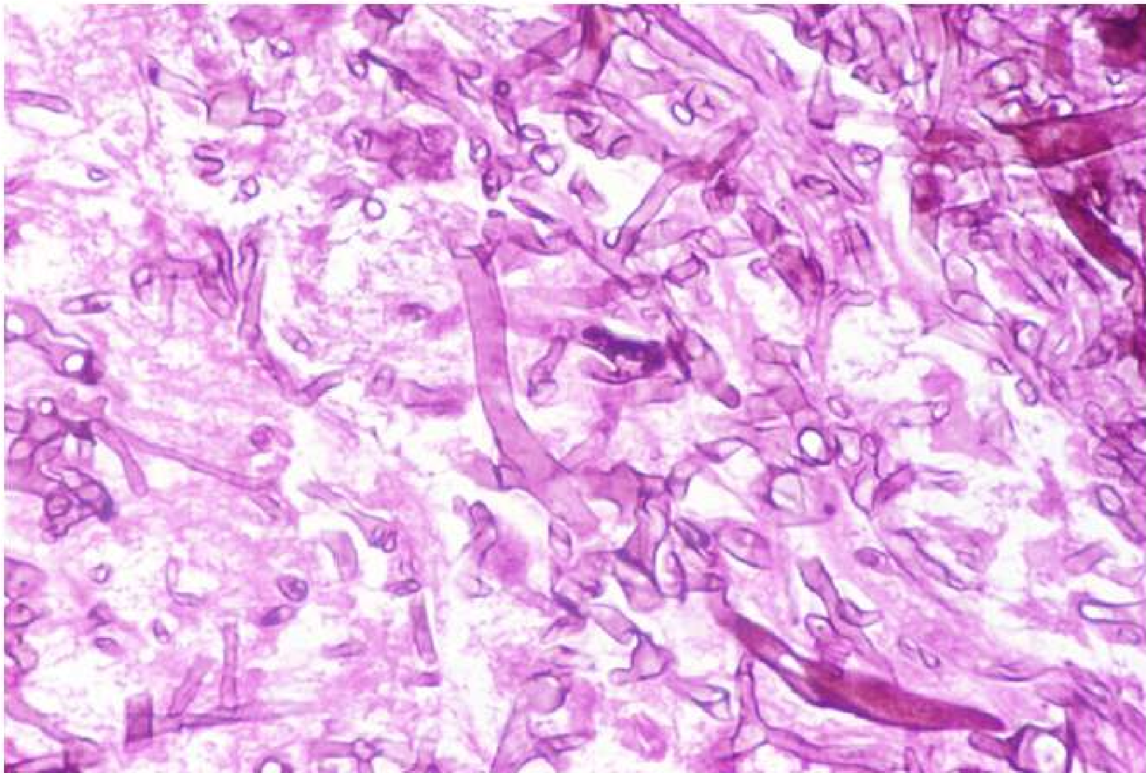
7. Direct microscopy of a skin scraping in 10% KOH and Parker ink solution showing broad, sparsely septate hyphae typical of a zygomycete.



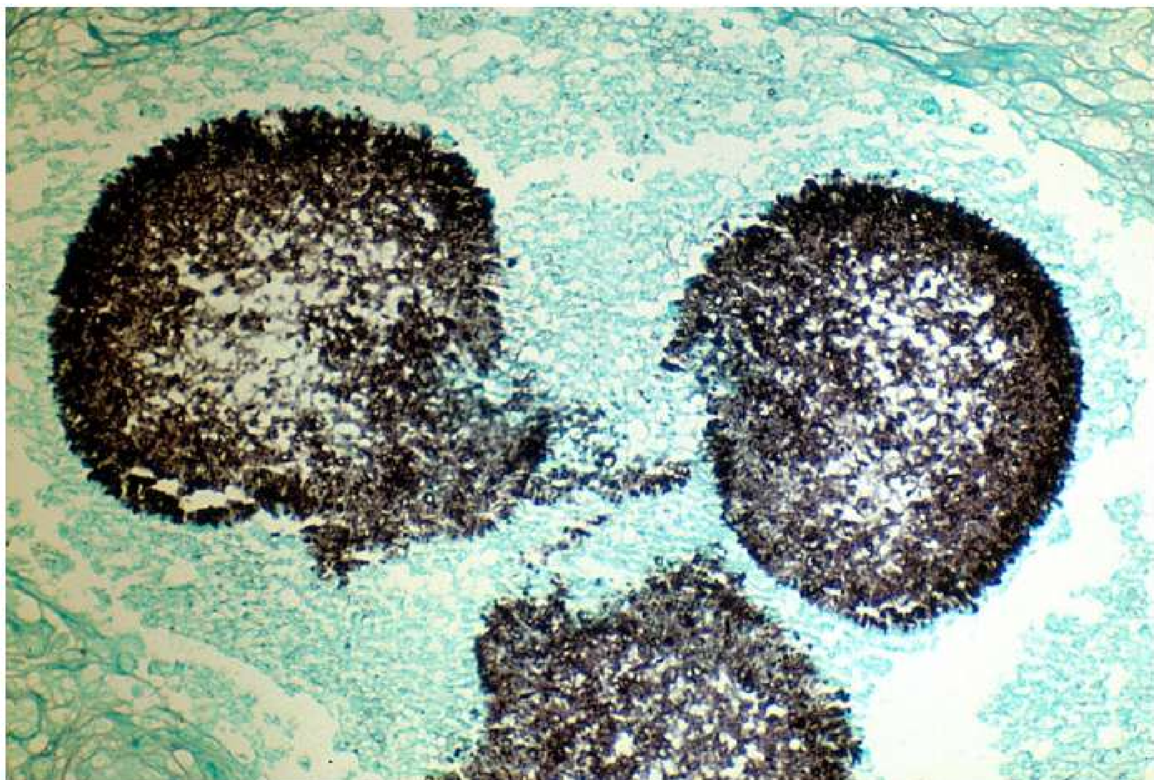
8. Direct microscopy of a skin scraping in KOH showing broad, sparsely septate hyphae with focal bulbous dilations typical of a zygomycete.



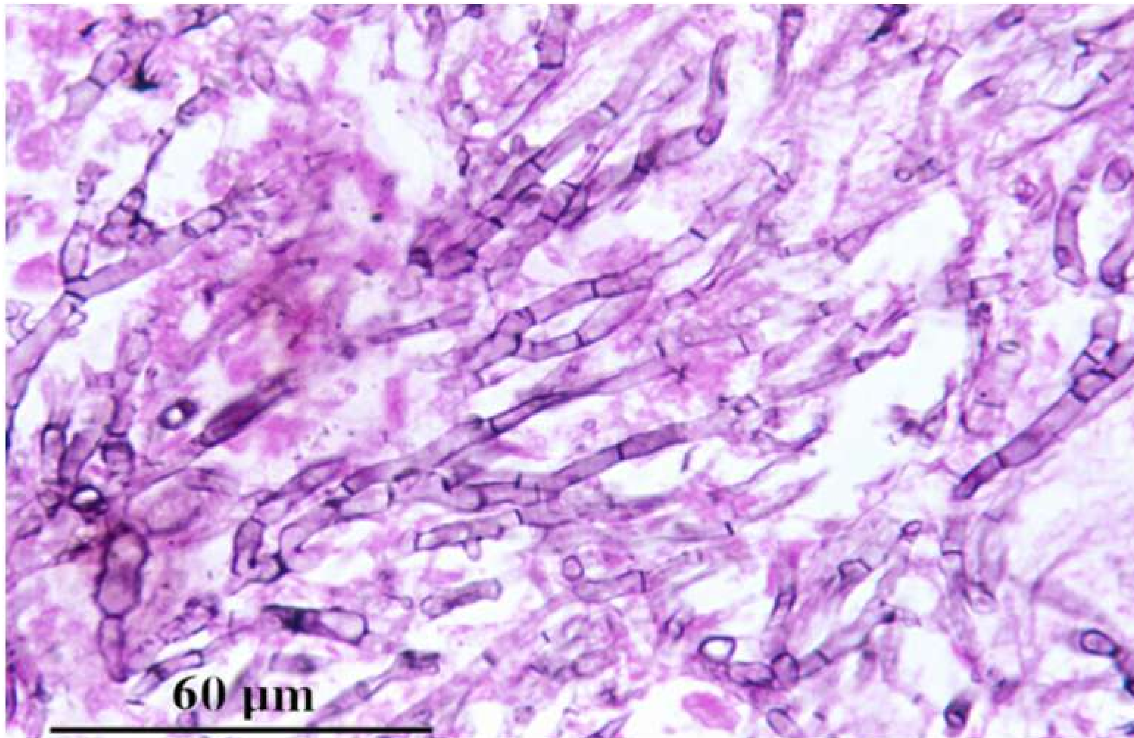
9. H&E stained section showing broad, infrequently septate, thin-walled hyphae with focal bulbous dilations and irregular, non-dichotomous, often right-angled, branching typical of a zygomycete.



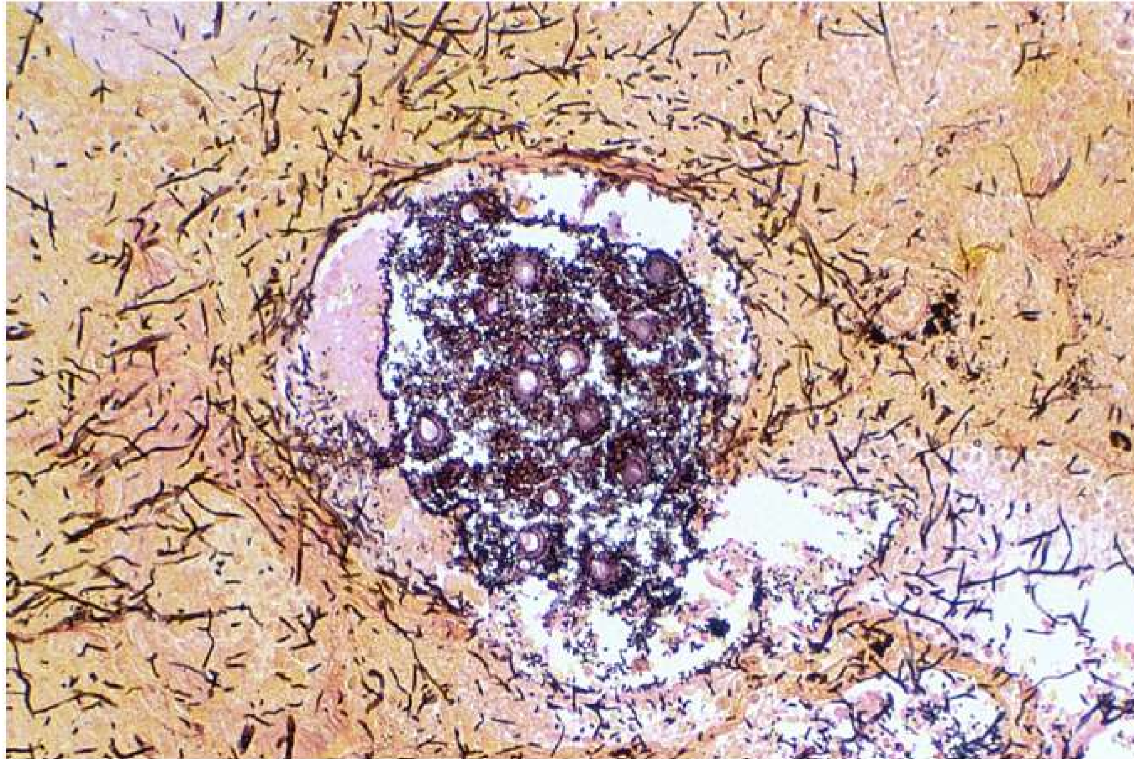
10. Grocott's methenamine silver (GMS) stained tissue section of lung showing fungal balls of hyphae of *Aspergillus fumigatus*.



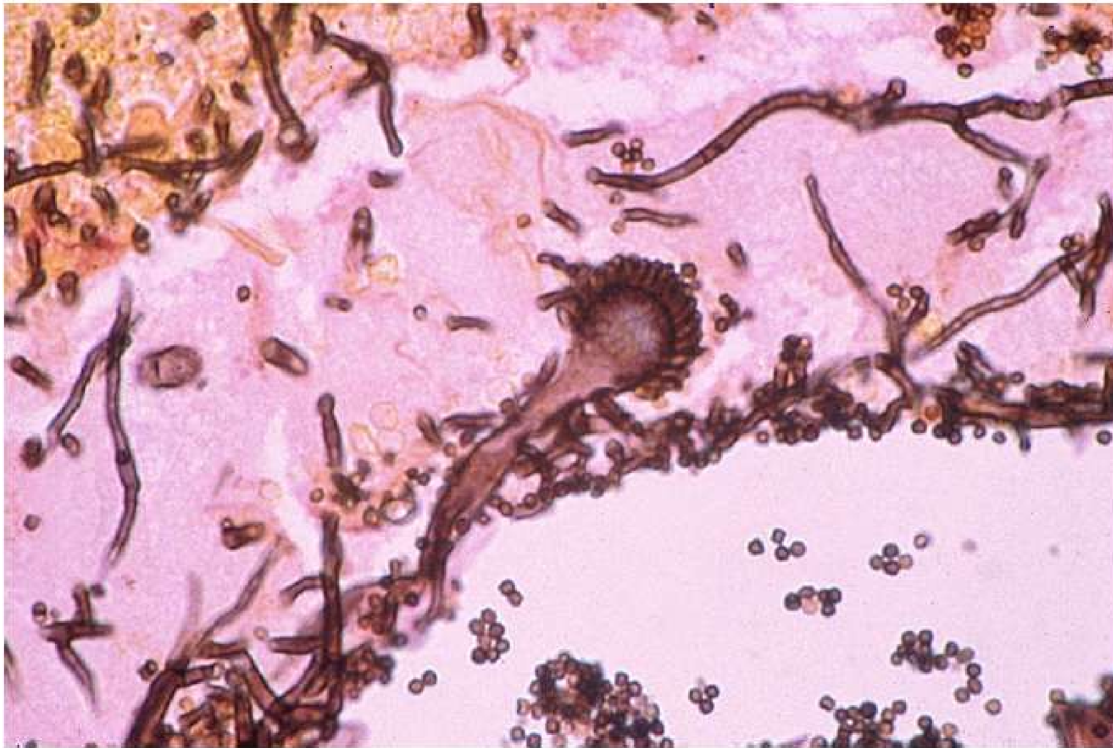
11. *Aspergillosis* of the lung. GMS stain showing dichotomously branched septate hyphae.



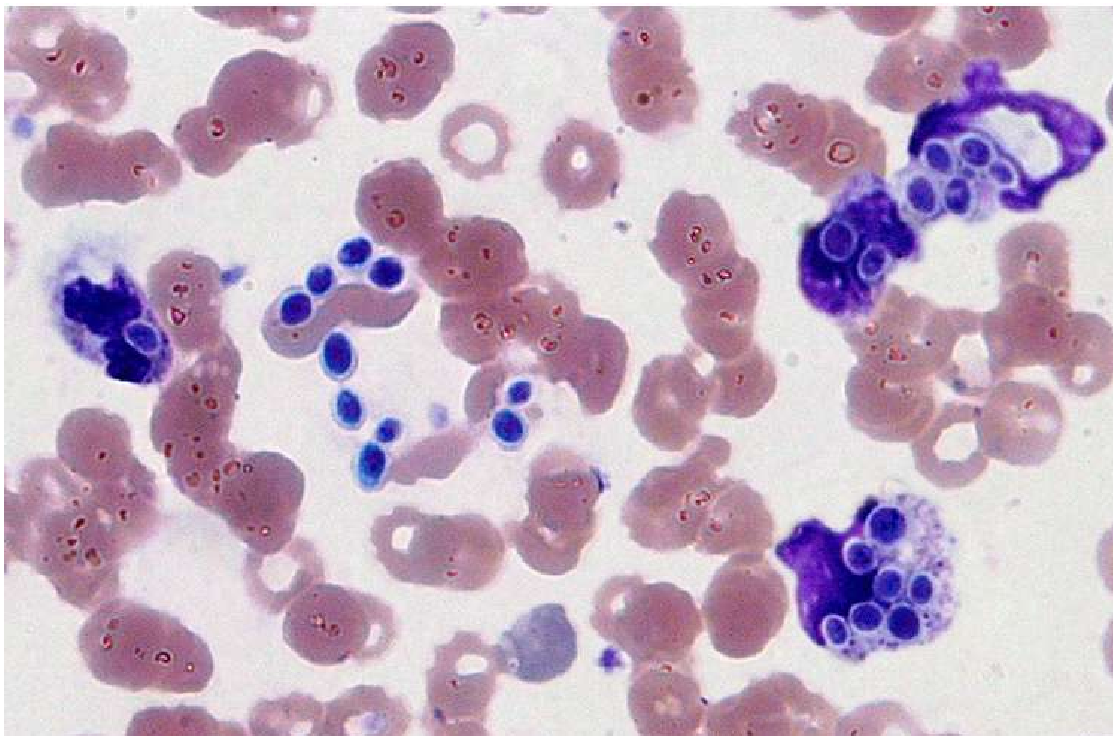

12. Grocott's methenamine silver (GMS) stained tissue sections showing *Aspergillus fumigatus* in lung tissue, note conidial heads forming in an alveolus.



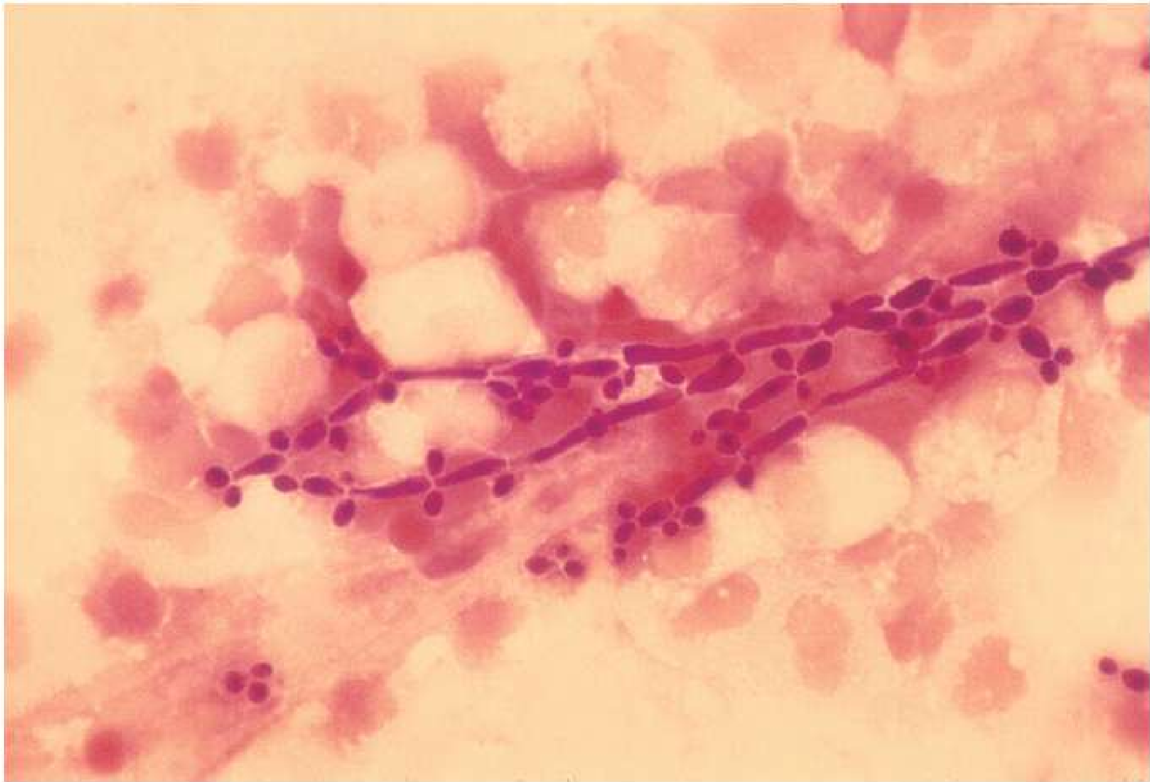
13. Grocott's methenamine silver (GMS) stained tissue sections showing *Aspergillus fumigatus* in lung tissue, note conidial heads forming in an alveolus.



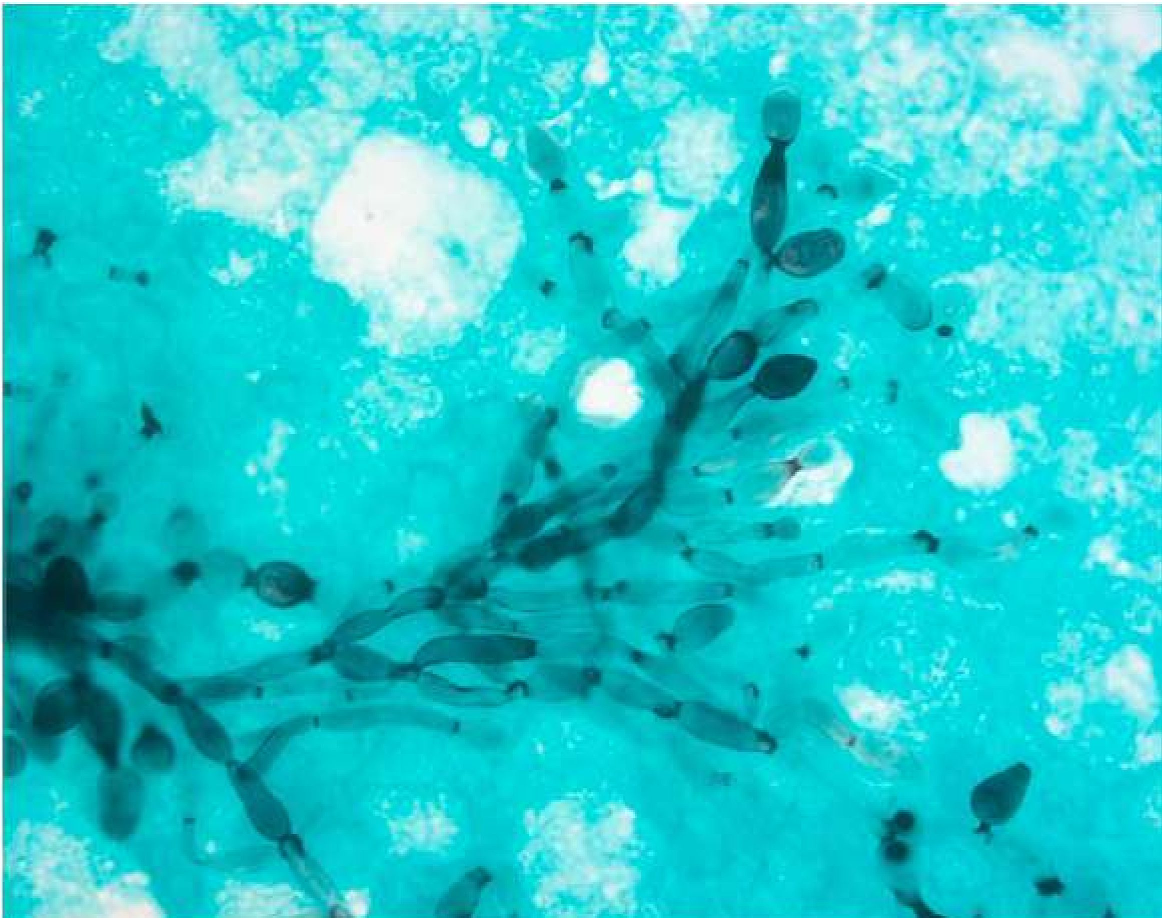
14. Candidemia: Giemsa stain showing *Candida* yeast cells.



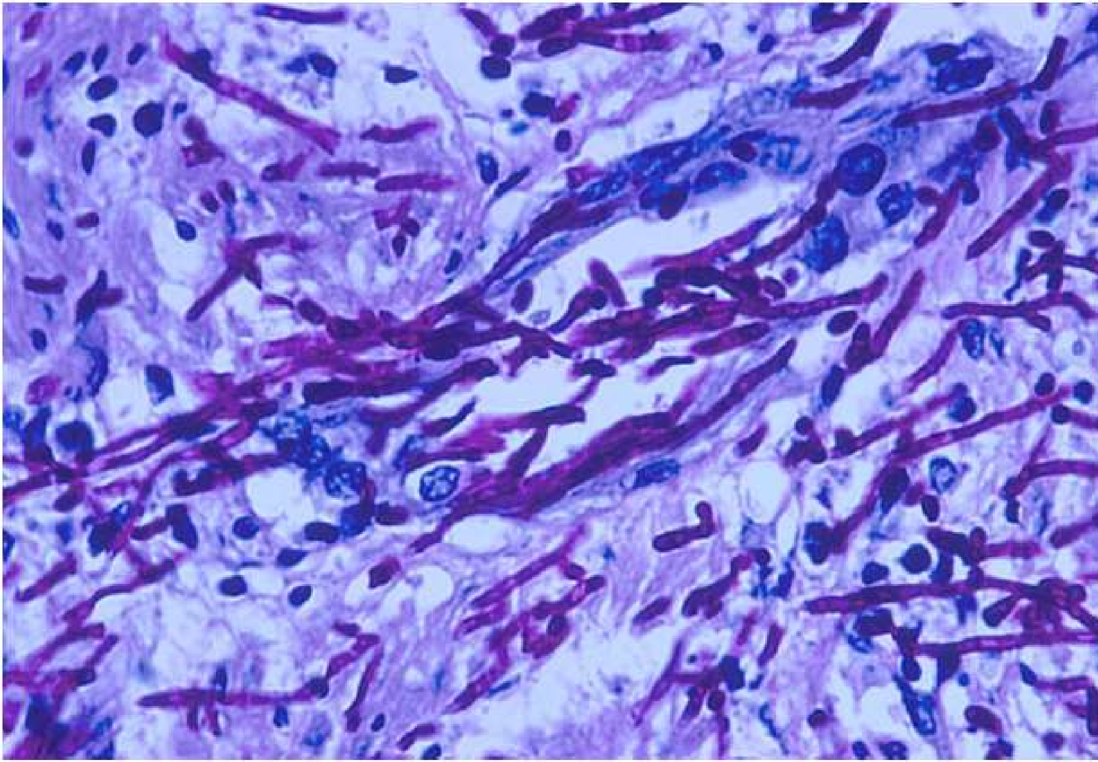
15. Direct smear of urine from a patient with candidiasis of the kidney showing *C. albicans* in mycelial or tissue phase with blastoconidia budding from the pseudohyphae.



16. GMS – showing pseudohyphae (elongating yeast cells) of *Candida albicans*.

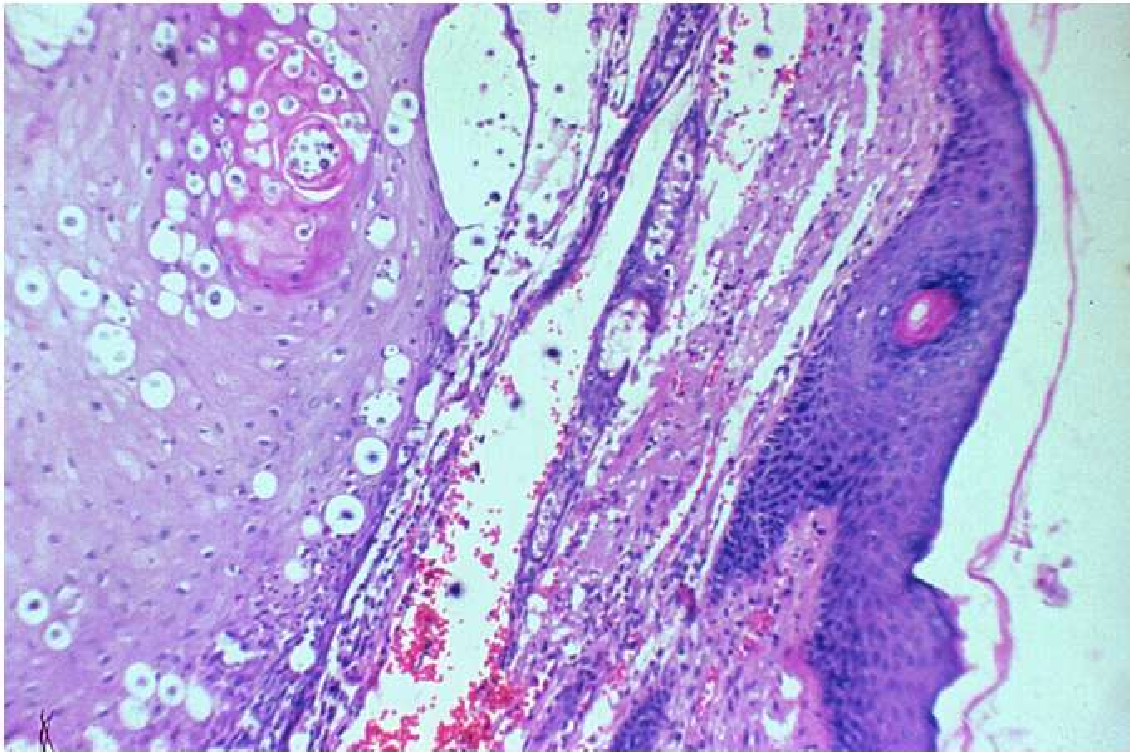


17. Periodic Acid-Schiff (PAS) stained section of post-mortem oesophagus showing invasion of blood vessel by *C. albicans*. Note blastoconidia and branched pseudohyphae.

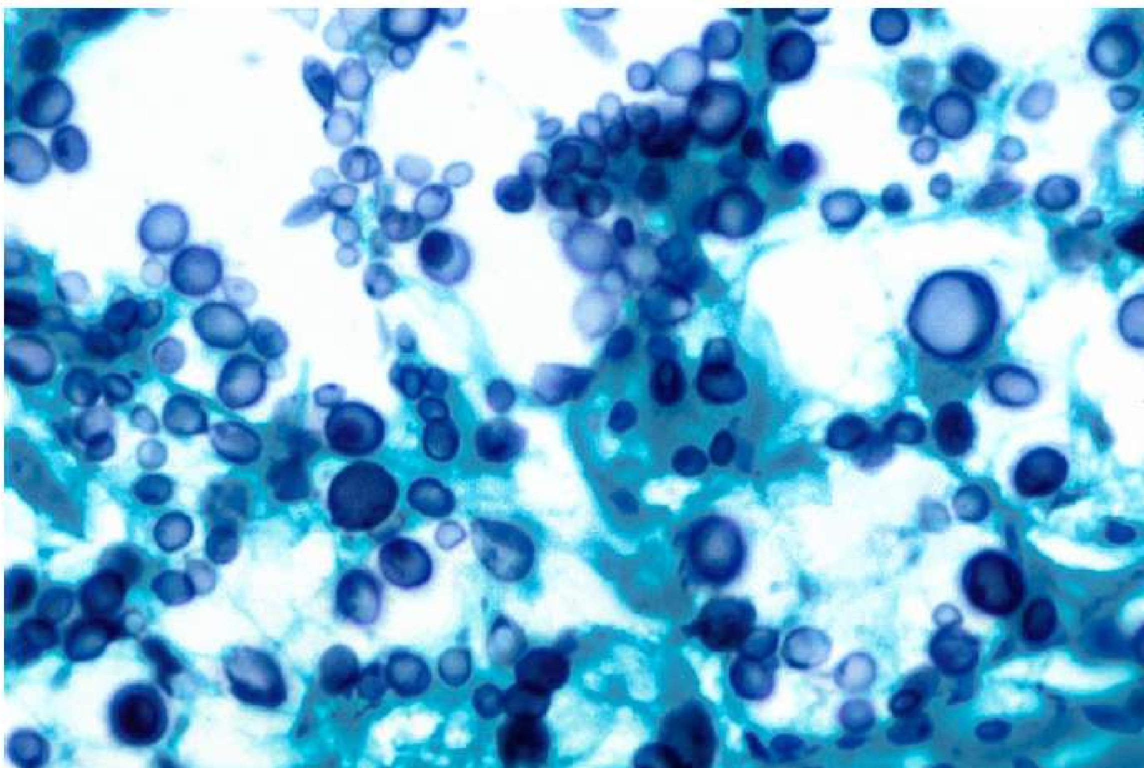


18. India ink preparation of CSF showing a budding yeast cell of *Cryptococcus neoformans* surrounded by a characteristic wide gelatinous capsule.

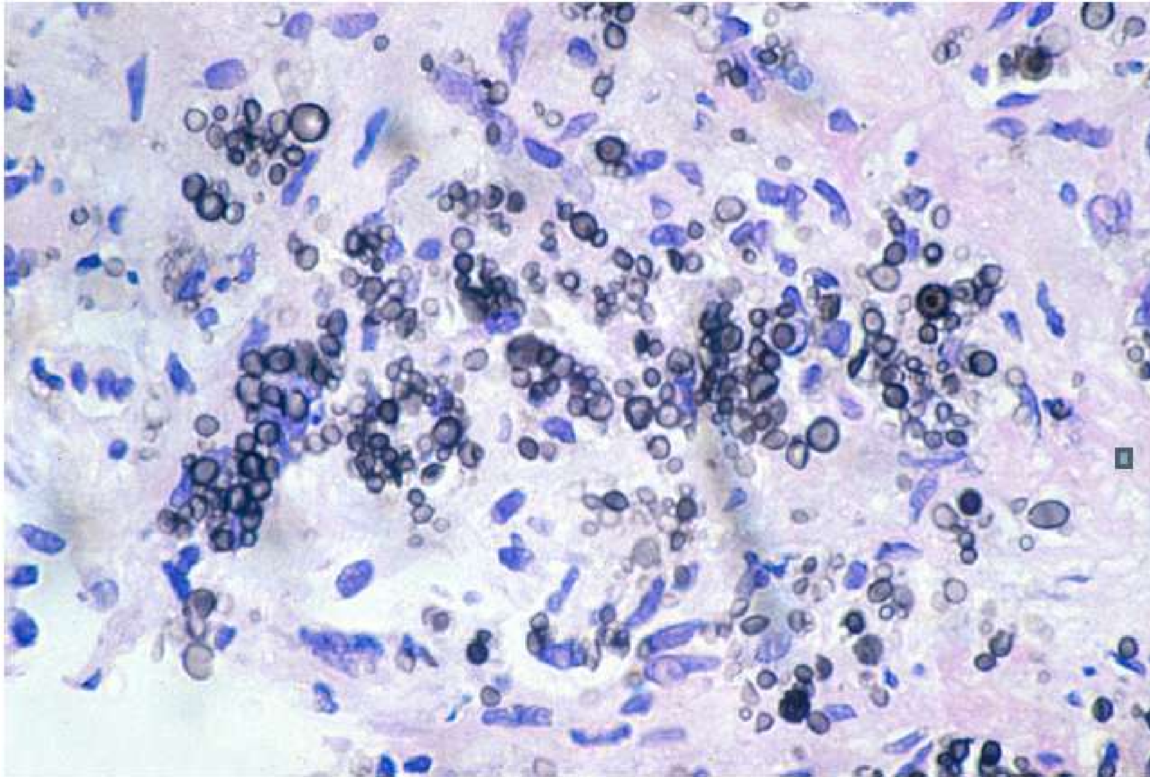
18. Tissue section stained by haematoxylin and eosin (H&E) showing numerous encapsulated yeast cells of *Cryptococcus neoformans.*



19. Grocott's methenamine silver (GMS) stained tissue section of lung showing typical encapsulated yeast cells of *C. neoformans*.



20. Grocott's methenamine silver (GMS) stained tissue section of lung showing atypical non-encapsulated yeast cells of *C. neoformans*.



## DISPOSAL OF CONTAMINATED MATERIALS

**Chemical Decontamination:**

• Use of an intermediate decontamination step during the storage or transport of waste, e.g., the addition of liquid chlorine bleach, iodophors, or phenolic disinfectants to pipette discard pans at work stations. The concentration of decontaminant for this use should be such that the addition of liquid waste will not interfere with its effectiveness.

• Gaseous decontamination of HEPA filters in biological safety cabinets. This procedure should be carried out prior to removal of the filter for replacement or prior to repairing the cabinet. Decontamination is usually carried out with formaldehyde sublimed by heat from paraformaldehyde flakes in the presence of high humidity. The cabinet must be sealed with plastic sheets and tape prior to initiating decontamination. Human contact

with the formaldehyde should be prevented because of the highly irritating, toxic, and possibly carcinogenic properties of the gas (the OSHA limit for permissible exposure is 2 ppm).

• Decontamination of large items of equipment that are to be removed from the laboratory for repair or discard. Care should be taken to avoid corrosion of sensitive parts if the equipment is to be reused rather than discarded. A disinfectant that has low corrosive properties and has been proven to be effective against the specific microorganism should be used for this purpose.

• Treatment of mixed hazardous waste such as combinations of infectious agents and radioisotopes. After an appropriate assessment of the waste, it may be prudent to use

chemically compatible decontaminants to avoid the release of potentially hazardous emissions.

Steam Autoclaving:

• Steam autoclaving usually is considered to be the method of choice for decontaminating cultures, laboratory glassware, pipettes, syringes, or other small items known to be contaminated with infectious agents. Location of the autoclave within the laboratory minimizes storage and transport problems. It provides a technically proved treatment method for rendering infectious material safe. Autoclaved waste can be disposed of as general waste.

**Incineration:**

• Incineration is the method of choice for treating large volumes of infectious waste, animal carcasses, and contaminated bedding materials. Because incinerators usually are located some distance from the laboratory, additional precautions for handling and packaging of infectious waste are necessary.

• Incinerators require approval and permits from local and state pollution control authorities. Although the initial capital costs and maintenance costs are high, incineration offers many advantages as a method for the treatment of infectious waste. Incineration significantly reduces waste volume and produces an unobjectionable end-product, ash. Proper design and operation can provide for energy (heat) recovery, making the operation more economical.

• Many modern incinerators achieve the proper conditions for complete and effective combustion by providing secondary combustion chambers or zones with burners to ensure that adequate conditions for time, temperature, and mixing are achieved. Primary combustion temperatures of at least 1600°F with good mixing and a gaseous retention time of about 2 seconds should provide for good burnout for the waste described in this chapter. All pathogens and proteinaceous materials are denatured at temperatures well below the mentioned temperature.

## KOH Mount

**Principle:** A KOH test is a simple, non-invasive procedure for diagnosing fungal infections of the skin or nails. KOH is a strong alkali. When specimen such as skin, hair, nails or sputum is mixed with 10% w/v KOH, it softens, digests and clears the tissues (e.g., keratin present in skins) surrounding the fungi so that the hyphae and conidia (spores) of fungi can be seen under a microscope. Microscopic examination of KOH preparation reveals the presence of fungal structure and aids in diagnosing mycoses.

**Materials:** Microscope slide and cover glass, 20% potassium hydroxide (KOH), and Microscope.

**Procedure:**

➢ The affected skin or nail is gently scraped with a small scalpel or the edge of a glass slide.

➢ The scrapings from the skin are placed on a microscope slide and a few drops of a potassium hydroxide (KOH) solution are added.

➢ The slide is heated for a short time and then examined under the microscope using lactophenol cotton blue.

**Results and Interpretation:** Potassium hydroxide (KOH) solution is alkaline and has the ability to dissolve keratin that is scraped from the outer layer of the skin. As the KOH dissolves the material binding the skin cells together, any fungus present is released. This allows for the identification of organisms such as dermatophytes.

**Fig.**- Fungal hyphae in a (KOH) preparation of skin scales as seen with the 10x objective.

## LCB Mount

**Principle:** The lactophenol cotton blue (LCB) wet mount preparation is the most widely used method of staining and observing fungi. It has the following constituents: 1) Phenol kills fungus; 2) Lactic acid acts as a clearing agent and helps preserve the fungal structures, 3) Cotton blue is an aniline dye that stains the chitin in the fungal cell walls which adds colour to the fungal preparation thereby enhancing and contrasting the structures; and 4) Glycerol is a viscous substance that prevents drying of the prepared slide specimen.

**Materials:** Microscope slide and cover glass, and Microscope.

**Procedure:**

1) After grease free of the glass slide, a drop of LCB was placed on the slide.

2) Aseptically add the supplied sample mixed to the dye and placed a cover glass on it.

3) Observed under microscope.

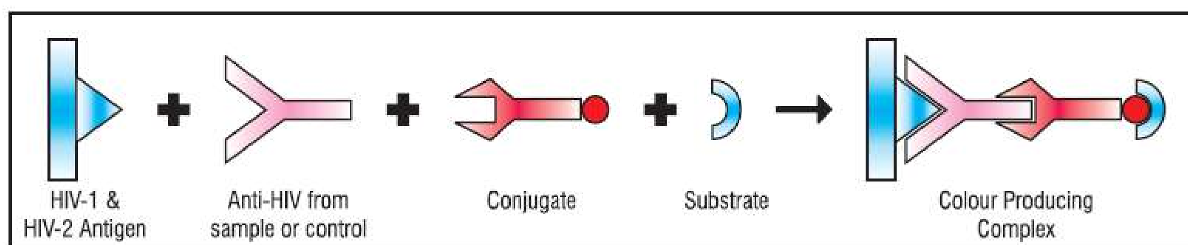
**Results and Interpretation:** Lactophenol cotton blue solution is a mounting medium and staining agent used in the preparation of slides for microscopic examination of fungi. Fungal spores, hyphae, and fruiting structures stain blue while the background stains pale blue.

Fig: *Aspergillus* spp in LPCB Mount

## SEROLOGICAL TEST RELATED TO VIRAL DIAGNOSIS

### 1. HIV-ELISA [Microwell ELISA Test for the Detection of Antibodies to HIV-1 and HIV-2 in Human Serum/ Plasma]

**Principle:** HIV envelope proteins proteins gp41, C terminus of gp 120 for HIV-1, and gp 36 for HIV-2 representing immunodominant epitopes are coated onto microtiter wells. Specimens and controls are added to the microtiter wells and incubated. Antibodies to HIV-1 and HIV-2 if present in the specimen, will bind to the specific antigens absorbed onto the surface of the wells. The plate is then washed to remove unbound material. Horseradish peroxidase (HRP) conjugated antihuman IgG is added to each well. This conjugate will bind to HIV antigen- antibody complex present. Finally, substrate solution containing chromogen and hydrogen peroxide is added to the wells and incubated. A blue colour will develop in proportion to the amount of HIV-1 and / or HIV-2 antibodies present in the specimen. The colour reaction is stopped by a stop solution. The enzyme substrate reaction is read by EIA reader for absorbance at a wavelength of 450 nm. If the sample does not contain HIV-1 or HIV-2 antibodies then enzyme conjugate will not bind and the solution in the wells will be either colourless or only a faint background colour develops.




**Procedure:**

**A. Preparation of Reagents:** Prepare the following reagents before or during assay procedures. Reagents and samples should be at room temperature (20-30ºC) before beginning

the assay and can remain at room temperature during testing. Return reagents to 2-8ºC after use. All containers used for preparation of reagents must be cleaned thoroughly and rinsed with distilled or deionized water. Prewarm the incubator at 37ºC.

Microlisa-HIV Strip: Bring foil pack to room temperature (20-30ºC) before opening to prevent condensation on the microwell strips.

a. Break-off the required number of strips needed for the assay and place in the well holder. Take the strip holder with the required number of strips, considering that two negative & three positive controls should be included in the run while opening the fresh kit. However, for one or two strips, one negative and two positive controls and for more strips at least two negative and three positive controls should be included in each subsequent run.

b. Unused wells should be stored at 2-8ºC, with desiccant in an aluminium pouch with clamp & rod. Microwells are stable for 30 days at 2-8ºC from the date of opening of sealed pouch, when stored with desiccant along with clamp & rod.

Caution: Handle microwell strip with care. Do not touch the bottom exterior surface of the wells.

**B. Sample Preparation:**

**I. Microwell Dilution:**

a) Pipette 100 µl of sample diluent in to the microwell.

b) Add 10 µl of serum sample to be tested.

c) Ensure thorough mixing of the sample to be tested.

**II. Preparation of Wash Buffer:**

a) Check the buffer concentrate for the presence of salt crystals. If crystals are present in the solution, resolubilize by warming at 37ºC until all crystals dissolve.

b) Prepare at least 50 ml. (2ml. concentrated buffer with 48 ml. water) of buffer for each microlisa strip used. Mix well before use.

c) Mix 20 ml. 25 X wash buffer concentrate with 480 ml. of distilled or deionized water.

Working Wash Buffer is stable for 2 months when stored at 2-8ºC.

**III. Preparation of Working Conjugate:** Dilute conjugate concentrates 1:100 in conjugate diluent. Do not store working conjugate. Prepare a fresh dilution for each assay in a clean glass vessel. Determine the quantity of working conjugate solution to be prepared from table given below. Mix solution thoroughly before use.

**IV. Preparation of working substrate solution:** Mix TMB substrate and TMB Diluent in 1:1 ratio to prepare working substrate. Do not store working substrate. Prepare a fresh dilution for each assay in a clean plastic/glass vessel. Determine the quantity of working substrate solution to be prepared from table. Mix solution thoroughly before use.

**V. Wash Procedure:**

1. Incomplete washing will adversely affect the test outcome.

2. Aspirate the well contents completely into a waste container. Then fill the wells completely with wash buffer avoiding overflow of buffer from one well to another and allow to soak (approx. 30 seconds). Aspirate completely and repeat the wash and soak procedure 4 additional times for a total of 5 washes.

3. Automated washer if used should be well adjusted to fill each well completely without over filling

4. Tap upside down on absorbent sheet till no droplets appear on the sheet, taking care not to dislodge the wells.

**VI. Test Procedure:** Once the assay has started, complete the procedure without interruption. All the reagents should be dispensed in the centre of the well and the tip of the pipette should not touch the wall of the microwell. Fit the strip-holder with the required number of Microlisa-HIV strips. The sequence of the procedure must be carefully followed. Arrange the assay control wells so that well A-1 is the reagent blank. From well A-1 arrange all controls in a horizontal or vertical configuration. Configuration is dependent upon reader software.

1. Add 100 µl sample diluent to A-1 well as blank.

2. Add 100µl Negative Control in each well no. B-1 & C-1 respectively. Negative Control is ready to use and hence no dilution is required.

3. Add 100µl Positive Control in D-1, E-1 & F-1 wells. Positive Control is ready to use and hence no dilution is required.

4. Add 100 µl sample diluent in each well star ting from G-1 followed by addition of 10µl sample. (Refer Microwell Dilution)

5. Apply cover seal.

6. Incubate at 37ºC + 2ºC for 30 min. + 2 min.

7. While the samples are incubating, prepare Working Wash Solution and Working Conjugate as specified in Preparation of Reagents.

8. Take out the plate form the incubator after the incubation time is over and, wash the wells 5 times with Working Wash solution according to the wash procedure given in the previous section (wash procedure).

9. Add 100 µl of Working Conjugate Solution in each well including A-1.

10. Apply cover seal.

11. Incubate at 37ºC + 2ºC for 30 min. + 2 min.

12. Aspirate and wash as described in step no. 8.

13. Add 100 µl of working substrate solution in each well including A-1.

14. Incubate at room temperature (20 - 30ºC) for 30 min. in dark.

15. Add 100 µl of stop solution.

16. Read absorbance at 450 nm within 30 minutes in ELISA READER after blanking A-1 well. (Bichromatic absorbance measurement with a reference wavelength 600 - 650 nm is recommended when available).

**Result: Calculation**

Abbreviations

| | |
|---|---|
| NC - | Absorbance of the Negative Control |
| $NC\bar{x}$ - | Mean Negative Control |
| PC - | Absorbance of the Positive Control |
| $PC\bar{x}$ - | Mean Positive Control |

**TEST VALIDITY:**

**Blank acceptance Criteria**

Blank must be <0.100 in case of differential filter being used. In case differential filter is not available in the reader the blank value may go higher.

**Negative Control Acceptance Criteria:**

NC must be $\leq$ 0.150. If it is not so, the run is invalid and must be repeated.

**Positive Control Acceptance Criteria:**

1. PC must be $\geq$ 0.50
2. Determine the mean ($PC\bar{x}$) value If one of three positive control values is outside of these limits, recalculate $PC\bar{x}$ based upon the two acceptable positive control values.
3. If two of the three positive control values are outside the limits, the assay is invalid and the test must be repeated.

**CUT OFF VALUE**

Absorbance (O.D.)

| | | | | | | |
|---|---|---|---|---|---|---|
| NC | - | 0.042 B1 Well | PC | - | 1.412 | D1 Well |
| | - | 0.040 C1 Well | | - | 1.392 | E1 Well |
| Total: | | 0.082 2 Wells | | - | 1.407 | F1 Well |
| | | | Total : | | 4.211 | 3 Wells |

$NC\bar{x} = 0.082/2 = 0.041$ $PC\bar{x} = 4.211/3 = 1.403$

The cut off value is calculated by adding Mean Negative Control ($NC\bar{x}$) and Mean Positive Control ($PC\bar{x}$) as calculated above and the sum is divided by 6.

$$\text{Cut off Value} = \frac{NC\bar{x} + PC\bar{x}}{6}$$

e.g. $NC\bar{x} = 0.041$, $PC\bar{x} = 1.403$

$$\text{Cut off Value} = \frac{0.041 + 1.403}{6} = \frac{1.444}{6} = 0.240$$

**Interpretation:**

1. Test specimens with absorbance value less than the cut off value are non-reactive and may be considered as negative for anti-HIV.

2. Test specimens with absorbance value greater than or equal to the cut off value are reactive for anti-HIV by Microlisa-HIV.

Note: Test specimens with absorbance value within 10% below the cut off should be considered suspect for the presence of antibodies and should be retested in duplicate.

3. Specimens with absorbance value equal to or greater than the cut off value are considered initially reactive by the criteria of Microlisa-HIV. Original specimen should be retested in duplicate.

4. If both duplicate retest sample absorbance value is less than cut off value, the specimen is considered non-reactive.

5. If any one of the duplicates retest sample absorbance value is equal to or greater than the cut off or both duplicate retest value are equal to or greater than the cut off, the specimen is considered reactive by the criteria of Microlisa HIV. Further confirmation by other EIA assays or confirmation assays including Western Blot or PCR is recommended.

## 2. GAC- ELISA Test for the Detection of Dengue IgG Antibodies in Human Serum/Plasma

**Principle:** Antibodies to Dengue if present in the specimen, will bind to the Anti-human IgG antibodies adsorbed onto the surface of the wells. The plate is then washed to remove unbound material. Horseradish peroxidase (HRPO) conjugated dengue antigen (DEN1-4) is added to each well. This dengue antigen conjugate will bind to Dengue specific IgG antibodies which is complexed with anti- human IgG antibodies. Finally, substrate solution containing chromogen and hydrogen peroxide is added to the wells and incubated. A blue colour will develop in proportion to the amount of Dengue antibodies present in the specimen. The colour reaction is stopped by a stop solution. The enzyme substrate reaction is read by EIA reader for absorbance at a wavelength of 450 nm. If the sample does not contain Dengue IgG antibodies then enzyme conjugate will not bind and the solution in the wells will be either colourless or only a faint background colour develops.

**Preparation of Reagents:**

Prepare the following reagents before or during assay procedures. Reagents and samples should be at room temperature (20-30ºC) before beginning the assay and can remain at room temperature during testing. Return reagents to 2-8ºC after use. All containers used for preparation of reagents must be cleaned thoroughly and rinsed with distilled or deionized water. Prewarm the incubator to 37ºC.

**i) Anti human IgG antibodies coated strips:** Bring foil pack to room temperature (20-30ºC) before opening to prevent condensation on the microwell strips.

a. Break-off the required number of strips needed for the assay and place in the well holder. Take the strip holder with the required number of strips, taking into account that one each of negative & positive control & three calibrators should be included in the run while opening the fresh kit. However, for one or two strips, one each of negative, positive control & two calibrator and for more strips one each of negative and positive control & three calibrator should be included in each subsequent runs.

b. Unused wells should be stored at 2-8oC, with dessicant in aluminium pouch with clamp & rod. Microwells are stable for 30 days at 2-8ºC from the date of opening of sealed pouch, when stored with desiccant along with clamp & rod.

Caution: Handle microwell strip with care. Do not touch the bottom exterior surface of the

wells.

**ii) Sample Preparation:**

**Tube Dilution**: Mark the tubes carefully for the proper identification of the samples. Dilute the serum samples to be tested, with sample diluent 1:100 in separate tubes (1 ml. sample diluent + 10 µl serum samples). Use a separate tip for each sample and then discard as biohazardous waste.

**iii) Preparation of Working Wash Buffer:**

a) Check the buffer concentrate for the presence of salt crystals. If crystals are present in the solution, resolubilize by warming at 37°C until all crystals dissolve.

b) Prepare at least 25ml. (1ml. concentrated buffer with 24 ml. water) of buffer for each strip used. Mix well before use.

c) Mix 20 ml. of 25X wash buffer concentrate with 480 ml. of distilled or deionized water. Working wash buffer is stable for 2 months when stored at 2-8°C.

**iv) Preparation of working substrate solution:** Mix TMB substrate and TMB Diluent in 1:1 to prepare working substrate.

Do not store working substrate. Prepare a fresh dilution for each assay in a clean plastic/glass vessel. Determine the quantity of working substrate solution to be prepared from table. Mix solution thoroughly before use. Discard unused solution. A deep blue color present in the substrate solution indicates that the solution has been contaminated and must be discarded.

**Test Procedure:**

Once the assay has started, complete the procedure without interruption. All the reagents should be dispensed in the centre of the well and the tip of the pipette should not touch the wall of the microwell.

Fit the strip-holder with the required number of Anti-human IgM coated strips. The sequence of the procedure must be carefully followed. Arrange the assay control wells in a horizontal or vertical configuration. Configuration is dependent upon reader software.

1. Add 100 µl Negative Control in A-1well.

2. Add 100 µl calibrator in B-1, C-1 & D-1 wells.

3. Add 100 µl Positive Control in E-1 well.

4. Add 100 µl of each sample diluted in sample diluent (1:100), in each well star ting from F-1-well. (Refer TUBE DILUTION).

5. Apply cover seal.

6. Incubate at 37ºC ± 1ºC for 60 min. ± 1min.

7. While the samples are incubating, prepare working Wash Solution as specified in preparation

of reagents.

8. Take out the plate from the incubator after the incubation time is over and, wash the wells

5 times with working Wash Solution.

9. Add 100 µl of Enzyme Conjugate Solution in each well.

10. Apply cover seal.

11. Incubate at 37ºC ± 1ºC for 60 min ± 1min.

12. Aspirate and wash as described in step no. 8.

13. Add 100 µl of working substrate solution in each well.

14. Incubate at room temperature (20-30ºC) for 30 min. in dark.

15. Add 50 µl of stop solution.

16. Read absorbance at 450 nm and 630 nm (reference filter) within 30 minutes in ELISA Reader.

**Calculation of Results:**

a. Cut off value = mean O.D. of calibrator × calibration factor

b. Calculation of sample O.D. ratio: Calculate sample O.D. ratio as follows:

Sample O.D.

Sample O.D. ratio $=\frac{Sample\ O.D.}{Cut\ off\ Value}$

c. Calculation of Dengue IgG units: Calculate by multiplying the sample O.D. ratio by 10.

Dengue IgG units = sample O.D. ratio × 10.

e.g.: Mean O.D. of calibrator = 0.75

Calibration factor = 0.7

Cut off value = 0.75 × 0.7 = 0.525

e.g.: sample absorbance (O.D.) = 0.925

Cut off value = 0.525

Sample O.D. ratio = 0.925 / 0.525 = 1.761

Dengue IgG units = 1.761 × 10 = 17.61

**Interpretation of Results:**

a. If the Dengue IgG Units is < 9 then interpret the sample as Negative for Dengue IgG antibodies.

b. If the Dengue IgG Units is between 9 - 11 then interpret the sample as Equivocal for Dengue IgG antibodies. Equivocal samples should be repeated in duplicate and calculate the average dengue units. Sample that remain equivocal after repeat testing should be repeated by an alternative method or another sample should be collected.

c. If the Dengue IgG Units is > 11 then interpret the sample as Positive for Dengue IgG antibodies.

**3. Measles (Rubeola) IgG ELISA**

**Introduction:** Measles is an acute, highly contagious viral disease. Although measles is usually considered a childhood disease, it can be contracted at any age. Measles is spread by direct contact with nasal or throat secretions of infected people or, less frequently, by airborne transmission. Measles symptoms generally appear in two stages. In the first stage, the individual may have a runny nose, cough and a slight fever. The second stage begins on the third to seventh day and consists of high fever and red blotchy rash lasting four to seven days. The rash usually begins on the face and then spreads over the entire body. Symptoms usually appear in 10-12 days, although they may occur between 8-13 days after exposure. The presence of IgG antibody to measles virus is indicative of previous exposure or vaccination. In individuals with acute measles, a significant increase in measles IgG antibody level is indicative of recent infection. IgM antibodies to measles virus are often detectable with onset of the rash and typically persist for 4 weeks. At least 80% of patients will be positive for measles IgM at 6 days and 100% at 16 days after onset of symptoms.

**Principle:** Diluted patient serum is added to wells coated with purified antigen. IgG specific antibody, if present, binds to the antigen. All unbound materials are washed away and the enzyme conjugate is added to bind to the antibody-antigen complex, if present. Excess enzyme conjugate is washed off and substrate is added. The plate is incubated to allow the hydrolysis of the substrate by the enzyme. The intensity of the color generated is proportional to the amount of IgG specific antibody in the sample.

**Specimen Collection and Handling:**

1. Collect blood specimens and separate the serum.

2. Typically, specimens may be refrigerated at 2–8°C for up to seven days or frozen for up to six months. Avoid repetitive freezing and thawing.

**Reagent Preparation:** Prepare 1X Wash buffer by adding the contents of the bottle (25 ml, 20X) to 475 ml of distilled or deionized water. Store at room temperature (20-25°C).

**Assay Procedure:**

Bring all specimens and kit reagents to room temperature (20-25°C) and gently mix.

1. Place the desired number of coated strips into the holder.

2. Negative control, positive control, and calibrator are ready to use. Prepare 1:21 dilution of test samples, by adding 10 µl of the sample to 200 µl of sample diluent. Mix well.

3. Dispense 100 µl of diluted sera, calibrator and controls into the appropriate wells. For the reagent blank, dispense 100 µl sample diluent in 1A well position. Tap the holder to remove air bubbles from the liquid and mix well. Incubate for 20 minutes at room temperature.

4. Remove liquid from all wells. Wash wells three times with 300 µl of 1X wash buffer. Blot on absorbance paper or paper towel.

5. Dispense 100 µl of enzyme conjugate to each well and incubate for 20 minutes at room temperature.

6. Remove enzyme conjugate from all wells. Wash wells three times with 300 µl of 1X wash buffer. Blot on absorbance paper or paper towel.

7. Dispense 100 µl of TMB substrate and incubate for 10 minutes at room temperature.

8. Add 100 µl of stop solution.

9. Read O.D. at 450 nm using ELISA reader within 15 min. A dual wavelength is recommended with reference filter of 600-650 nm.

**Calculation:**

1. Check Calibrator Factor (CF) value on the calibrator bottle. This value might vary from lot to lot. Make sure you check the value on every kit.

2. Calculate the cut-off value: Calibrator OD x Calibrator Factor (CF).

3. Calculate the Ab (Antibody) Index of each determination by dividing the O.D. value of each sample by cut-off value.

## *Candida albicans*

**Division:** Deuteromycotina

**Class:** Blastomycetes

**Order:** Cryptococcales

**Family:** Cryptococcaceae

**Genus:** *Candida*

**Species:** *albicans*

(i) *Candida albicans* is small, oval, and yeast like unicellular fungus.
(ii) It shows pseudomycelial growth and multilateral budding. The cell elongates and develops into pseudomycelium.
(iii) The genus comprises of imperfect forms of ascomycetous and basidiomycetous yeasts of various genera.
(iv) *C. albicans* is the imperfect state of Syringospora, which is a member of basidiomycotina.
(v) They form chlamydospores and produce spherical clusters of blastospores.
(vi) The colonies are moderate in size, smooth and pasty. The older colonies have honey comb like appearance in the centre and develop radical furrows.
(vii) The possess a capsular form of polysaccharide that shows pyrogenic activity.
(viii) Examples are *C. albicans*, *C. utilis*, etc.

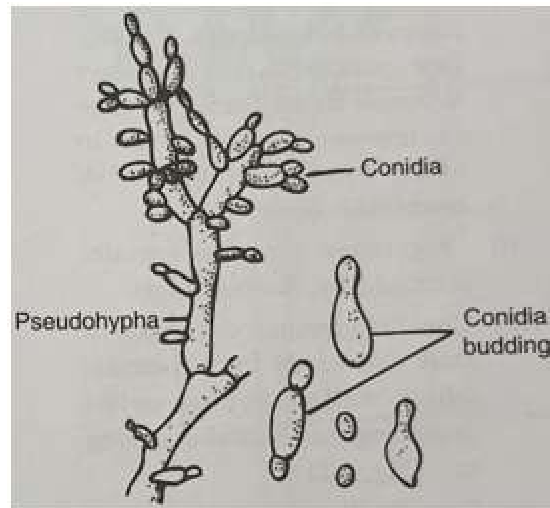



Fig.-Structure of *Candida* sp.

## *Aspergillus*

**Class:** Deuteromycetes

**Order:** Moniliales

**Family:** Moniliaceae

**Genus:** *Aspergillus*

(i) Colonies on Czapek Dox agar are white (*A. versicolor*) on white at first and becoming yellowish (*A. flavipes*), blue green (*A. sydowi*), lime green (*A. flavus*), cinnamon to deeper brown shades with age (*A. terreus*), blackish brown to black with slight yellowish mycelia (*A. niger*).

(ii) Vegetative mycelim septate branched hyphae colourless.

(iii) Conidial apparatus developed as stalk and heads from footcells (thick-walled hyphal cells) producing conidiophores at long axis.

(iv) Conidiophores septate or un-septate, broadening into elliptical, hemispherical or globose fertile vesicles.

(v) Vesicles bear phialides in one series (uniseriate), or two series (biseriate).

(vi) Phialides clustered in terminal groups or radiating from entire surface.

(vii) Conidia (conidia bearing cells) elliptical, globose, smooth walled, rough or spinulose walls produced in chains.

(viii) Some species produce cleistothecia e.g. *A. versicolor*, *A. ruber*, some strains produce sclerotia e.g. *A. niger*; some species produce irregularly globose, ovoid or elongated heavily walled abundant hulls cells e.g. *A. granulosis*.

(ix) They play a significant role in production of amylase (a. niger), diastase (*A. flavipes*, *A. parasiticus*), otomycosis in humans (*A. niger*), etc.

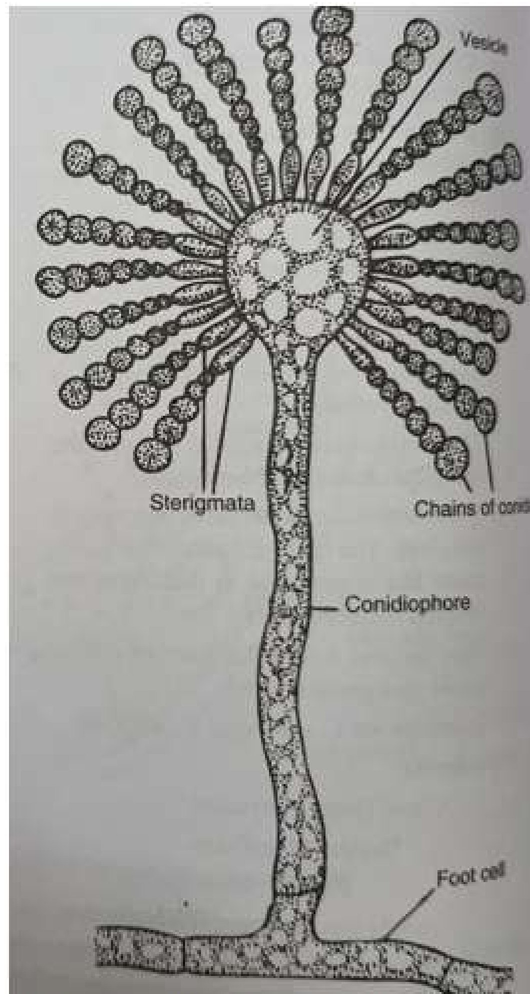



Fig.-*Aspergillus:* Conidiophore and chains of conidia

**DIAGNOSTIC TESTS IN VIROLOGY, ANIMAL CELL CULTURES, MEDIA, STERILIZATION, DEMONSTRATION OF CELL LINE.**

**Animal Cell Culture:** Cell culture refers to the removal of cells from an animal or plant and their subsequent growth in a favorable artificial environment. The cells may be removed from the tissue directly and disaggregated by enzymatic or mechanical means before cultivation, or they may be derived from a cell line or cell strain that has already been already established.

**Primary Culture:** Primary culture refers to the stage of the culture after the cells are isolated from the tissue and proliferated under the appropriate conditions until they occupy all of the available substrate (i.e., reach confluence). At this stage, the cells have to be subcultured (i.e., passaged) by transferring them to a new vessel with fresh growth medium to provide more room for continued growth.

**Cell Line:** After the first subculture, the primary culture becomes known as a cell line or subclone. Cell lines derived from primary cultures have a limited life span (i.e., they are finite), and as they are passaged, cells with the highest growth capacity predominate, resulting in a degree of genotypic and phenotypic uniformity in the population.

**Cell Strain:** If a subpopulation of a cell line is positively selected from the culture by cloning or some other method, this cell line becomes a cell strain. A cell strain often acquires additional genetic changes subsequent to the initiation of the parent line.

**Cell Culture Equipment**

**Basic Equipment**

• Cell culture hood (i.e., laminar-flow hood or biosafety cabinet)

• Incubator (humid $CO_2$ incubator recommended)

• Water bath

• Centrifuge

• Refrigerator and freezer (–20°C)

• Cell counter

• Inverted microscope

• Liquid nitrogen ($N_2$) freezer or cryostorage container

• Sterilizer (i.e., autoclave)

**Expanded Equipment**

• Aspiration pump (peristaltic or vacuum)

• pH meter

• Confocal microscope

• Flow cytometer

Additional Supplies

• Cell culture vessels (e.g., flasks, Petri dishes, roller bottles, multi-well plates)

• Pipettes and pipettors

• Syringes and needles

• Waste containers

• Media, sera, and reagents

• Cells

**Culture media:** Today, with standardized media and sophisticated incubation conditions, culturing animal cells is considerably easier than it used to be. Since 1950s, tissue culture media were developed and conditions were worked out which closely simulate the situation in vivo. In particular, the environment is regulated with regard to the temperature, osmotic pressure, pH, essential metabolites (such as carbohydrates, amino acids, vitamins, proteins and peptides), inorganic ions, hormones and extracellular matrix. Among the biological fluids that proved successful for culturing cells, serum is the most significant. Five to twenty percent of serum is usually added to media for optimal cell growth.

Serum is an extremely complicated mixture of compounds including undefined components, therefore much work has gone towards creating a chemically defined alternative to serum. A wide variety of culture media is currently available. The choice of culture media is dependent on the requirements of cells. The components of suitable culture media include:

**Basic media:** The most basic media are balanced salt solutions (BSS), e.g., phosphate-buffered saline (PBS), which may be used for washing cells and for short incubations in suspension. More complex defined media are used for growth and maintenance. Defined media can also vary in complexity, by the addition of a number of constituents, e.g., from Eagle's minimum essential medium (MEM) which contains essential amino acids, vitamins and salts, to McCoy's medium, which contains a larger number of different amino acids, vitamins, minerals and other extra metabolites (such as nucleosides).

**Buffering capacity:** A number of supplements to the basic media are necessary to enable them to be used for culturing cells. Cell cultures have an optimum pH for growth, generally between pH 7.4- 7.7. The type of buffering that is used for the media depends on the growth conditions. When cells are incubated in a $CO_2$ atmosphere equilibrium is maintained between the medium and the gas phase. A bicarbonate−$CO_2$ buffering system is most often used due to its low toxicity towards the cells. HEPES, a much stronger buffer, may also be used, however, in this case much greater concentrations of HEPES than bicarbonate are required when used in a $CO_2$ atmosphere. Each type of media has a recommended bicarbonate concentration and $CO_2$ tension to achieve the correct pH and osmolarity. Nevertheless, this may vary slightly among laboratories, therefore, a sample of media should be left under the normal incubating conditions and monitored overnight, the buffering can then be adjusted accordingly. HEPES buffer should normally be used in conjunction with bicarbonate for which a relationship between the HEPES and bicarbonate exists for differing $CO_2$ levels, although, HEPES alone can maintain pH in the

absence of exogenous $CO_2$. The addition of 1-5 mM pyruvate to the medium increases the endogenous cellular production of $CO_2$ and limits the need for a $CO_2$ atmosphere. Some defined media have been devised for this purpose, e.g., Leibovitz L-15 medium. Cells which produce large amounts of endogenous $CO_2$ under certain incubation conditions may require HEPES to buffer this $CO_2$ product. The density of the culture may affect the $CO_2$ requirement, however, in general phenol red in the medium will indicate the state of the pH at any given time.

**Glutamine and other Amino acids:** In addition to buffering the medium, there are other growth requirements including amino acids, the requirement for which may vary with cell culture type. Commonly the necessary amino acids include cysteine and tyrosine, but some non-essential amino acids may be needed. Glutamine is also required by most cell lines and it has been suggested that cultured cells use glutamine as an energy and carbon source in preference to glucose, although glucose is present in most defined media. Glutamine is usually added at a final concentration of 2 mM, however, once added to the medium the glutamine is only stable for about 3 weeks at 4°C.

**Serum:** Although there is much research aimed at attempting to reduce the requirement of cells for serum, by alternative supplementation of the media, it is apparent that most cell lines still require serum for adequate growth. Various sources of serum may be used such as calf, fetal calf, and horse. Many continuous cultures utilize calf serum, but often fetal calf serum

provides the best growing conditions. The level of serum used depends on the particular cell line and should be determined empirically. Batches of serum may vary considerably in their ability to support cellular growth. It is therefore important to test batches of serum and have sufficient quantities of a batch that shows suitable growth supporting characteristics stored at -20°C. To check these properties, cloning efficiency and growth characteristics (morphology, growth patterns) should be carried out over a concentration range of 2% to 20% serum. This wide concentration range will disclose if the alteration in the concentration of serum is possible to give optimal growth characteristics for a particular cell line. Antibiotics and antimycotics: Unless good sterile conditions can be maintained (e.g., using laminar flow hoods) it is necessary to incorporate antibiotics and antimycotics into the media. A wide range of suitable preparations are available from relatively specific antibiotics, e.g., penicillin/streptomycin solutions, to broader spectrum antibacterial/antimycotic agents such as kanamycin or amphotericin B. The antibiotics chosen should clearly not to be toxic to the cells in culture and may depend on the type of contamination experienced in the individual laboratory.

**Supply and Preparation of Culture Media:** The choice of culture media used will depend on the type of primary cell, cell line, and the incubation conditions. However, it is best to start with the medium recommended by the original supplier of the cells. Changing the medium necessitates the investigation on growth characteristics such as growth curves and cloning efficiency. To render a change in culture conditions from one medium to another it is advisable to 'condition' the cells, by increasing the ratio of new to old medium with successive passages. Culture media have a limited storage life and the recommendations indicated by the supplier should be followed. Liquid defined media may have a storage life at 4°C of up to one year, while glutamine lasts only 3 weeks at 4°C. Serum lasts for about one year at - 20°C. Culture media can be supplied in powdered form which requires dissolving and filter sterilizing, as a 10x concentrate liquid which requires dilution prior to use, or as a 1x liquid media. Media preparation, in all cases, requires high quality water. Bottles of media should be prepared in small batches, for instance two weeks supply at a time. This will ensure that constituents such as glutamine do not have time to deteriorate and also that if contamination should occur it is confined to a few bottles.

**Culturing Animal Cells**

**Primary Cultures or Continuous Cell Lines:** If you remove tissue from an embryo, dissociate it into a suspension of single cells, and plate them out onto a culture dish, a series of characteristic events occurs. Firstly, cells are in a lag phase, usually no more than 1-2 days in length, during which there is little or no increase in cell number. During this time, cells are "conditioning" the medium, undergoing internal cytoskeletal and enzyme changes and adjusting to the new medium. Secondly, the cells undergo a period of rapid division, so-called log phase growth. Then, as they approach confluency and form contacts with one another, their rate of division slows and they begin to express a program of differentiation characteristic of their tissue of origin. Muscle cells fuse and acquire cross-striation, epithelial cells from the kidney or gut become linked by junctional complexes and transport ions from one surface to another, heart cells begin to beat spontaneously. Cultures such as those just described are referred to as primary cultures, because they are prepared from cells taken directly from the animal. The cells divide or not (depending on what they are accustomed to), acquire differentiated characteristics, and ultimately die. For the next experiment, it's back to

the animal again to obtain new tissue and prepare new culture. Alternatively, in the case of cells that divide in culture, it is possible to 'passage' or 'subculture' them by inducing them to detach from the substrate, 'splitting' them (i.e., diluting them several-fold in medium and re-plating them into new dishes), and allowing them to re-enter log phase growth. However, the properties of the cultured cells often change gradually with passaging, as more rapidly dividing cell populations come to predominate and more 'differentiated' cells, which divide more slowly, are lost. When cells are repeatedly sub-cultured, most cease division after a finite number of generations, typically between 20 and 80. This is thought to reflect the same process of senescence that occurs in cells in situ. However, it is possible to develop populations of cells that can be passaged indefinitely and that express a reasonably stable phenotype. These are referred to as established or continuous cell lines. Some cell lines have arisen spontaneously in normal cells being passaged in culture, but the majority has been obtained by culturing tumor cells. In addition to their infinite life span (their 'immortality'), such cell lines frequently share several additional properties that distinguish them from 'normal' cells in culture. They divide more rapidly, they do not require attachment to the substratum for growth, and when reintroduced into animals, they form tumors. Cell lines with these properties are sometimes referred to as transformed cell lines.

**Cultivation of Viruses in the Cell lines**

**Principle:** Viruses infect healthy cells grown in the laboratory. When susceptible cells are used for inoculation of viruses, they show pathological changes and the viruses can be harvested from the cells for further tests. The growth of viruses in the cell lines can be known by a) cytopathic effects, b) immunofluorescence, c) haemagglutination and haemadsorption, and d) interference. Many viruses kill the infected viral cells in which they grow and bring about detectable changes in morphology of the cells. These changes are collectively known as cytopathic effects. Some viruses however do not produce any cytopathic effect (e.g., rubella virus). The most important precaution to be taken during maintenance of cell lines is sterility. Contamination of cell lines should be prevented and even cross contamination among cell lines should be avoided.

**Requirements**

**I Equipments**

Inverted microscope, incubator, haemocytometer and biological safety cabinet.

**II Reagents and Lab Wares**

Sterile glassware, pre-sterilized tissue culture plasticware, Pasteur pipettes and measuring pipettes, membrane filter, syringes, vials, discard jar, Eagle’s, minimum essential medium (MEM), sodium bicarbonate ($NaHCO_3$), EDTA trypsin mixture, foetal calf serum (FCS), sterile double distilled water, virus inoculum, spirit and sodium hypochlorite. Monolayer of a cell culture in a culture flask is treated with trypsin or versene to disperse cells.

**III Specimen**

Suspected virus infected specimen like the cerebrospinal fluid (CSF), stool, rectal swab, and throat swab.

**Procedure**

1 Discard the trypsin versene mixture and add a small amount of MEM with 10% FCS to the monolayer of cells.

2 Count the cells with the medium in a hemocytometer for appropriate splitting.

3 Inoculate the cells into sterile flasks or tubes for viral inoculation

4 Fill the new flasks with MEM and incubate in horizontal position.

5 Select a healthy monolayer, which is also confluent, for viral inoculation.

6 Inoculate the monolayer of cells with virus using sterile Pasture pipette, and incubate at 37°C.

7 Observe for the cytopathic effect (CPE) 7 days after inoculation.

**Observations**

After incubation, the flasks are observed for confluency and healthy monolayer of cells and virus infected cells are classified. Viruses are known to produce cytopathic effects are identified by observing the same in the infected cell lines. Non-cytopathogenic viruses are identified by other methods like immunofluorescence, haemagglutination and haemadsorption, and interference

**Results and Interpretation**

The cell lines are observed for any cytological alterations that are diagnostic of viral infections