LESSON ASSIGNMENT

LESSON 8  
Mycological Procedures.

TEXT ASSIGNMENT  
Paragraphs 8-1 through 8-12.

TASK OBJECTIVES  
After completing this lesson, you should be able to:

8-1. Identify the characteristics of procedures used to process specimens for mycological studies.

8-2. Select the statement that correctly describes principles guiding application of specific methods of identifying mycological organisms.

8-3. Select the statement that describes procedures for specific methods of identifying mycological organisms.

8-4. Select the statement that describes interpretation of specific procedures for identifying mycological organisms.

SUGGESTION  
After completing the assignment, complete the exercises of the end this lesson. These exercises will help you to achieve the lesson objectives.
LESSON 8
MYCOLOGICAL PROCEDURES

Section I. SPECIMEN PROCESSING

8-1. INTRODUCTION

Accuracy of results obtained from mycological studies in a clinical laboratory depends on the correct application of procedures for processing specimens and identifying organisms. To apply these procedures properly, a laboratory specialist must understand the scientific principles on which those procedures are based.

8-2. POTASSIUM HYDROXIDE WITH GLYCEROL

a. Principle. Potassium hydroxide (KOH) serves as a clearing agent for observation of fungal elements in clinical specimens. The addition of 10 percent glycerol decreases precipitation of KOH in reagent bottles and allows the KOH preparation to be kept for 18 hours or longer.


c. Procedure.

(1) Place a drop of KOH in the center of a clean, glass, and microscope slide.

(2) Place a fragment of the tissue, purulent material, or scrapings in the KOH, and tease apart with teasing needles to produce a thin KOH preparation.

(3) Cover preparation with a cover slip, gently heat the slide by passing it through a bunsen burner flame several times.

NOTE: Use caution to ensure you do not overheat the slide.

(4) Examine the preparation using the low power (10x) objective of the microscope. Use high dry (45/47x) objective to confirm observations.

d. Interpretation.

(1) DO NOT REPORT AS "POSITIVE" OR "NEGATIVE"!!!

(2) If any fungal elements are seen, report as "Positive for (insert the structure/element identified)." Example--"Positive for true hyphae."

(3) If no fungal elements are seen, report as "No fungal elements seen."
8-3. INDIA INK REAGENTS AND TEST

a. Principle. The capsule on yeast cells will repel the carbon particles in India ink that results in a clear, capsular "halo" around cells.

b. Reagents. India ink used for this procedure is available from many commercial sources and can usually be obtained wherever art or drawing supplies are sold. For routine use, dilute the commercial item with water, 2:1. Commercially prepared ink capsules are also available.

NOTE: India ink is easily contaminated with bacteria, yeast, or fungal spores. The reagent should be checked periodically for these cells by preparing a mount without adding specimen.

c. Procedure.

(1) Place two or three loopfuls (one small drop) of diluted India ink in the center of a clean, glass slide.

(2) Add a drop of the specimen to be studied (body fluids, urine, and so forth). Tissue should be homogenized before using. Only a small portion should be used if working with an unknown yeast colony.

(3) Mix well. Preparation should be brownish, not black, in color. It may be diluted with sterile distilled water, if necessary, and should be covered with a cover slip.

(4) Examine the preparation using the low power (10x) objective of a microscope. Use high dry (45/47x) objective to confirm observations.

d. Interpretation. Presence of budding yeast cells with a clear halo is indicative of capsular material and considered a POSITIVE preparation. If no yeast cells are observed or cells without the halo are observed, a NEGATIVE report may be submitted.

NOTE: When encapsulated yeasts are observed in a preparation of spinal fluid in India ink, *Cryptococcus neoformans* may be suspected. Cultural confirmation is required before the agent can be positively identified, because encapsulated yeasts other than *C. neoformans* may be seen in specimen preparations.

8-4. LACTOPHENOL COTTON BLUE STAIN

a. Principles. Cotton blue (China blue) stains chitin and cellulose. Since cell walls of fungi are primarily chitin, this stain is an excellent choice for observing fungi in clinical specimens.

c. **Procedure.**

(1) Place a small drop of lactophenol cotton blue (LPCB) in the center of a clean glass slide. The slide should be placed on a light box or sheet of white paper for easier manipulation.

(2) Remove a fragment of fungus culture with a teasing needle and place in the LPCB.

**NOTE:** Since a colony grows from the center outwards, it is usually advisable to take the specimen from an area 4-5 mm from the edge. This area should show characteristic structures required to identify the organism properly. The center, being the oldest section of the culture, usually shows an abundance of sterile hyphae, while the periphery will not have aged sufficiently to produce any characteristic structures. If identifiable structures are not seen in this area (4-5 mm from the edge), use a fragment 8-10 mm from the outer edge and repeat the procedure. If characteristic structures are still not evident, the colony may be too young, or the particular organism may not sporulate on that medium. If only sterile hyphae are seen, reincubate the culture.

(3) After removing residual carbon from the teasing loops with steel wool or a towel (after flaming), gently tease apart.

(4) Gently lower a cover slip onto the preparation. Heat from the microscope lamp will spread the medium evenly. **DO NOT PRESS OR TAP THE COVER SLIP** because this tends to break the conidia from the conidiophores and make identification more difficult, or impossible.

(5) The preparation may be preserved indefinitely by sealing the edges with colored nail polish after wiping off excess mounting medium. Colored polish has been found to last longer than clear polish.

(6) Examine the preparation using the low power (10x) objective of a microscope. Use a high dry (45/47x) objective to confirm observations.

d. **Interpretation.** If possible, report identification of organisms based on observed structures. If reportable structures are not seen or structures seen are nonspecific, additional testing, such as a slide culture and/or biochemical testing, may be required.

### 8-5. **SLIDE CULTURE**

a. **Principle.** Occasionally, conidia from a culture may not be observed in a Lactophenol Cotton Blue preparation. By growing the organism in a way that allows periodic observation, the precise time that conidia are at prime identifiable stage can be determined.
b. **Equipment.**

(1) Sterile petri dish (15 x 100 mm).

(2) Filter paper (100 mm diameter).

(3) Sterile, distilled water.

(4) Sterile pipets (graduated or Pasteur).

(5) Sterile, rimless test tube (approx. 15 mm in diameter) -- this is an optional item.

(6) Sterile scalpel.

(7) Petri dish with potato dextrose agar (PDA) or other medium of choice. Plate should contain 15 ml of agar.

(8) Sterile microscope slides and cover slips.

c. **Procedure.**

(1) Flame rim of sterile test tube and allow to cool. Use tube or scalpel to cut plugs or squares in agar plate.

(2) With flat side of scalpel, transfer agar plug to center of sterile slide. Set slide atop a piece of filter paper placed in the bottom of a sterile petri dish.

(3) With a sterile probe, inoculate the edges of the agar in 3 or 4 areas with small fragments of the fungal colony.

(4) Use sterile forceps to place a sterile coverslip on top of the agar plug.

(5) Moisten the filter paper by placing approximately 1.5 ml of sterile distilled water along the edge of the petri dish and allowing the filter paper to absorb it. Do not allow water to get on the surface of the slide.

(6) Incubate culture as required (usually room temperature).

d. **Interpretation.**

(1) Examine culture every other day. Remove slide from petri dish and wipe off any condensation on bottom of slide. Place slide on stage of microscope and observe using low power (10x) and/or high dry (45/47x). Examine for development of identifiable structures.
(2) If culture has not reached the proper growth stage yet, replace culture in moist chamber and reincubate. Additional water may be added when necessary; however, if too much water is added, culture may become contaminated.

(3) If slide culture has developed identifiable structures, gently lift cover slip off agar surface with pair of sterile forceps and place on slide containing drop of LPCB. If agar block sticks to coverslip, gently remove with a sterile scalpel or probe. Preparation may be preserved indefinitely by sealing edges with nail polish after removing excess mounting media.

(4) Examine preparation using low power (10x) objective of a microscope. Use high dry (45/47x) objective to confirm observations.

(5) If possible, report organism identification based on observed structures. If reportable structures are not seen or structures seen are nonspecific, additional testing such as repeat slide culture and/or biochemical testing may be required.

Section II. ORGANISM IDENTIFICATION

8-6. GERM TUBE TEST

a. Principle. This procedure is used to differentiate Candida albicans and Candida stellatoidea from other yeasts.


c. Principle.

(1) Emulsify a small amount of yeast culture in a tube of germ tube medium.

(2) Incubate the tube for 1 hour at 35°C.

(3) Examine the culture by placing a loopful of the serum culture on a clean glass slide. Coverslip and observe microscopically for germ tube formation.

(4) If germ tubes are not seen, reincubate as long as an additional 2 hours and repeat proceeding step (3) every 15 minutes.

NOTE: The test is examined within 3 hours because yeasts develop pseudohyphae after incubating three hours.

d. Interpretation.

(1) Positive--Formation of germ tubes.

(2) Negative--Nonformation of germ tubes.
e. **Quality Control.**
   
   (1) Positive--*Candida albicans*.
   
   (2) Negative--*Candida tropicalis*.

8-7. **STERILE RICE GRAIN TEST**

a. **Principle.** This procedure is used for differentiation of *Microsporum* spp, *H. canis* and *H. gypseum*, and for sporulation of other dermatophytes. *H. audouinii* produces only negligible growth.

b. **Reagents.** See Lesson 9.

c. **Procedure.**

   (1) Inoculate a flask of rice grain medium with several fragments of fungal culture. Be careful not to transfer any portion of the SDA medium.

   (2) Incubate at room temperature for 1-2 weeks.

   (3) Examine for growth on a periodic basis.

   (4) Optional: When growth is sufficient, prepare a tease mount and examine for the presence of spores.

d. **Interpretation.**

   (1) Positive--growth.

   (2) Negative--no growth.

e. **Quality Control.**

   (1) Positive--*Microsporum canis*

   (2) Negative--*Microsporum audouinii*.

8-8. **UREASE TEST**

a. **Principle.** This test determines the ability of an organism to hydrolyze urea.

b. **Reagents.** Christensen's urea agar--prepare according to manufacturer's instructions.
c. **Procedure.**

   (1) Inoculate two tubes of urea agar with fragments of mold or yeast culture being tested.

   (2) Incubate one tube at 25ºC and the other at 35ºC.

   (3) Observe daily for one week for change in color to red.

d. **Interpretation.**

   (1) Positive--pink to red color developed within 7 days.

   (2) Negative--no pink to red color developed or color developed after 7 days.

e. **Quality Control for Molds.**

   (1) Positive--*Trichophyton mentagrophytes* var. *mentagrophytes*.

   (2) Negative--*Trichophyton rubrum*.

f. **Quality Control for Yeasts.**

   (1) Positive--*cryptococcus albidus*.

   (2) Negative--*Candida albicans*.

**8-9. IN VITRO HAIR TEST**

a. **Principle.** This test is used to differentiate *Trichophyton mentagrophytes* and *Trichophyton rubrum*.

b. **Reagents.**

   (1) Sterile, distilled water.

   (2) Sterile petri dishes (glass or plastic).

   (3) Sterile glass bottle (if glass petri dishes are not available).

   (4) Sterile forceps.

   (5) CLEAN human hair. Ensure it has not been sprayed with hair.

   (6) Ten percent sterile yeast extract (prepared fresh monthly). Filter sterilize.
c. **Procedure.**

   (1) Put short strands of CLEAN human hair in glass petri dish or bottle.

   **NOTE:** Best results are obtained if hair is from a pre-adolescent child.

   (2) Sterilize in an autoclave for 15 minutes at 121°C.

   (3) If hair is sterilized in a glass bottle, transfer aseptically to plastic petri dish with sterile forceps.

   (4) Add 25 ml of sterile, distilled water.

   (5) Add 0.1 ml (2 or 3 drops) of sterile yeast extract (10 percent).

   (6) Add several fragments of fungal culture being tested.

   (7) Incubate at room temperature.

   (8) Examine at 7 and 14 days.

      (a) Remove several hair strands with sterile forceps for

      (b) Mount in LPCB and examine. Gentle heating will aid stain penetration of the mycelium.

d. **Interpretation.**

   (1) Positive--wedge shaped perforations of the hair.

   (2) Negative--no perforation of hair during the 28-day test.

   **NOTE:** Perforations usually are not abundant until 10th to 14th day of test.

e. **Quality Control**

   (1) Positive--Trichophyton mentagrophytes.

   (2) Negative--Trichophyton rubrum.

8-10. **PERIODIC ACID-SCHIFF STAIN**

   a. **Principle.** The periodic acid-Schiff (PAS) procedure is used to stain fungi as well as tissue. Stain reacts with polysaccharides found in cell walls of fungi. Periodic acid, acting as an oxidizing agent, breaks the C-C bonds at the 1:2 glycol sites within
polysaccharides. Hydroxyl groups are converted to aldehyde radicals. Basic fuchsin combines with the aldehyde groups and forms a bond that is strong enough to withstand the bleaching effect of the sodium metabisulfite.

b. **Reagents.**

(1) See Lesson 9 for PAS.

(2) Spread into a thin smear Ethanol solutions; aqueous, 70 percent, 85 percent, AND 95 percent.

(3) Absolute ethanol.

(4) Xylene.

(5) Mounting medium.

(6) Sterile, wooden applicator sticks.

(7) Microscope slides with frosted ends.

(8) Coplin jars or other staining dishes.

(9) Slide warmer.

(10) Incubator, 35°C.

(11) Microscope.

(12) Cover slips.

(13) Control slides prepared from tissue or sputum known to contain yeast.

c. **Procedure.**

(1) **Preparation of smears from clinical specimens.**

   (a) Skin and nail scrapings.

      1 Place a thin film of albumin on a clean, glass slide.

      2 Embed several thin skin or nail scrapings in the film by placing them on top of the albumin and gently pressing down with another clean, glass slide.
3. Allow smear to air dry for several hours. Drying may be speeded up by placing the slide on a slide warmer or in an incubator. **DO NOT HEAT FIX OVER A FLAME!**

(b) Tissue.

1. Prepare a homogenate from the tissue.
2. Place a drop in the center of a clean glass slide.
3. Spread into a thin smear.
4. Allow to air dry.

(c) Exudates/purulent material.

1. Place a drop of water the center of a center glass slide.
2. Emulsify a loopful of material in the water and spread across the slide.
3. Allow to air dry.

(2) Staining procedure

(a) Place fungal smear in absolute ethanol for 1 minute.
(b) Drain alcohol, and immediately place slide in 5 percent Periodic Acid solution for 5 minutes.
(c) Wash in running water for 2 minutes.
(d) Place in basic fuchsine solution for 2 minutes.
(e) Wash in running water for 2 minutes.
(f) Place in sodium metabisulfite for 5 minutes.
(g) If employing counterstain see technique listed below.

(h) If counterstain is not used, dehydrate by passing through 70 percent, 85 percent, 95 percent, and absolute alcohol for 2 minute intervals.

(i) Place in xylene for 2 minutes and mount with cover slip and Permount. Do not allow slide to dry before mounting cover slip.
(3) Technique for staining employing counterstain.

   (a) Following removal of slide from metabisulfite, wash 5 minutes in running water.

   (b) Place slide in light green stain for 5 seconds.

   (c) Wash for 5-10 seconds.

   d) Dip slide for 5 second intervals in 85 percent, 95 percent, and absolute alcohols.

   (e) Dip in xylene and mount with Permount.

d. Interpretation.

   (1) Observe control slides first. Fungal elements stain a magenta color and background appears pink to red, depending on thickness of preparation. Potential problems areas include:

       (a) Periodic acid may deteriorate resulting in a lack of staining of fungal elements. Periodic acid solution should be kept in a dark bottle.

       (b) Sodium metabisulfite may deteriorate, resulting in lack of bleaching and causing the background to be as darkly stained as fungal elements.

       (c) If light green counterstain is used, background will appear to be green; fungal elements will appear to be magenta.

   (2) Report results of the control slide and, using criteria outlined above, interpret the test slide.

NOTE: Bacteria and neutrophils may retain the basic fuchsin along with fungal elements, but this should not cause any interpretation difficulties

8-11. SALINE WET MOUNT

   a. Principle. Saline mounts are used to examine clinical specimens and pure cultures of yeasts.

   b. Reagents and Equipment.

       (1) Sterile saline.

       (2) Sterile, glass microscope slides.
c. **Procedure.**

(1) Place a small drop of saline on a clean, glass slide.

(2) Suspend a small amount of clinical specimen or culture in the saline.

(3) Gently cover slip the preparation.

d. **Interpretation.** Examine microscopically for presence of ascospores as well as size, shape, and other characteristics of blastoconidia. Describe the fungal elements observed.

8-12. **CUT-STREAK METHOD FOR MORPHOLOGY OF YEASTS**

a. **Principle.** Many yeasts procedure similar biochemical test results, making differentiation difficult. These same yeasts, however, may have very distinctive microscopic morphologies. By inoculating them to a "starvation medium" such as cornmeal agar, sporulation may be induced, which will allow observation of these characteristic morphologies.

b. **Reagents and Equipment.**

(1) See Lesson 9 for preparation of starvation medium.

(2) Inoculating needles.

(3) Sterile slides and cover slips.

(4) Forceps.

c. **Procedure.**

(1) Touch an inoculating needle to a 24 to 72 hour old yeast culture.

(2) Make two, parallel scratches, several inches long, just below the surface of the agar. **DO NOT CUT THE AGAR DEEPLY!**

(3) Make several zigzag streaks across the original scratches.

(4) Place a sterile coverslip over a portion of the scratches.
(5) Incubate at room temperature for 18 to 24 hours. Some isolates may require incubation for as long as 72 hours.

(6) Remove lid of petri dish and observe microscopically (10x or 45/47x).

d. **Interpretation.** Examine for characteristic microscopic morphologies of yeasts, and report structures observed, such as arthroconidia, blastoconidia, pseudohyphae, chlamydospores, and so forth. It may also prove helpful to observe macroscopic colony morphology.

Continue with Exercises
EXERCISES, LESSON 8

INSTRUCTIONS: Answer the following exercises by marking the lettered response that best answers the exercise, by completing the incomplete statement, or by writing the answer in the space provided at the end of the exercise.

After you have completed all of these exercises, turn to "Solutions to Exercises" at the end of the lesson and check your answers. For each exercise answered incorrectly, reread the material referenced with the solution.

1. Potassium hydroxide (KOH) with glycerol acts as a/an:
   a. Preservative.
   b. Clearing agent.
   c. Stain.
   d. Antifungal agent.

2. A positive India ink test is indicative of the presence of:
   a. Encapsulated yeast.
   b. Pathogenic mold.
   c. Nonencapsulated yeast.
   d. Pseudohyphae.

3. Lactophenol cotton blue is useful for microscopic examination of fungal cultures because it:
   a. Acts as a clearing agent.
   b. Confirms results of the India ink test.
   c. Stains walls of the fungi.
   d. All of the above.
4. The reason for using a slide culture procedure is:

______________________________________________________________.

5. Which of the following represents the usual temperature use to incubate a slide culture?
   a. 0°C.
   b. 30°C.
   c. 37°C.
   d. Room temperature.

6. A positive germ tube test indicates:
   a. Any *Candida spp*.
   b. *Candida albicans*.
   c. *Candida tropicalis*.
   d. *Microsporum audouinii*.

7. Which phase of fungal growth is studied in the Urease test?
   a. Mold phase.
   b. Yeast phase.
   c. Both mold and yeast phases.
   d. None of the above.
8. Which of the following colors is developed as a result of a positive urease test?
   a. Red.
   b. White.
   c. Yellow.
   d. Black.

9. The "in vitro" hair test requires the sterilization of several strands of clean hair.
   a. True.
   b. False.

10. The period of observation for an "in vitro" hair test is:
    a. 5-10 days.
    b. 10-14 days.
    c. 16 days.
    d. 28 days.

11. What organism produces a positive sterile rice grain test?
    a. \textit{Microsporum canis}.
    b. \textit{Candida albicans}.
    c. \textit{Microsporum audouinii}.
    d. \textit{Trichophyton rubrum}.
12. In the periodic acid-Schiff stain procedure, sodium metabisulfite functions as an:
   a. Oxidizing agent.
   b. Bleaching agent.
   c. Staining agent.
   d. Dehydrating agent.

13. The fuchsin stain, on slides processed with the Periodic Acid-Schiff Stain procedure, is retained by:
   a. Fungi cell walls.
   b. Bacteria.
   c. Neutrophils.
   d. All of the above.

14. Which of the following represents the category of culture media used for the Cut-Streak procedure?
   a. Nutrient media.
   b. Isolation media.
   c. Starvation media.
   d. Enhanced media.

15. The cut-streak procedure induces __________________, thereby allowing for microscopic observation of characteristic ____________________.

Check Your Answers on Next Page
SOLUTIONS TO EXERCISES, LESSON 8

1. b (para 8-2a)
2. a (para 8-3d)
3. c (para 8-4a)
4. Growing an organism in a manner that will allow for periodic observation of its microscopic morphology. (para 8-5a)
5. d (para 8-5c(6))
6. d (para 8-6e)
7. b (para 8-8e, f)
8. c (para 8-8d)
9. a (para 8-9c)
10. a (para B-9d)
11. d (para B-7e)
12. a (para a-10a)
13. d (para 8-10d)
14. c (para 8-12a)
15. Sporulation, Morphology (para 8-12a)

End of Lesson 8