LABORATORY 11: MYCOLOGY

I. Overview of Major Groups of Pathogenic Fungi.

Although the Kingdom Fungi have been undergoing considerable phylogenetic reorganization we still find value in classifying these somewhat unique pathogens according to the more common clinical presentation or pathogenesis.

A. **Cutaneous mycoses** include: Dermatophytes (“ringworm” causing *Microsporum* and *Trichophyton* spp.) as well as the monomorphic yeast *Malassezia* spp. and less commonly the yeast-like *Geotrichum* spp. in reptiles, amphibians and terrestrial animals.

B. The **subcutaneous Mycoses** are represented primarily by the thermally dimorphic *Sporothrix schenckii* and the less commonly encountered Dematiaceous fungi and others that often manifest as chronic eumycotic mycetomas.

C. The **Systemic Mycoses** include three thermally dimorphic genera (*Histoplasma capsulatum*, *Coccidioides* spp. and *Blastomyces dermatitidis*) as well as the monomorphic yeast *Cryptococcus neoformans*.

D. **Opportunistic fungi** include: *Candida* spp., *Aspergillus* spp. and several genera within the aseptate Phylum Zygomycota. These include the more common Mucorales (ie. *Mucor* and *Rhizopus*) as well as the exotic (but possibly emerging) *Mortierella wolfii* from the order Mortierellales.

E. **Fungal-Like** pathogens include the achorophyllic algae *Prototheca* spp., *Pneumocystis* spp. and *Pythium insidiosum*.

II. Some fungal media and stains.

A. **SAB** - Sabouraud agar is a good routine selective medium for primary isolation of many fungi. It has peptone and 2% dextrose and an acidic pH of 5.6 that inhibits the growth of many bacteria. Antibiotics can be added to increase the selectivity. *Mycosel™ Agar* is a commercially available (BD BioSciences) that includes cycloheximide (inhibits rapidly growing saprophytic molds) and chloramphenicol and is useful for primary isolation of pathogenic fungi from samples that may have a complex fungal and/or bacterial community.

B. **DTM** - Dermatophyte test medium is a selective (chloramphenicol, gentamicin and cycloheximide) and partially differential media (Phenol red) for the presumptive
identification of Microsporum and Trichophyton species. Cycloheximide inhibits many of the nonpathogenic saprophytic molds and phenol red detects increasing alkalinity of the media (yellow-to-red) around dermatophytes within 10-14 days.

C. **BA or BHI (fungal formulation)** - Brain Heart Infusion agar (with chloramphenicol and gentamicin) with 10% sheeps blood will support the growth of a number of fungi.

D. **CHROMagar™** - is a commercially (BD BioSciences) available selective (chloramphicol) and differential (chromogenic) media for identification of primarily Candida spp. C. albicans, tropicalis and krusei colonies are: light green, dark blue and light purple (mauve), respectively.

E. **10-20 % KOH** - (with or without LPCB): The addition of potassium hydroxide to opaque clinical samples (hair, skin, mucosal scrapings) or extremely mucoid sputum results in “clearing” of the sample within 5-30 minutes. Fungal elements are left intact and more easily visualized.

F. **LPCB** - Lactophenol cotton blue is commonly used for the microscopic examination of fungal cultures prepared by scotch tape method. The aniline dye component (cotton blue) lightly stains the envelope.

G. **India ink** is used to demonstrate the characteristic large capsule of Cryptococcus neoformans in clinical material such as CSF.

H. **Giemsa** stain is useful for intracellular detection of Histoplasma capsulatum in bone marrow or buffy coat smears. The small oval yeast are cytoplasmic and blue with a hyaline (transparent) halo that arises from a poorly staining envelope.

I. **PAS** - Periodic acid-Schiff stain stains yeast and hyphae bright pink or purple against a light green or orange background.

J. **Methenamine silver** stain is very useful and results in black or brown fungal elements against a light green background. Like PAS this stain is technically involved and typically used in clinical pathology or histology labs.

**III. Basic Terminology**

The following terms are used to describe some basic fungal elements.

A. **Arthroconidia**: An asexual spore formed by disarticulation of the hyphae, e.g. Dermatophytes, Coccidioides immitis.

1. **Ectothrix**: Arthroconidia outside the hair shaft, **most dermatophytes of veterinary importance are ectothrix**.
2. **Endothrix**: Arthroconidia inside the hair shaft, some *Trichophyton* species are endothrix.

B. **Chlamydoconidia/spore**: Thick-walled, resistant spores formed by direct differentiation of hyphae, e.g. *C. albicans*, *H. capsulatum*.

C. **Conidium**: An asexual spore: these arise in a variety of ways.
   1. **Macroconidia**: Large, sometimes multicellular spores, e.g. Dermatophytes.
   2. **Microconidia**: Small, single-celled conidia borne laterally on hyphae (mold-phase of *Sporothrix schenckii*), or terminally on stalks (conidiophores - *Aspergillus* spp.).

D. **Conidiophore**: A stalk-like branch of the hyphae on which conidia develop (*Aspergillus* spp.). A sporangiophore is the equivalent structure in the Mucorales.

E. **Pseudophyphae**: Arise from chains of budding yeast cells that have not separated. Typically seen in *Candida albicans* that have penetrated deeper tissues - the germ tube test is pseudohyphal development in the presence of serum.

F. **Septate**: Having cross-walls or septate hyphae (most excluding Mucorales/Mortierellales and true yeasts) in vegetative fungal filaments.

G. **Sporangium**: Closed, spherical structure in which asexual endospores (sporangiospores) are produced. Fungi in the order Mucorales reproduce asexually in this manner.

H. **Sterigmata (Phialide)**: Specialized structures, short or elongated, born on a vesicle and producing conidia, e.g. *Aspergillus* spp.

I. **Thermally Dimorphic**: Two morphological phases; typically a yeast at 37 °C (body temp) and a mycelial (mold) phase at room or environmental temperatures. Examples of thermally dimorphic fungi include: *Blastomyces dermatitidis*, *Histoplasma capsulatum* and *Sporothrix schenckii*. *Coccidioides immitis* is also thermally dimorphic however at body temperature an **endosporangium** (spherule) containing large numbers of endospores develops rather than a yeast.

J. **Yeasts**: Unicellular, monomorphic, fungi that reproduce by asexual budding. The true yeasts include *Malassezia* spp. and *Cryptococcus* spp.. Colonies can be smooth and “bacterial” like.
IV. Initial fungal characterization depends to a large extent such things as: growth on selective and/or differential media, unique gross morphological features of colonies, pigment production, the microscopic recognition of sexual and asexual spores, the type of hyphae (pseudohyphae, septate, aseptate) and the demonstration of thermally dimorphic forms (Sporothrix, Histoplasma, Coccidioides and Blastomyces). In addition many fungi and yeasts are contaminants or commensals of an animal's body, consequently sampling stringency is required in order to establish causation for a number of the opportunistic fungal pathogens (ie. Mucorales, Candida spp. and Aspergillus spp.).

While a number of the fungal pathogens are cultured with relative ease and growth is rapid (ie. Candida spp., Aspergillus spp., Cryptococcus neoformans and members of the Mucorales), still others can be fastidious and/or slow (Malassezia spp., dermatophytes) while others are serious risk group 3 pathogens (Histoplasma capsulatum, Blastomyces dermatitidis and Coccidioides immitis) that must be diverted to facilities with appropriate biosafety facilities.

Direct or histological examination of tissues or samples collected from animals with suspect mycoses can be a very useful, time saving and possibly life saving presumptive diagnostic tool. Colony and microscopic morphological diversity present considerable diagnostic challenges. New technologies such as PCR as well as MALDI-TOF mass spectrometry-based identification for fungal pathogens provide some advantages over conventional morphology-based identifications.

V. Mycological Examination of Tissues

A. Direct examination of skin/hair mucosal scrapings

Because of their size (5 -20 uM) fungal hyphae and/or spores and some yeast can be demonstrated in wet mounts of thick mucoid samples or opaque keratinous material such as skin or hairs by clearing with warm 10% KOH for 5 minutes or longer.

B. Many fungi do not stain well with gram stain (exceptions would be Candida or Malassezia spp. which stain dark blue to black or brown). Direct examination of body fluids (blood smears, CSF, oral, nasal, exudates) or aspirates (trantstracheal,
lymph node, bone marrow etc) can be carried out in a clinic or microbiology laboratory using phase-contrast microscopy, India ink (for suspect Cryptococcosis), Wright’s or Giemsa (Histoplasmosis) stains.

C. **Histological Examination**

Stains that very useful to visualize fungi in tissue smears or fixed sections but are technically involved include **Periodic acid-Schiff (PAS)** which stain fungal elements pink or purple often against a light green background. **Methenamine silver stains** are very useful for visualizing fungi (stain dark brown or black) against a pale green background.

VI. **Fungal Culture**

A. BHI with chloramphenicol or gentamicin, Sabouraud dextrose agar (SAB), or the commercially available Mycosel™ Agar (BD- Diagnostic systems) are used for routine culture. Cultures are typically incubated at room temperature and/or 37°C for up to 6 weeks.

B. Mold type (mycelial) colonies can be examined by preparing a Scotch tape mount and staining with lactophenol cotton blue (LPCB).

1) **Technique**

a) Place a drop of lactophenol cotton blue on a microscope slide. Hold a 1.5 inch piece of Scotch tape between the thumb and index finger. Press the sticky side against the fungal colony and pull gently away. Now place the strip with the aerial mycelium into the lactophenol blue on the microscope slide. Examine under the microscope.

b) It is also possible to tease out a colony with forceps, add lactophenol cotton blue and a cover slip, and examine.
**VII. Laboratory Demonstrations**

**A. ZYGOMYCOSIS** (i.e. *Mucor, Rhizopus spp.* or *Mortierella wolfii*), 
**ASPERRILLOSIS AND DERMATOPHYTOSIS** (*Microsporum + Trichophyton*)

1. Examine the methenamine silver stained slide of the placenta of a cow which aborted (11-1). Sketch the structures. Is it septate or aseptate? Is it dichotomous (division of hyphae into two equal branches of the same width as the original branch)? How could you demonstrate fungal hyphae rapidly in clinical or pathological material?

2. Examine a PAS stained section of a skin biopsy from a dog (11-2) showing ectothrix arthrospores in hair and in hair follicles.

3. Examine the colonies on three day old blood and SAB agar plates inoculated with material from a dog with **chronic sinusitis**. Examine some colony material prepared using scotch tape and LPCB microscopically.
   a. What is the most likely genus and what is this disease called?
   b. What other diagnostic tests could be used?
   c. What other diseases does this fungus cause?

4. Examine a methenamine silver stained section of bovine placenta taken from a case of abortion. Locate hyphae? Are they septate or aseptate?

5. Examine the 2-day-old culture of *Mucor* provided and the LPCB mount.
   a. What media is being used? Why?
   b. List some diseases this group of fungi can cause.
Zygomycetes, Aspergillus and Dermatophytes

Aspergillus fumigatus

Phialide

Conidia

Vesicle

Conidiophore

Microsporum canis

Macroconidium

Microconidia

Rhizopus spp.

Sporangium

Sporangiospore

Sporangiospore

Rhizoids

Ringworm parasitised hair

Fungal hyphae

Arthroconidia

HAIR
6. Examine the infected hair, from a case of ringworm (Dermatophytosis) in a cow, that has been cleared for 10 minutes with 10% KOH. Locate the arthropores. What is the likely species? Is this a useful diagnostic test?

7. Examine the cultures of the following Dermatophytes: *M. canis, M. gypseum* and *T. mentagrophytes* grown on Sabouraud's agar. Are these distinctive? How? How are *Trichophyton* species often identified? Why?

**B. YEASTS AND THERMALLY DIMORPHIC FUNGI**

1. **YEASTS**
   a. *Candida*

   The main species of importance are *C. albicans*, *C. krusei* and to a lesser extent *C. tropicalis*. They grow readily on BA or Sabouraud agar at 37° C. A new commercially available chromogenic agar (CHROMagar™) is selective and differential and can presumptively distinguish these three species based on colony color. The “gold-standard” test for speciation of *C. albicans* is the **germ-tube test**. Other species can be identified using commercially available systems (ie. API-20C Yeast). **A growing number of yeast can be rapidly identified using the MALDI-TOF mass spectrometry identification system at AVC.**

Examine the BA culture and gram stain (11-4), incubated 2 days at 37° C, inoculated with a uterine swab taken from a mare with chronic metritis.

   Colonial character and smell (presumptive diagnostic):

   What bacterium might you confuse this with?

   What is the characteristic feature of a yeast?

   What other infections do *Candida* cause?
b. *Malassezia pachydermatis*

This yeast is isolated from cases of *otitis externa* and *dermatitis* in dogs. It is thought to be an opportunist pathogen, since it may be found in the ears and on the skin of normal healthy dogs. Most species within this genera require a lipid source for growth typically provided as olive oil on the surface of solid media. *M. pachydermatis* can grow in the absence of this lipid supplement and can be readily seen on gram stained smears from dog’s ears.

Examine the gram stained smear (11-5) provided from a case of chronic otitis in a dog. Identify *Malassezia pachydermatis* (*foot-print shaped*). How would you treat this infection?

c. *Cryptococcus neoformans*

i. Examine H and E stained preparation from a dog which showed pneumonia and mediastinal lymphadenopathy at post mortem.

How did this infection arise?

ii. A SAB culture (48 hours, 37oC) from lung tissue in (i) above and an India-ink wet mount of bronchiolar aspirate are provided.

Can this organism cause zoonotic disease?
Yeasts and Thermally Dimorphic Fungi

*Coccidioides immitis* in tissue

*Sclerocystis* in tissue

*Histoplasma capsulatum*

...in culture

...in the body

*Blastomyces dermatitidis* in tissue

Large daughter cell

Thick cell wall

Often seen within macrophages

*Cryptococcus neoformans* in tissue

Capsule visible in India ink preparation

*Sporothrix schenckii*

in tissue

*Candida albicans*
2. **Thermally Dimorphic Fungi**

These are fungi that (as the name above implies) have two forms: a yeast (*Sporothrix schenckii*, *Histoplasma capsulatum* and *Blastomyces dermatitidis*) or sporangium (*Coccidioides immitis*) form in clinical material (body temperature), and a mold form at environmental temperatures.

a. Examine the micrographs of LPCB stained *H. capsulatum* prepared from colonies grown at 20 and 37°C. Sketch the microscopic appearance of both forms. Examine the PAS stained tissue section from an animal that died from histoplasmosis. How do these organisms appear in this tissue sample?

b. Examine the methenamine silver stained tissue section from a dog with blastomycosis (*Blastomyces dermatitidis*). Draw the organism. How does the size compare to *H. capsulatum* in tissue? What material or sites would be sampled to demonstrate them in clinical material?

c. Examine the PAS stained histological preparation from a dog with coccidioidomycosis (*Coccidioides immitis*). Can you find the spherules? Under what circumstances would you see this infection in Canada?

d. SAB and BHI plates inoculated with exudate material from a case of *equine ulcerative lymphangitis* and cultured at 20°C and 37°C for 7 days. An LPCB scotch tape slide was prepared from colonies taken from the 20°C culture. Examine this sample and suggest a pathogen (this is the only Risk Group 2 pathogen within these thermally dimorphic fungi).
C. FUNGAL-LIKE PATHOGENS

1. Several cows developed thin watery milk with visible flakes. One animal had enlarged supramammary lymph nodes. Apart from the milk changes the cows appeared clinically healthy. Milk samples were plated on BA, MAC and Edwards. There was no growth on MAC or Edwards - the BA plate is provided as well as a gram stain from the colonies.

What is the most likely pathogen? Hint - this is the only “plant” known to cause infections in animals.

How does this infection arise?

What intervention strategies might the farmer consider?

D. QUESTIONS

1. What does endothrix and ectothrix mean when describing dermatophytes? Are the veterinary dermatophytes “ecto-“ or “endo-“?

2. What is the most common dermatophyte of cats? How common is it? How could you diagnose and control infection in a cattery?

3. Can *M. canis* infect humans?

4. What drugs are used to treat ringworm?
5. What methods are used to demonstrate fungi in tissue?

6. What media might be used to isolate the following?
   a. Dermatophytes
   b. *Candida albicans* (what is the “**germ-tube test**”?)
   c. Dimorphic fungi

7. What is the natural habitat of
   a. *Malassezia pachydermatis*
   b. *Histoplasma capsulatum*
   c. *Microsporum canis*
   d. *Microsporum gypseum*
   e. *Cryptococcus neoformans*