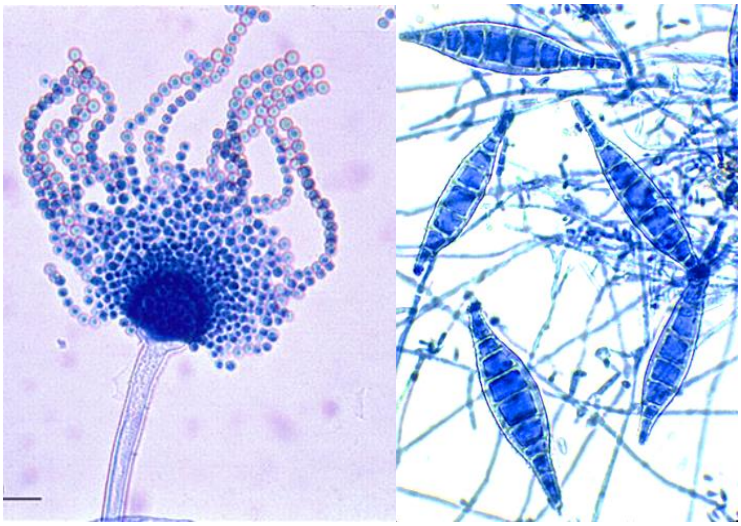
Laboratory Manual

VETERINARY MICROBIOLOGY

VMC Unit-II



Department of Veterinary Microbiology

College of Veterinary & Animal Sciences, Udgir

MAHARASHTRA ANIMAL & FISHERY SCIENCES UNIVERSITY, NAGPUR

LABORATORYMANUAL
FOR
VETERINARY MICROBIOLOGY

VMC

(New Syllabus As Per MSVE 2016)

Unit – II

Veterinary Microbiology

Compiled
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LABORATORY MANUAL
FOR
VETERINARY MICROBIOLOGY

CERTIFICATE

Certified that this is a bonafide record of practical work done in the laboratory for the course of **VETERINARY MICROBIOLOGY (VMC) Unit II** during the year.

Name of the student:_____

Registration No.: _____

Exam seat No.:_____

CourseTeacher

ANNUAL EXAMINATION

Evaluated the practical record submitted for the Annual Practical Examination held on_____.

Course Teacher **Sectional Head**

Examiner

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Practical 1

COLLECTION, TRANSPORTATION, PROCESSING OF SAMPLES AND PREPARATION OF MEDIA FOR ISOLATION OF FUNGI

Collection of clinical material for diagnosis of fungal infection

1. Aspergillosis: Affected tissue, nodular lesions from the lungs in case of Brooder's Pneumonia, under refrigeration. Milk in a sterile container in case of fungal mastitis.

2. Blastomycosis/ Candidiasis/ Coccidiodomycosis: Affected tissue under refrigeration.

3. Cryptococcosis: Affected tissue under refrigeration and brain tissues should be sent in Zenker's fluid.

4. Histoplasmosis: Smears and swabs from lesions and affected tissues under refrigeration.

5. Rhionosporiodosis: Affected tissues (Polyps) fresh.

6. Ringworm: Skin scabs or dead tissue (keratinised tissue), hairs, nails etc to be dispatched in an envelope.

Preparation of media for cultivation of fungi:

The media recommended for various fungal pathogens are as follows:

1. Media with or without cyclohexamide (Cycloheximide is added to inhibit the growth of rapidly growing contaminating molds.)
2. Media with or without an antibacterial agent (Chloramphenicol, Gentamicin and Ciprofloxacin are commonly used antibacterial for this purpose).

Media recommended:

1. **Brain-heart infusion (BHI) agar**: It is a non-selective fungal culture medium that permits the growth of virtually all clinically relevant fungi. It is used for the primary recovery of saprophytic and **dimorphic fungi**

2. **Czapek's agar**: It is used for the subculture of *Aspergillus* species for their differential diagnosis.
3. **Inhibitory mold agar (IMA):** Primary recovery of dimorphic pathogenic fungi. Saprophytic fungi and dermatophytes will not be recovered.
4. **Mycobiotic agar:**
    1. It is generally Sabouraud's dextrose agar with cycloheximide and chloramphenicol added.
    2. It is used for the primary recovery of dermatophytes.
    3. Niger Seed Agar: It is used for the identification of *Cryptococcus neoformans*.
5. **Potato Dextrose Agar (PDA):** It is a relatively rich medium for growing a wide range of fungi.
6. **Sabouraud's Heart Infusion (SABHI) agar:** Primary recovery of saprophytic and dimorphic fungi, particularly fastidious strains.
7. **Sabouraud's dextrose agar (SDA):**
    1. Sabouraud's agar is sufficient for the recovery of dermatophytes from cutaneous samples and yeasts from vaginal cultures.
    2. Not recommended as a primary isolation medium because it is insufficiently rich to recover certain fastidious pathogenic species, particularly most of the dimorphic fungi.
    3. Sabouraud's dextrose agar (2%) is most useful as a medium for the subculture of fungi recovered on enriched medium to enhance typical sporulation and provide the more characteristic colony morphology.

Exercise

1. Write the composition of Sabouraud's dextrose agar medium.

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Practical 2

KOH MOUNT, LACTOPHENOL COTTON BLUE STAINING OF MOULDS AND DERMATOPHYTES

Fungus are eukaryotic organism and they are classified into two main groups that is yeast and molds. Its cell wall is made up of chitin. The fungal structures include mycelium, sporangiospore, spores etc. The Lactophenol Cotton Blue wet mount is simple and widely used staining method for fungi.

Lactophenol Cotton Blue Stain (LCB)

Cotton Blue	0.05g
Phenol Crystals	20g
Glycerol	40ml
Lactic Acid	20ml
Distilled water	20ml

Method

Preparation of staining requires two days.

1. Dissolve the Cotton Blue in distilled water and leave overnight to eliminate insoluble dye.
2. Next day, add phenol crystals to the lactic acid in a glass beaker and stir it on magnetic stirrer until the phenol is dissolved.
3. Add the glycerol and filter the cotton blue solution into the Phenol + Glycerol + lactic acid solution.
4. Mix and store at room temperature.

The main components of LCB staining are :

1. Phenol: Fungicidal in nature
2. Lactic Acid :Preserves fungal structures
3. Cotton Blue: Stains the chitin in the fungal cell walls & the cytoplasm (in light blue).

Staining of Clinical Specimens (Non-keratinized) Procedure (LCB Staining)

1. Place a drop of 70% alcohol on the slide.
2. Add the specimen to the drop of alcohol.
3. Add one or two drops of Lactophenol Cotton Blue Stain before alcohol gets off.
4. Place the coverslip on the drop avoiding air bubbles to be trapped.
5. Examine under Microscope using 10X and 40X objective.

Staining of fungus from culture

1. Take a grease free slide.
2. Add a drop of lactophenol cotton blue solution on a slide.
3. Sterilize the inoculation loop or needle and cool it then transfer mycellial growth onto the LCB stain and press it gently so that it easily mix with the stain.
4. Take a clean cover slip and with the help of a forcep place the cover slip on mycellial gowth + LCB.
5. With the help of blotting paper, wipe the excess stain .
6. Observe the preparation under low & high power objectives of the microscope.

Exercise

Q1. Explain KOH mount for direct sample staining.

Q2. Write the microscopic picture of Trichophyton and Microsporum stained with Lactophenol Cotton Blue.

References

Leck Astrid, 1999. Preparation of Lactophenol Cotton Blue Slide Mounts, *Community Eye Health*. 1999; 12(30): 24

http://www.generalmicroscience.com/microbial-laboratory-techniques/staining- fungus-using- lactophenol-cotton-blue/

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Practical 3

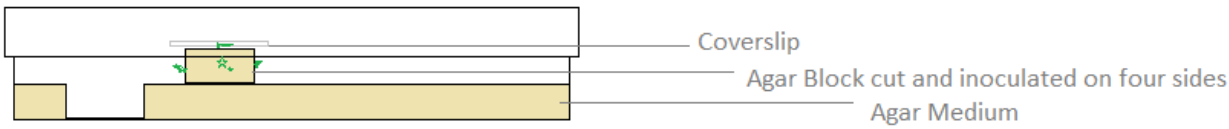
SLIDE CULTURE TECHNIQUE, CULTURAL CHARACTERISTICS AND ANTIFUNGAL SENSITIVITY TESTING OF FUNGI

Slide Culture Technique

For accurate identification of fungi, it is required that the precise arrangement of the conidiophores and the way in which the spores are produced is essential. The simple method of slide culturing used widely is described here, which permits fungi to be studied virtually *in-situ* with as little disturbance as possible.

Procedure:

1. With the help of a sterile blade cut out an agar block (6 x 6 mm) enough to fit under the coverslip.
2. Flip the block up onto the surface of the agar.
3. Inoculate the sides of the agar block with spores or mycelia of the fungus to be grown.
4. Flame the coverslip and place it on agar block.
5. Incubate at 26^{O}C until growth and sporulation take place.
6. After attaining the growth, remove the cover slip from the agar block.
7. Apply a drop of 95% alcohol as a wetting agent and gently lower the coverslip onto a small drop of Lactophenol cotton blue on a grease free glass slide.
8. The slide can be left overnight to dry and later sealed with nail polish.
9. When sealing with nail polish use a coat of clear polish followed by one coat of red coloured polish.



Slide Culture Technique

Cultural characteristics

Different types of fungi will produce different colonies, some colonies may be coloured, some colonies are circular in shape, and others are irregular. A specific terminology is used to describe common colony types. These are:

- Form - The basic shape of the colony e.g., circular, filamentous, etc.
- Size – The diameter of the colony. Tiny colonies are referred to as punctiform
- Elevation - This describes the side view of a colony. Turn the Petri dish on end.
- Margin/border – The edge of a colony. - magnified shape of the edge of the colony?
- Surface - How does the surface of the colony appear? For example, smooth, glistening, rough, wrinkled, or dull.
- Opacity - For example, transparent (clear), opaque, translucent (like looking through frosted glass), etc.
- Colour - (pigmentation) - For example, white, buff, red, purple, etc.

The fungal cultural characteristics from reverse side of the plate specifically in dermatophytes is taken in account, especially pigmentation.

Yeast colonies are very similar to bacterial colonies. Moulds often have fuzzy edges. They usually turn into a different colour, from the centre outwards.

Antifungal susceptibility testing

Antifungal susceptibility testing can be used for drug discovery and epidemiology, to predict therapeutic outcome.

Disk diffusion method:

1. Organisms are subcultured on potato dextrose agar (PDA) or oatmeal agar (for *T. rubrum*) at 30°C for 4 to 15 days.
2. Following growth, conidia were harvested in sterile saline, and using a hemacytometer, the conidial suspension was adjusted to 1.0×10^6 conidia/ml.
3. Mueller-Hinton (MH) agar plates are streaked evenly with a swab dipped into the standardized inoculum suspension.

4. Lids are left ajar for 3 min in a laminar flow cabinet to allow for any excess surface moisture to be absorbed into the agar before the drug-impregnated disks were applied.
5. Disks containing the test agents were applied to the surfaces of inoculated plates.
6. Plates were inverted and incubated at 30°C for 4 to 7 days to allow for fungal growth.
7. Inhibition zone diameters (IZD) were measured in millimeters.

Table: *In vitro* activities of 5 antifungal agents (10mg concentration) against *T. rubrum*

Antifungal agent	IZD range (mm)
Ketoconazole	20-50
Miconazole	20-45
Itraconazole	30-45
Fluconazole	0-50
Griseofulvin	30-55

- Each drug testing was performed with fresh inoculum in triplicate on three different days.

Exercise

1. Enlist other methods of antifungal susceptibility testing.

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Practical 4

DIAGNOSIS OF ASPERGILLOSIS AND CANDIDIASIS

Aspergillosis

Brooder pneumonia is a common respiratory problem during brooding period of broiler chicken in high humid region. It is caused by fungal genus Aspergillus, specially *Aspergillus fumigatus.*

Aspergillus is also responsible for mastitis in cattle and buffaloes.

Material collection: yellowish spherical caseous granulomatous lungs nodules, milk sample in mastitis cases.

Agar Medium used: Sabouraud's agar plates / Potato dextrose agar medium. Incubated at 37^{O}C for 6-7 days.

Cultural characteristics & Microscopic : Colonies of *Aspergillus fumigatus* are granular to cottony with shades of green, greenish-brown pigment. Microscopically the conidiophores are relatively long (300-500um), the vesicles are 30-50 um in diameter, club shaped and covered on top half with only a single row of sterigmata (Uniseriate), giving rise to long chains of spherical to slightly ovoid conidia tend to sweep towards the central axis.

Aspergillus flavus

Colonies of Aspergillus flavus are granular to woolly and with shade of yellow or yellow brown.

Microscopically: Conidiophores are long 400-800um.Vesicles are 25-45 um in diameter. The sterigmata arise from ¾ or the entire circumference of the vesicle and may have one row or two rows. Conidia are spherical smooth slightly roughened with maturity and form long chains.

Candida albicans:

Candida albicans occurs naturally as a commensal of mucous membranes and in the digestive tract of humans and animals. It accounts for up to 70% of *Candida* species isolated from sites of infection and has been reported as a causative agent

of all types of candidiasis i.e., **Thrush** in poultry and **mastitis in cattle and buffalo** caused by *Candida albicans*.

Collection of clinical material:
Milk from mastitis caused by *Candida albicans* in a sterile milk sampling bottle.

Cultural characteristics:
Corn Meal Agar / Sabouraud's dextrose agar : colonies are white to cream colored, smooth, glabrous and yeast-like in appearance.

Microscopic morphology shows spherical to subspherical budding yeast-like cells or blastoconidia, 2.0-7.0 x 3.0-8.5 um in size.
Staining: Apply either Gram's staining /Simple staining method.

Biochemical /Physiological Tests:
Germ Tube test is Positive within 3 hours,
Hydrolysis of Urea is Negative,
Growth on Cycloheximide medium is Positive,
Growth at $37^{O}C$ is Positive.

Exercise
1. Explain the Germ tube test.

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Practical 5

DEMONSTRATION OF OTHER IMPORTANT YEAST

Yeasts are fungi that grow as single cells, producing daughter cells either by budding or by binary fission. They differ from most fungi, which grow as thread-like hyphae. Include several yeasts that cause disease in animals and humans.

Here we consider several examples of yeasts: ***Saccharomyces cerevisiae-True yeast,*** the common baker's yeast. Genus ***Cryptococcus***, which includes *C. neoformans*, a pathogen of animal and humans

Cryptococcus neoformans

Habitat

- Cryptococcus is encapsulated yeast that is found in soil contaminated with pigeon droppings or eucalyptus trees and decaying wood.

Microscopic

- A characteristic polysaccharide capsule of variable thickness (1-30µm) surrounds these yeasts. In its natural environment the capsule is thinner and the yeast smaller, while thicker capsules tend to be found from infected tissues. The capsules stain pink by the Meyer's mucicarmine technique.
- *Cryptococcus neoformans* var. *neoformans* and *Cryptococcus neoformans* var. *gattii*- encapsulated yeast
- The species has **4** serotypes (A,B,C,D) based on capsular polysaccharide antigen.

Cultural Characteristics:

- Culture: 37^0C, 1-2 days
- SDA with out cyclohexamide: creamy, white and mucoid, Urease positive.
- **Birdseed agar**: Brown to black colony- C.neoformans produces phenoloxidase enzyme that results in production of melanin and thus a brown to black discoloration of the colony when it is grown on **caffeic acid agar or bird seed agar.**

Exercise

1. Name the disease conditions caused by pathogenic yeasts in animals.

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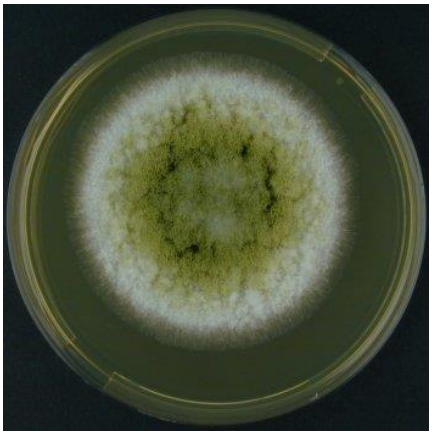
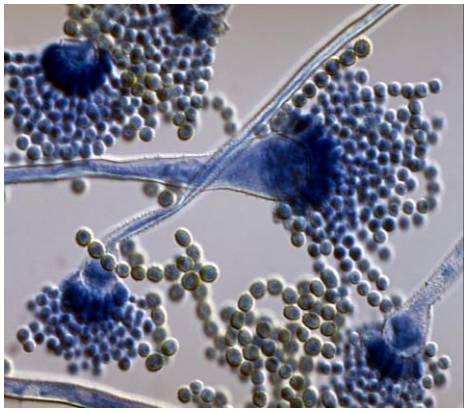
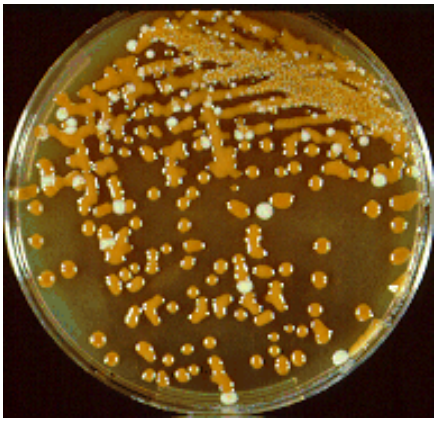
PLATE 1



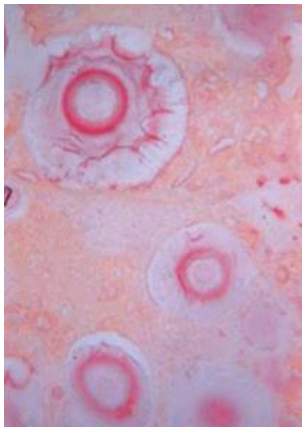
1. *Aspergillus flavus*



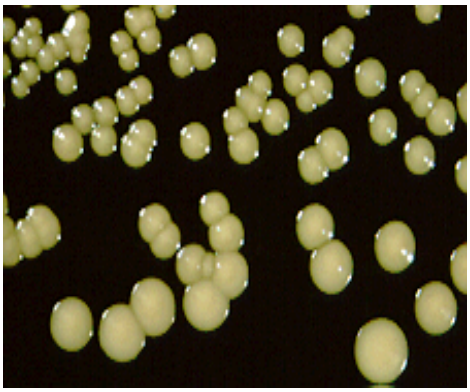
2.*Aspergillus flavus*- Microscopic



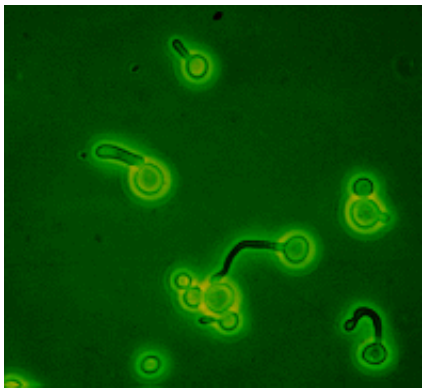
3.*Cryptococcus neoformans*-Bird Seed Agar medium



4. *Cryptococcus neoformans*: Mucicarmine stain



5. Colony characteristics of *Candida albicans*



6. Germ Tube Test – *Candida albicans*

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COVASU

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