www.jolsc.com e ISSN: 2455-0930

General Article

Basic Guidelines and Procedures for the Clinical and Laboratory Diagnosis of Mycotic Infections in Animals: A General Overview of Veterinary Importance

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Abstract

There are various mycotic diseases of veterinary importance affecting the domestic animals. The diagnosis of the causative fungi or yeast responsible for those infections is essential in veterinary microbiological laboratories for confirmative diagnosis followed by its treatment and control. The specimens collected for diagnosis and isolation and identification of the etiology are the clinical materials generally obtained from the skin and / or tissues. The fungal infection may be either superficial or generalized in nature. Proper diagnosis of the fungal agents most often poses a problem because of secondary bacterial infection during the course of its diagnosis. The present article was constructed to provide an overview to the readers and students on the preliminary tests employed for the accurate and pin-point clinical diagnosis of mycotic diseases in animals in veterinary microbiology laboratories.

Key words: *Clinical specimen, Fungal, Laboratory diagnosis*

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Dermatophytosis and Dermatomycosis (Ringworm)

Laboratory diagnosis

Preliminary examination: If possible, the patient's lesions should be examined in a darkened room using ultra-violet light rays of 3660 Å (Wood's lamp). Since the hair infected by certain Mycrosporum spp. Produces a marked yellowish-green fluorescence under such light, the precise localization of infected area may be ascertained. Infections caused by zoophilic dermatophytes such as *M. canis* and *M. distortum* can be detected by these means, but almost all *Trychophyton spp*. and many *Mycrosporum spp*. are not fluorescent in hair. The flourescence is due to Trychophyton metabolites produced due to breakdown products of keratin and not due to the mold. The metabolite is produced only by the fungi that have invaded actively grow in hair and cannot be elicited from and in vitro infection of hair.

Direct microscopic examination:

A direct examination involves placing the skin scrapings or hair in 20% KOH on glass slide and heating gently without boiling. Boiling may cause precipitation and crystal formation that will make examination of specimens difficult. Parker superchrome blue-black ink mixed 1 part in 9 parts KOH will help to outline fungus elements in scrapings. A cover slip is placed on the preparation and examined under low power microscope.

Culture:

Whether arthrospores have been detected or not, the materials collected should be inoculated on Sabouraud agar with cyclohexamide and chloramphenicol. The acidity of Sabouraud agar inhibits most bacteria. Chloramphenicol further restricts bacterial growth and cyclohexamide inhibits most saprophytic (contaminant) fungi. Vitamin enrichment of the media is necessary to

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grow certain dermatophytes. *Tricophyton equinum* requires nicotinic acid and *T. verrucosum* require thiamine and inositol. *T. verrucosum* grows much faster at 37^{o}C than at room temperature.

Additionally, dermatophyte test medium (DTM) may be used as primary culture medium. The medium selectively inhibits most bacteria and contaminant fungi, while encouraging dermatophyte growth. It can also be used as differential agar. The dermatophyte produces an alkaline (red) reaction on DTM through oxidative deamination, while most of the other organisms that are capable of growing on DTM produce an acidic (yellow) reaction. Approximately, 98% of the fungi which produce an alkaline reaction on the medium are dermatophytes.

Rhinosporidiosis

Diagnosis

The presence of large sporangia on the polyps which are composed of friable pink or red granulomatous tissue and bleed easily are diagnostic feature of rhinosporidiosis. The spores and sporangia can be demonstrated in the nasal exudates and tissue sections. These can be squeezed with a forceps from biopsy of the polyps into a drop of water and observed microscopically.

In the tissue sections stained by H & E stain, various forms of sporangia can be seen. Young trophic forms 10-100 u in diameter with central basophilic karyosomes and amorphous cytoplasm. Mature forms 100-300 µ in diameter containing sporangiospores which develop on the side and fill the centre as they mature and escape through break in sporangial wall. Then third form is empty and collapsed form of sporangia.

R. seeberi has not been grown in mycelium form. However, maturation of spores and sporangia in biopsy material placed in liquid medium (T.C. 199) has been obtained.

Sporotrichosis

Diagnosis

Direct microscopic examination:

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It is almost impossible to diagnose sporotrichosis by direct examination of smears of pus from lesions because the parasitic yeast form cells of *S. schenki* are observed very rarely or only with difficulty in stained clinical specimens.

Histopathological diagnosis using stained histologic sections prepared from biopsy materials is also difficult because only a few yeast form cells appear in the infected tissues and their histopathological features are not pathognomonic.

Culture:

This method is reliable diagnostic procedure for the identification of *S. schenki*. Exudative material to be cultured should be aspirated from an unopen lesion employing the usual aseptic techniques and place on Sabouraud agar supplemented with both chloramphenicol (50 mg/litre) and cyclohexamide (500 mg/litre) and brain heart infusion agar. *S. schenki* is dimorphic. It appears as budding yeast in host tissue and on enriched medium when grown at 37^{o}C. It grows in mycelia form when cultured at room temperature. Sabouraud agar adequately supports mycelia growth and is a useful isolation medium

Mycelial colonies are first moist (3-7 days) and have a wrinkled or folded appearance. A white to grey aereal mycelium then initially at (periphery) and with age becomes yellow and eventually black. There is considerable variation in colony color.

Microscopically, the mycelium is composed of fine branching septate hyphae. Delicate tapered conidiophores rise at right angles from the hyphae. Initially conidia are borne at the tip of the conidiophores in a manner which resembles 'flower like petals'. Each conidium is borne on very delicate sterigma. As sporulation progresses, spores are formed along the sides of conidiophores and eventually along the hyphae. Conidia are pyriform to oval or nearly spherical.

The tissue or yeast form of *S. schenki* grows adequately on blood enriched BHI agar when incubated at 37^{o}C. Colonies appear in a few days. They are yeast like with pasty consistency and with color variations from creamy white to grey to yellowish.

Microscopic examination reveals small round or oval budding cells. Some cells are elongated and have been described as cigar shaped and are termed cigar bodies. The cells are positively stained by Gram's Method.

Pathogenicity tests:

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Purulent material obtained especially from unopened lesions may be inoculated into experimental animals like rats hamsters or mice. The intraperitonal inoculation of pus or suspension of cells from cultures into mice causes peritonitis. In male mice, orchitis may occur within 10 days. The disease in mice tends to be progressive and disseminated lesions, particularly involving the bones are characteristic. Microscopic examination of material obtained from infected sites reveal numerous cigar shaped Gram positive organisms.

Histoplasmosis

Microscopic examination of animal tissues parasitized with *H. capsulatum* reveals that the organisms are found largely intracellularly, particularly in reticulo-endothelial cells (macrophages and epitheloid cells). The fungus grows and reproduces as yeast n the cellular system. The organism is round or slightly oval and measures from 1-4 µ in diameter. In sections stained with H & E, a central, spherical usually basophilic body is surrounded by an unstained zone of a capsule around the central body, but the organisms has no true capsule. The clear halo is actually an artifact due to contraction of the fungus methods. He wall is stained selectively, usually leaving its cytoplasm unstained. Thus the organisms appear as an empty red ring. These stains are particularly useful in visualising organisms when only a few are present and differentiating them from other phagocytosised particles, especially tissue debris. The cytoplasm may contain so many organisms that the phagocytocising cell is greatly enlarged. Proliferation of the parasitized cells causes displacement of normal tissues and eventual impairment of physiologic function.

Laboratory diagnosis

Usually, the isolation of *H. capsulatum* is done from samples of tracheal exudates, blood, urine, peritoneal fluid and sternal bone marrow. Biopsy specimens from affected lymph nodes are useful both for culture and mouse inoculation. Fecal materials should be routinely cultured. All specimens for cultures should be freshly obtained. The clinical material to be refrigerated as the *H. capsulatum* does not remain viable in clinical materials for many hours unless refrigerated or frozen. Wherever, possible, dry ice should be used for shipment of the materials.

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Direct microscopic examination:

The examination of unstained clinical material is unrewarding due to the small size and difficulty of the differentiation of the organism. Geimsa or Wright stain of nodal smears of blood and bone marrow smears in acute disseminated cases may delineate the invading agent. The organisms occur intracellularly (usually in monocytic cells) as small, round or oval budding yeast (2-4u diameter). A clear halo is seen around the darker staining central material.

Mouse inoculation:

The organisms can be recovered from grossly contaminated specimens by mouse inoculation. Material is ground in physiological saline containing chloramphenicol (0.05 mg/ml). Each of the four mice are inoculated intraperitoneally with graded doses (e.g. 0.2, 0.5 and 1 ml). After 2-4 weeks, the mice are sacrificed and materials obtained from liver are spleen is incoculated into media and tissues are examined histopathologically.

Blastomycosis

Direct laboratory examination

The finding of typical lesion and organisms in tissue sections or clinical specimens supports the identification. The large, spherical and thick-walled yeasts (15-20 µ diameter) are readily seen in potassium hydroxide wet mounts and have a refractile wall. The yeast can also be demonstrated in tissue sections with histopathological stains in which he wall remains colorless. A single bud is frequently seen connected to the larger mother cell by a wide base.

Pathogenicity test:

Intraperitoneal injection of 1 ml of saline suspension of either mycelia or yeast form cells usually produces demonstrable infection in mice. More extensive infections results from the addition of 5% gastric mucin to the inoculated material. Clinical materials should be treated with antibacterials/ antibiotics prior to inoculation to prevent bacteria from killing the experimental animal. If the experimental mice survive, they should be sacrificed after 3 weeks.

Gross lesions are usually confined to the peritoneal cavity caseous material, pus or nodules may be found in the abdominal viscera. Nodules may be present in the lungs in extensive infections.

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Microscopic examinations of fresh preparations show the characteristic thick walled, single budded forms of *Blastomyces dermatidis*.

Coccidiomycosis

Laboratory diagnosis

The mycelia forms of *Coccidioides immitis* should be handled carefully because the inhalation of spores from the mycelial forms is hazardous. Mature mycelial filaments fragment into tiny arthrospores which readily become airborne following the removal of cotton plug or the lifting of petridish cover. The handling of mold form of the fungus can be eliminated by inoculating suspected clinical materials into mice. The resulting infected tissues or exudates which are less dangerous to handle can be collected and examined for spherules. Clinical material should be treated with antibacterials/ antibiotics before it is inoculated tom prevent contaminating bacteria from killing the experimental animal.

Direct microscopic examination:

Direct, unstained mounts of clinical materials such as urine, exudates and pleural or peritoneal fluids may contain characteristic spherules. These when mature, have a thick refractile wall and contain endospores.

Aspergillosis

Diagnosis:

The diagnosis of aspergillosis requires a cautious approach is aspergilli are the most common contaminants in the laboratory and they can be routinely cultured from the skin and upper respiratory tract. Repeated isolation of *Aspergillus spp*. from clinical specimens in the absence of other pathogenic agents is presumptive evidence of aspergillosis. Recovery of organisms from the unexposed tissues helps to support a diagnosis. Sabouraud dextrose agar medium containing chloramphenicol is used for the isolation of Aspergillus organisms. The colonies of *Aspergillus fumigatus* are rapidly growing and flat. They are at first white and slightly fuzzy, but a the conidia develop they become dark-bluish, green and appear powdery. Microsopically, a vesicle

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like inverted flask with a rounded bottom and a long drawn neck is the characteristic feature of the organisms. The appearance of characteristic nodular lesions on the affected organs is almost diagnostic, but it is necessary to eliminate tuberculosis and granulomatous diseases. Differential diagnosis is conclusive by the demonstration of hyphae in the nodules which may be carried out by crushing the nodule under a cover slip on a side in a drop of 20% potassium hydroxide or in lactophenol cotton blue.

Candidiasis (Moniliasis or Thrush)

Diagnosis

1. **Direct examination of clinical materials:** Masses of budding cells and fragments of mycelium often with budding cells and fragments of mycelium often with budding cells attached may be demonstrated.
2. Isolation of the yeast on Sabouraud dextrose agar with chloramphenicol at 30^{o}C. *Candida albicans* show yeast like budding cells and pseudomycelium.
3. The production of characteristic chlamydospores on corn meal agar or on special chlamydospore agar using Dalmu's technique.
4. Another identification technique with at least equal specificity is the 'serum tube' test. It is based on the production of filamentous outgrowths (pseudogerm tubes/ pseudomycelium) by cells of *Candida albicans* when inoculated into serum and incubated at 37^{o}C from one to two hours.
5. Histopathological findings are helpful in diagnosis.
6. **Animal inoculation:** Mice, rabbits and other laboratory animals are susceptible to infection.

Cryptococcosis

Microscopical examination reveals globose cells 5-20 µ in diameter with occasional buds, ach cell having a wide mucilaginous capsule. The examination of CSF, pus and other fluids from

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siuspected cases of cryptococcosis is facilitated by mounting with India ink. Sections of tissues show the closely packed yeast cells often without any marked tissue reaction.

Formation of thick mucilaginous capsule is characteristic of *Cryptococcus neoformans* when growing in tissue and under proper condition in culture. *Cryptococcus neoformans* lacks the ability to form a true mycelium. This important identifying characteristic can be tested on chlmydospore agar or on corn meal agar.

Pathogenicity test

The mice, intracerebrally inoculated with 0.02-0.03 ml of saline suspension of the organism usually die in four days to two weeks.

Phycomycosis

Laboratory identification:

Aspirates of scrapings are examined as KOH preparations for the presence of broad, aspirate refractile mycelium with tick walls. The broad hyphae have a collapsed, ribbon-like appearance and may be distorted.

Samples of material collected for direct examination should be inoculated to SDA and SDA with chloramphenicol and incubated at 37^{o}C.The Mucorales are sensitive to cyclohexamide and this antibiotic should be incorporated into the medium. Isolation of Mucorales from sputum is however, not considered clinically significant, since it is so frequently seen in sputum without evidence of pathology.

Histopathologic examination is best in tissues stained with H & E, whereas other special fungus strains such as PAS and Gridley methods are much less satisfactory. The characteristic is reversed for most fungi and may be useful in differentiating the Mucorales.

The mucorales in tissues are seen as broad, rarely septate hyphae that branch haphazardly and stain deeply with haematoxylin. The hyphae are usually abundant and the tissue reaction is acute and pyogenic with abundant polymorphonuclear neutrophil infiltration and necrosis. The hyphae have a tendency to invade in the blood vessels and cause thrombi.

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