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Special Issue: Manual guidance of veterinary clinical practice and laboratory

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ANNEX

General Characteristic and Miscellaneous Examination of Microorganism

Some important characteristics of the organisms are given in each genera which may serve as key for their identification.

Gram Positive Cocci

* *Staphylococci:* Gram positive cocci arranged in bunches, catalase positive, acid from glucose. Pathogenic staphylococci are coagulase positive.
* *Streptococci:* Gram positive cocci, arranged in chains, catalase negative, beta hemolytic.
* *Micrococcus:* Gram positive cocci, single or small clusters, catalase positive, maltose (-), mannitol (-), coagulase (-), nonhemoly tic.

Gram Positive Rods

* *Bacillus anthracis:* Gram positive rods, cylindrical rods, spores are centrally located, short chains, square ends of rods, capsulated, reduce methylene blue, non-hemoly tic, produces dull, opaque, greyish colour colonies.
* *Listeria:* Beta hemolytic, catalase (+), nitrate not reduced, motile at 25OC, acid from glucose. Single or short chains, noncapsulated, on blood agar circular, transparent colonies, pathogenic for guinea pigs leading to death in 3-4 days.
* *Eysipelothrix:* Alpha hemolytic, small round, translucent, discrete colonies with entire edge, 1 mm dia, organisms are short rods, tluckened filament, non-motile, guinea pigs are resistant, glucose fermented, catalase negative, nitrate not reduced. *Actinomyces:* Gram positive coccoid, branching filaments in media; in tissues sulfure granules, club like process.
* *Corynebacteria:* Gram positive, coccoid or pleomorphic, non motile, clumps or single, on blood agar minute pin point colony, beta hemoly tic, cream to orange coloured colonies.
* *Lactobacillus:* Long, slender, Gram positive rods, motile, catalase negative.
* *Nocardia:* Gram positive rod, acid fast, branching filaments, rod or coccoid, catalase positive.
* *Clostridium:* This group consists of anaerobic bacilli with rounded ends, spore formers, Gram positive rods.

➢ ***Clostridiurn chauvoei:*** Gram positive rods with rounded ends, single or short chains, long filament, spores are elongated or oval and sub terminally or terminally located and are wider then width of organism which gives it a tennis racket like appearance.

➢ ***Cl. septicurm:*** Cylindrical Gram positive rod, single filaments.

➢ ***CI. Novyi:***Largest size, parallel edge, rounded ends, long jointed filaments, Gram positive.

➢ ***C1. Perfringe:*** Gram positive rods with square ends, nonmotile.

➢ ***Cl. botulinum:*** Gram positive large rods with rounded ends, short chains, oval spores and terminally located.

➢ ***C1. tetani:*** Gram positive large slender rod, short chains, rounded ends; spore 2-3 times larger than diameter of rod, located terminally giving the organism drum stick like appearance.

➢ ***Cl. hemolyticum:*** Gram positive rods, oval or elongated, spore terminally situated.

Gram Negative Rods

* *Yersinia:* Small, Gram negative rods or coccobacilli, catalase (+), oxidase (-), motile at 25OC, urease (+), reduces nitrate.
* *Pasteurella:* Small Gram negative rods or coccobacilli,

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oxidase (+), opaque whitish colonies on blood agar, bipolar staining from blood or tissues.

* *Brucella*: Gram negative coccobacilli, non-motile, noncapsulated, oxidase positive (Br. ovis oxidase (-). Urease positive (Br. ovis urease (-), produces small delicate semitransparent colonies on tryptose soya agar.

* *Pseudomonas*: Gram negative rods, catalase positive, oxidase positive, motile (*pseudomonas*allei non-motile), produces translucent large, irregular and spread colonies, blue-green water soluble pigment in media.

* *Actinobacillus*: Gram negative rods, oxidase positive, catalase positive, non-motile, produces flat colonies, transparent, slightly bluish in colour.

* *Moraxella*: Gramnegative rods, non-motile, catalase positive, oxidase (+), does not grow on McConkey agar, short, diplococcoid rods, produces small, round, greyish white colonies, translucent, beta hemolytic.

* *Escherich coli*: Produces circular, convex, smooth translucent colonies having wet appearance, lactose fermenter, non-motile, Gram negative coccobacilli, Indole (+), MR (+), VP (-), citrate not utilized, produces metallic sheen in EMB agar.

* *Salmonella*: Gram negative rods, non-lactose fermenter, produces white colonies on McConkey agar and pink on brilliant green agar (BGA), produces homogenous, smooth, glistening, greyish colonies.

* *Protcus*: Gram negative rod, motile, thin transparent colonies, swarming on agar plates, MR (+), VP (-), citrate not utilized, 3s (+), urease positive.

* *Klebsiella*: Gram negative, coccobacilli, mucoid colonies, indole (+), MR (i), VP (+), citrate utilization (+),H,S (-), ireases (+).

* *Shigela*: Gram negative rod, non-motile, pleomorphic, long filaments, occurs in chain, rough, dry, circular, mucoid colonies, opaque, raised colony on agar surface.

* *Hemophilus:* Gram negative rod or coccobacilli, non-motile, semitranslucent, flattened, circular, greyish colony with sharp edges.

* *Vibrio or Campylobacter:* Gram negative, non-capsulted,'s' shaped, bluish pin point size colonies, slightly raised on thiol medium or blood agar.

* *Mycobacteria*: An acid fast bacillus, capsulated, slow growth, takes at least a week's incubation period for growth.

* *Leptospira*: Spirochete Long spiral rods, having many curves, ends look like hook, demonstrated in dark field microscope, difficult to grow.

* *Rickettsia*: Rod shaped, coccoid or pleomorphic, Gram negative, intracellular organism, does not grow on artifical media. Grows well in yolk sac of 5-7 days old chicken embryo; after 4-5 days' incubation inclusions in yolk sac smears stained with Geimsa or Macchiavello stain. Intra-cytoplasmic inclusions can be demonstrated in liver impression smears of guinea pig inoculated I/P with rickettsia.

* *Chlamydia:* Intra-cellular organisms, produce intracellular inclusions, do not grow in artificial media, and grow in seven day old chicken embryo through yolk sac route, inclusion in yolk sac membrane.

* *Mycoplasma*: Pleomorpluc, small coccobacilli, ring form, filamentus, produces fried egg colony, grows on PPLO broth or agar containing horse serum, produces nipple shaped colonies, small colonies seen by stereoscope, for cultivation incubated at 2-3°C for 24-48 hours in PPLO broth, then transferred 0.1 ml to solid media. Incubated in humid chambers at 37.5OC. Growth occurs in 2-4 days, flood the plate with 1-2 ml PPLO brolh, incubate further for 2-3 days, if no growth for 14 days then declare as negative.

Some importance veterinary laboratory test

* **Catalase test**: On a clean and dry glass slide, place a drop of 30% hydrogen peroxide. Take bacterial culture with loop from a slant or colony of plates and mix it with hydrogen peroxide on glass slide. The production of gas characterized by appearance of bubbles is indication of a positive reaction.

* **Coagulase test**: Take 0.5 ml of rabbit plasma diluted (1:5) with sterile normal saline solution. Add 2-3 drops of overnight incubated broth culture and mix well. Incubate at 370C a positive reaction is indicated by coagulation of plasma within 2-3 hrs.

* **Oxidase test**: Prepare a 0.5% solution of N, N, N: N Tetramethyl-p-phenylenediamine dihydrochloride and put a drop of this on suspected colony on the plate. The colour of colony will become dark purple in case the oxidase test is positive.

* **Nitrate reduction test**: In a test tube, take 5 ml trypticase broth culture and add 1 ml of 0.8% sulfanilic acid in 5N acetic acid. Add drop by drop 0.5% alpha naphthylamine in 5N acetic acid. Development of red/pink/maroon colour is an indication for the positive nitrate utilization reaction. However, in negative test reaction add zinc dust in the tube of test culture as well as in broth for control. The colour of positive reaction is characterized as red or pink while the control will remain the same.

* **Indole test**: Take a 48 hour growth of organism in brain heart Infusion (BHI) broth. Add 1 ml ether in 5 ml culture and mix well. Keep the tube on stand for a few minutes. Add 0.5 ml of Kovac's reagent slowly on the top of the test tube contents. If indole is present, a red colour ring is formed just below the ether layer in the culture.

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* **MR Test**: Take 5 ml growth of organism in MR-VP broth and incubate for 4-5 days. To tlus add a few drops of MR reagent. In positive test, red colour will develop in the culture. Yellow colour is indication of a negative reaction.

* **VP test**: Take 2 ml growth of organism in MR-VP broth incubated for 2 days and to this add 0.6 ml of 5% alpha naphthol in ethanol and 0.2 ml of 40% potassium hydroxide containing 0.3% creatine and shake well. Place the test tube on stand for 5-10 min. In positive reaction, orange red colour will develop.

* **Urease test**: Inoculate the organism on urea agar slants under sterile conditions. Incubate for 12 hours at 37 °C. In case of positive test, the media turns into pink colour.

* **Citrate Utilization test**: Inoculate the organisms on Simmon's citrate agar slants and incubate for 24 hours at 37 °C. The development of a blue colour is indication of citrate utilization.

* **Hydrogen sulfide gas production test**: The organism is inoculated on TSI agar slants with a straight wire of the bacteriological loop. Inoculate the slant as well as butt and incubate the culture for 18-24 hours at 37 °C

* **Hemolysis**: Inoculate the organism in pure culture on blood agar plate in straight line and incubate for 24 hours at 37 °C. If there is greenish narrow zone around the colony, it is alpha hemolytic. Clear zone indicates beta hemolysis and no zone indicates gammahemolysis.

* **Sugar fermentation tests**: The organisms are inoculated in the sugar fermentation media (sugar + peptone water) (Apyendix). The fermentation of sugar is indicated by development of pink colour in the sugar tube. The development of gas is detected by placing Durham's tube in the sugar tube. The sugars which are generally used for fermentation tests are glucos, lactose, fructose, mannose, mannitol, dulcitol, sucrose, rhamnose, etc.

Fungi

Skin scrapings or suspected clinical samples are inoculated on Sabouraud's dextrose agar containing anti-bacterial dmgs and cycloheximide in order to prevent the growth of bacteria and saprophytic fungi. Media may be placed in plates or tubes.

Incubate the culture media at room temperature for several days and examine ths daily for fungal growth.

* *Trichophyton*: It takes 7-10 days to grow on Sabouraud's dextrose agar. Colonies are whte which become tanned after sometime and have a granular pigment. Smear made from the colonies may show the presence of numerous microconidia with some macroconidia; these canbe stained with lactophenol cottonblue whichenhances the visibility of fungus.

* *Microsporidium*: It grows well in 3-4 days and produces flat colony, white or yellow. Microscopically, few microconidia and numerous macroconidia seen; macroconidia are large, spindle shaped, multiseptate, thick walled bodies having a knob at the tip.

* *Candida*: It produces soft, cream coloured colonies, with yeasty odour.

* **Blastomyces**: White colonies which turn brown after some time. Branching septate hyphae containing lateral oval conidia.

* *Cryptococcosis*: Thick walled, oval to spherical budding cells surrounded by gelatinous capsule, growthin 10-15 days, colonies are white, glistening, mucoid, which turn brown with age.

* *Histoplasma*: On Sabouraud's dextrose agar, it produces white, cottony colonies which become brownish after some time. Branclung septate hyphae having microconidia; in older cultures macroconidia are seen.

* *Coccidioids*: It produces fluffy white colonies on Sabouraud's dextrose agar. The hyphae contain rectangular arthrospores, which float in the air. It should be grown only in sophisticated laboratories because there is danger of air trasmissin.

Virus

The virus can be cultured in diagnostic laboratories, but it requires trained and specialized personel. The culture of virus can be done in embryonated chicken eggs or in cell culture systems.

Chicken Eggs

The clinical samples (feces/ tissues/secretion/excretion) are collected in sterile containers. These are processed in order to prepare bacteria free filtrate for inoculation in chicken eggs or cell culture. For this, prepare 1:5 or 1:10 suspension of clinical samples in HBSS, which is centrifuged at l0000X for 30 min at 4°C. The supernatant is collected and filtered through EK grade Seitz filter. If the material is less in quantity (5-10 ml), it can be filtered through nitrocellulose membrane filter having 0.22 micrometer pore size. The filtrate is stored at -20°C till use. It can be inoculated (0.1ml) in embryonated chicken eggs through following routes:

- ✓ Chorioallantoic membrane (CAM)
- ✓ yolk
- ✓ Allantoic cavity

The eggs are inoculated under aseptic conditions preferably in laminar flow or sterile hoods. The inoculated eggs are incubated for 2-5 days at 37°C in incubator. During incubation, the eggs are seen daily through candling and dead embryos (within 24 hrs) are discarded. The growth of virus on CAM is observed by appearance of tuckering, congestion, hemorrhage, pock lesions, and necrosis. The allantoic or yolk sac fluid is examined for the presence of virus by using indirect tests like HA or Hl. The growth of embryos and lesions on various organs of embryo are also observed.

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Cell culture

The cell cultures are primary or continuous cell lines. The primary cell culture can be prepared from chicken fibroblasts, calf kidneys, calf tests, lamb kidneys and lamb testes. For this the tissues or organs are collected under sterile conditions in HBSS. These are cut into small pieces with sterile scissors and forceps. The cell suspension is kept on magnetic stirrer with a bar after addition of trypsin which dissociates the cells. After stirring, the cell suspension filtered through gauze or muslin cloth and the cells are washed with HBSS three times and one time with culture medium (Eagles MEM or medium 199) having 10% fetal calf serum.

A 10% cell suspension is prepared and is poured in cell culture bottles. The bottles are incubated at $37^{0}C$ to form a sheet on flat surface of glass bottle; it is ready within 2-3 days. Check the cell culture for contamination. After completion of sheet, clinical sample can be inoculated on the cell culture. The presence of virus in cell culture can be detected by appearance of cytopathic effects, including necrosis, desquamation, aggregation, syncytia formation, plaques and presence of inclusions in the cell. The growth of virus is also observed by immunofluorescent-staining, immunoperoxidase staining and cell culture fluid can be examined for the presence of virus by ELISA, DIA or CIEP. Electromicroscopically, one can demonstrate the virus in cells or in cell culture fluid.

Continuous Cell Lines

These are immortal cancerous cells, which grow fast if proper nutrition is provided. Now a days, these cells are available comrnercially or in some developed institutes which supply them on request free of cost or with some nominal charge. The cells are maintained and cultured in same way as in primary cell culture. The relevant details of media and/or other growth requirements are supplied with the cells which should be followed for their culture.

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