18/MHS06/052
M.L.S
MCB
ANSWERS

1.Streak-stab technique
Often when inoculating a BAP to observe hemoloysis patterns, investigators will also stab several times through the agar using an inoculating loop. This stab allows for the detection of streptolysin O, a specific hemolysin produced by Streptococcus pyogenes. This hemolysin is inactivated by O2 and is only seen subsurface (in an anaerobic environment) around the stab mark. Note the oval-shaped areas of clearing around the stab marks in the picture below; these are caused by streptolysin O.
Mannitol Salt Agar (MSA)

This type of medium is both selective and differential. The MSA will select for organisms such as Staphylococcus species which can live in areas of high salt concentration (plate on the left in the picture below). This is in contrast to Streptococcus species, whose growth is selected against by this high salt agar (plate on the right in the picture below).

The differential ingredient in MSA is the sugar mannitol. Organisms capable of using mannitol as a food source will produce acidic byproducts of fermentation that will lower the pH of the media. The acidity of the media will cause the pH indicator, phenol red, to turn yellow. Staphylococcus aureus is capable of fermenting mannitol (left side of left plate) while Staphylococcus epidermidis is not (right side of left plate).

Oxidase Test

This test is used to identify microorganisms containing the enzyme cytochrome oxidase (important in the electron transport chain). It is commonly used to distinguish between oxidase negative Enterobacteriaceae and oxidase positive Pseudomadaceae.

Cytochrome oxidase transfers electrons from the electron transport chain to oxygen (the final electron acceptor) and reduces it to water. In the oxidase test, artificial electron donors and acceptors are provided. When the electron donor is oxidized by cytochrome oxidase it turns a dark purple. This is considered a positive result. In the picture below the organism on the right (Pseudomonas aeruginosa) is oxidase positive.

Coagulase test

Coagulase is an enzyme that clots blood plasma. This test is performed on Gram-positive, catalase positive species to identify the coagulase positive Staphylococcus aureus. Coagulase is a virulence factor of S. aureus. The formation of clot around an infection caused by this bacteria likely protects it from phagocytosis. This test differentiates Staphylococcus aureus from other coagulase negative Staphylococcus species.

Taxos A (bacitracin sensitivity testing)

This is a differential test used to distinguish between organisms sensitive to the antibiotic bacitracin and those not. Bacitracin is a peptide antibiotic produced by Bacillus subtilis. It inhibits cell wall synthesis and disrupts the cell membrane. This test is commonly used to distinguish between the b-hemolytic streptococci: Streptococcus agalactiae (bacitracin resistant) and Streptococcus pyogenes (bacitracin sensitive). The plate below was streaked with Streptococcus pyogenes; notice the large zone of inhibition surrounding the disk.

Taxos P (optochin sensitivity testing)

This is a differential test used to distinguish between organisms sensitive to the antibiotic optochin and those not. This test is used to distinguish Streptococcus pneumoniae (optochin sensitive (pictured on the right below)) from other a-hemolytic streptococci (optochin resistant (Streptococcus mitis is pictured on the left below)).

Urease test

This test is used to identify bacteria capable of hydrolyzing urea using the enzyme urease. It is commonly used to distinguish the genus Proteus from other enteric bacteria. The hydrolysis of urea forms the weak base, ammonia, as one of its products. This weak base raises the pH of the media above 8.4 and the pH indicator, phenol red, turns from yellow to pink. Proteus mirabilis is a rapid hydrolyzer of urea (center tube pictured here). The tube on the far right was inoculated with a urease negative organism and the tube on the far left was uninoculated.

Motility agar

is a differential medium used to determine whether an organism is equipped with flagella and thus capable of swimming away from a stab mark. The results of motility agar are often difficult to interpret. Generally, if the entire tube is turbid, this indicates that the bacteria have moved away from the stab mark (are motile). The organisms in the two tubes pictured on the right are motile. If, however, the stab mark is clearly visible and the rest of the tube is not turbid, the organism is likely nonmotile (tube pictured on the left).

Spirit Blue agar

This agar is used to identify organisms that are capable of producing the enzyme lipase. This enzyme is secreted and hydrolyzes triglycerides to glycerol and three long chain fatty acids. These compounds are small enough to pass through the bacterial cell wall. Glycerol can be converted into a glycolysis intermediate. The fatty acids can be catabolized and their fragments can eventually enter the Kreb ' s cycle. Spirit blue agar contains an emulsion of olive oil and spirit blue dye. Bacteria that produce lipase will hydrolyze the olive oil and produce a halo around the bacterial growth. The Gram-positive rod, Bacillus subtilis is lipase positive (pictured on the right) The plate pictured on the left is lipase negative.

Simmon's Citrate Agar

This is a defined medium used to determine if an organism can use citrate as its sole carbon source. It is often used to differentiate between members of Enterobacteriaceae. In organisms capable of utilizing citrate as a carbon source, the enzyme citrase hydrolyzes citrate into oxaoloacetic acid and acetic acid. The oxaloacetic acid is then hydrolyzed into pyruvic acid and CO2. If CO2 is produced, it reacts with components of the medium to produce an alkaline compound (e.g. Na2CO3). The alkaline pH turns the pH indicator (bromthymol blue) from green to blue. This is a positive result (the tube on the right is citrate positive). Klebsiella pneumoniae and Proteus mirabilis are examples of citrate positive organisms. Escherichia coli and Shigella dysenteriae are citrate negative.

2• Staining techniques

Gram Staining

Gram stain is a key starting point to identify microbial species.The stain differentiate mem- brane structures between gram-positive and gram-negative microorganisms. Gram-positive microbes have a thick cell wall made up of pep-tidoglycan(50–90%),which are stained purple by crystal violet,where as gram-negative microbes have a thinner layer(10% ofcelwal), which are stained pink by the counter-stain safranin[24,30,33–36].

1.Apply two drops of crystal violet on smear for 30s.
2.Wash with tap water.
3.Add two drops of Gram's iodine for 30s.
4.Repeat step2.
5.Add 95% ethanol.
6.Repeat step2.
7.Add two drops of safranin.
8.Repeat step2.
9.Observe in microscope under oil immersion.
10.Yeasts are gram-positive,but poorly stained; Cryptococcus neoformans is a notable exception(gram-negative).

Giemsa Staining

A variety of "Romanowsky-type" stains with mixtures of methylene blue and azure eosin compounds have been used successfully for many years on diverse fungi with various procedures and modifications. Giemsa stain is a member of the Romanowsky group of stains, which are defined as being the black precipitate formed from the addition of methanol[37]. In this stain, eosin ions are negatively charged and stain basic components of cells orange to pink.It was also originally designed to incorporate cytoplasmic (pink)staining with nuclear (blue) staining and fixation as a single step for smears and thin films.This stain has widely been used to examine Pneumocystis jiroveci, Rhinosporidium seeberi,and Histoplasma capsulatum [38–40].

1.Flood the smear with methyl alcohol and leave fo r3–5min for fixation. 2.Add prepared Giemsa stain and leave for 45min.
3.Wash slide thoroughly with running tap water.
4.Blot dry with absorbent paper.
5.Observe under oil immersion. 6.Look for intracellular budding yeasts;fungi stain with

purplish-blue.

Wright Staining

The Wright stain is an alcoholic solution of methylene blue, azureA, thiamin, and eosinY. Methyl groups are activated and react with charged components of the cell to produce coloration.It is used to detect blood parasites, viral and chlamydial inclusion bodies, yeast cells, and species of Pneumocystis. Eosin ions are negatively charged and stain basic components of cells orange to pink, where as other dyes stain acid iccelstructures to various shades of blue to purple

1.Cover the smear with freshly filtered Wright stain and leave for 1–3min.

2.With out removing the stain, pour on buffer solution(pH) Gently mix buffer and stain;upon proper mixing, metallic green sheen(green scum)rises to the surface of the fluid.

4.Leave for 3min or longer.

5.Wash the slide gently with flowing tap water and wipe the bottom of the slide with a clean filter paper.

6.Air dry the slide and observe under the microscope.

7.Intracellular yeast cells are typically stain blue and species of Pneumocystis stain purple.

2b

Identification techniques

Wet Mount Techniques

Fungal specimens can be visualized using wet mount techniques through suspension of culture in either water or saline, mixed with alkali to dis- solve background material [21] or mixed with a combination of alkali and contrasting dye (e.g., lactophenol cotton blue or India ink) [22, 23].

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The dyes nonspecifically stain the fungal mate- rial, which increases contrast with the background and permits examination of the detailed structures. A variation is the India ink method, in which the ink darkens the background rather than the fungi.

Potassium Hydroxide Wet Mount

Potassium hydroxide (KOH) is used to dissolve proteinaceous material and facilitate detection of fungal elements that are not affected by strong alkali solution. It is a strong alkali used as a clear- ing agent to observe fungi in a wet mount preparation. The concentration of KOH is usually based on the specimen that is being used. Normally, 10–20% KOH is used; occasionally, 40% is used when the specimen is not cleared by 10–20% of KOH. In this method, the fungal structures, such as hyphae, large yeasts (Blastomyces), spherules, and sporangia, are well distinguished. In unstained preparations (KOH without stain), the fungal structures may be enhanced by using a phase contrast microscope [18, 24]. The clearing effect throughout the specimen can be accelerated by gently heating the KOH preparation.

Visualization of fungi can be further enhanced by the addition of dyes to the preparation. This method is quick, simple, and inexpensive [25, 26].

1. Place a large drop of KOH solution with a

Pasteur pipette.

2. Transfer small quantity of the culture with a

loop or the tip of a scalpel into the KOH drop.
3. Put a clean coverslip over the drop gently so
that no air bubble is trapped.
4. Clearing can be hastened by gentle heating of
the slide, but it is best avoided.
5. Observe under 20× and 40× objective of light
or phase contrast microscope.
6. Look for budding yeast cells; branching
hyphae; type of branching; and the color, separation, and thickness of hyphae (see Note 12).

Lactophenol Cotton Blue Wet Mount

Lactophenol cotton blue (LCB) is a mounting medium commonly used in microbiology laboratories for preparing mounts of fungal cultures. LCB is used as both mounting fluid and stain. In this method, phenol will kill the organisms, and
the lactic acid preserves fungal structures; chitin in the fungal cell wall is stained by the cotton blue. It can be used alone or in conjunction with KOH. Library slides may be made by allowing the mount to dry for 3 weeks and then sealing with collodion [24, 27, 28].

1. Put a large drop of LCB with a Pasteur pipette.
2. Transfer a small quantity of the culture to the drop.
3. Tease the culture well with teasing needles, so as to get a uniform spread.
4. Put on a coverslip gently to avoid entrapment of air bubbles.
5. Examine under the 20× and 40× objectives of light microscope.
6. Observe the morphological features carefully. 7. Fungal elements will stain deep blue against a clear pale-blue background.

India Ink Wet Mount

India ink can be added to specimens to provide dark background that will highlight hyaline yeast cells and capsular material. This method is used to detect microorganisms that are surrounded by capsules. The dye is excluded by the capsule, cre- ating a clear halo around the yeast cell. It is a rapid method for the preliminary detection and identification of specimens containing species of Cryptococcus [29].

1. Add a small drop of India ink on a smear.
2. Place a coverslip over the smear and press it
gently to obtain a thin mount.
3. If India ink is too thick (dark), dilute it by 50%
with saline.
4. Allow the preparation to stand for few minutes
to settle.
5. Scan under low power in reduced light; switch
to high power, if necessary.
6. Organisms possessing a capsule appear highly
retractile, surrounded by a clear zone against a dark background