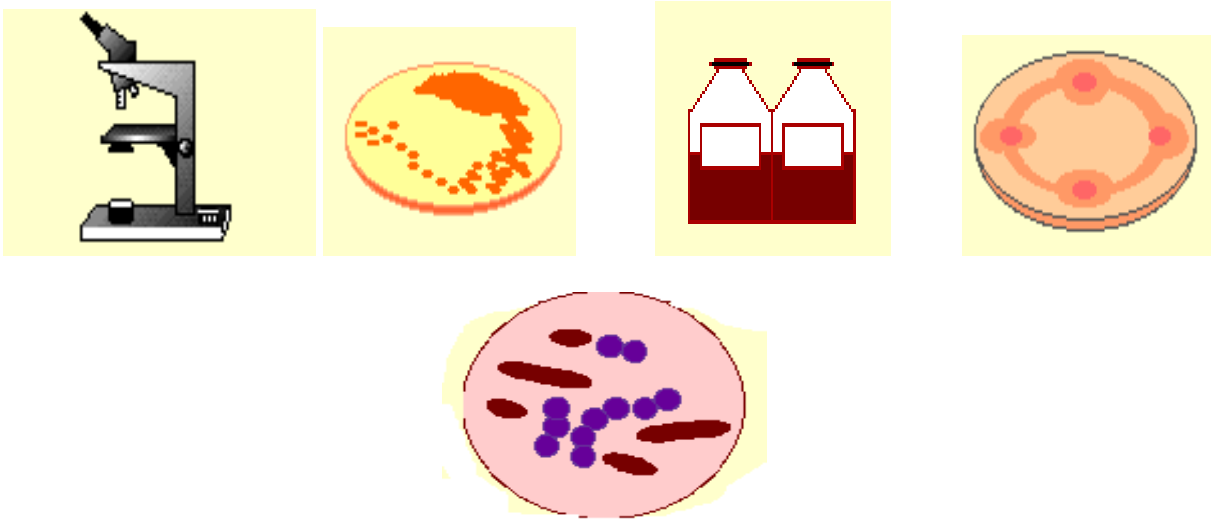
KING ABDUL AZIZ UNIVERSITY
FACULTY OF APPLIED MEDICAL SCIENCES
MEDICAL LABORATORY TECHNOLOGY

MLT 303

Diagnostic Microbiology Laboratory Manual



Student Name: _____

Computer NO.: _____

CONTENTS

BACTERIOLOGY PRACTICAL

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Cultur
unkno
Do **NO**

Briefcase and clothing must never be placed on the bench, or on the floor near the bench where they may cause accidents.

A laboratory coat should be worn whilst in the laboratory, and may be left there at the end of a class.

Hand-to-mouth transmission of infection

The hands may become contaminated with pathogenic bacteria whilst handling specimens or cultures, or merely by contact with a previously contaminated bench. Therefore NO materials should be conveyed from hand to mouth whilst in the laboratory.

(1) Do not eat, drink or smoke.
(2) Do not suck a pen or pencil.
(3) Do not pipette cultures, chemicals or even distilled water by mouth.

The hands should be washed immediately, if consciously contaminated with a known pathogenic organism.

At the end of each class, the hands should be washed thoroughly with detergent and water before leaving the laboratory.

Accidents

In the event of a laboratory accident, such as spilling or dropping a live culture, remain calm and do the following:

(1) Report the accident to your instructor as soon as possible.
(2) Place paper towels over the spilled material.
(3) Pour disinfectant liberally over the towels.
(4) After 15 minutes, remove and dispose of the towels in the receptacle used for the disposal of contaminated materials.

Disposal of contaminated material

A container of disinfectant is provided on the bench for the disposal of Pasteur pipettes and microscope-slides.

Bacterial cultures or specimens that are no longer required should be left on the bench.

Sampling and Transfer of Bacterial Cultures

The wire-loop

Nichrome or platinum wire is recommended. The wire should be perfectly straight and in line with the handle. The loop at the end of the wire must form a complete circle so that it can withdraw a sample from a liquid-culture.

For sterilization of the loop, the air-control of the Bunsen burner should be adjusted to give a flame with a short, blue, central cone. With the aluminum handle held as nearly vertical as possible without burning the fingers, the loop should be placed in the hottest part of the flame (just above the blue cone) until the whole length of the wire is heated to redness. Such flaming ensures complete sterilization of the wire and the chuck of the handle. The loop should now be allowed to cool thoroughly before use, or the bacteria that it touches will be killed.
The recently sterilized loop may be used for transferring bacteria from a clinical specimen, or previously grown culture, to a glass-slide for microscopic examination, or to fresh culture medium. After use, the loop should be re-sterilized and allowed to cool before it is put down.

Disposable sterile plastic inoculating loops are in use.

Pipettes

They may be used for transferring larger amounts of liquid-culture than can be held in the wire-loop. (e.g. Pasteur pipette, plastic pipette).

Screw-capped containers for bacterial cultures

Bacterial specimens and cultures may be kept in either plugged test tubes or screw-capped bottles. The latter have the advantage that, when the caps (with their rubber liners) are screwed on tightly, the content of the bottle do not dry or spill. The bottles most commonly used are:

a. “Bijou", holding about 7 ml.
b. “universal", holding about 25 ml.
c. “medical flat” of various sizes, holding 50 ml., 100 ml., etc.

The most satisfactory way to remove and hold the cap is by **firm pressure between the little finger and palm** of the right-hand (left-hand if left-handed), so that the other fingers and thumb are free to manipulate a loop or pipette which can then transfer material to or from the opened bottle.

As on all occasions when bacterial specimens or cultures are exposed, this procedure should be carried out beside a Bunsen flame, so that a current of air is flowing upwards, and particles containing bacteria or fungi are then less likely to fall into the open bottle. Similarly, the breath should be held, so as to avoid contamination of the cultures with droplets from the upper respiratory tract.

Hanging-Drop Technique
(Motility test)

Purpose
To determine bacterial motility.

Principle
Bacteria move by means of flagella. Flagellar stains are available for this determination but are not commonly used in clinical laboratories. Bacterial motility can be observed under a microscope using hanging drop technique, which allows the examination of unstained living bacteria under higher magnification without danger of lowering the objectives onto the contaminated drop.

Materials

Supplies

1. Microscope slides
2. Inoculating loop
3. Bunsen burner
4. Cover slip
5. A lead pencil for labeling the slide
6. Vaseline

Cultures

Positive control of bacterial suspension.
Negative control of bacterial suspension.
Unknown 1, unknown 2, and unknown 3 of bacterial suspension.

Method

1. With a piece of cloth (or your laboratory coat) remove every trace of dust and grease from a microscopic-slide and a cover-slip, and place the latter flat on the bench.
2. Apply a little Vaseline to the four corners of the cover-slip.
3. With a recently sterilized (and cooled) loop, transfer a loopful of liquid-culture to the center of the cover-slip.
4. Place the microscopic-slide over the cover-slip so that contact is made with the Vaseline but not with the droplet of culture.
5. Invert the preparation smoothly but quickly, so that the droplet of culture hangs below the cover-slip, but is prevented from touching the slide by the depth of the Vaseline at the corners.

Examination under the microscope

The high-power (dry) objective lens provides adequate magnification for this purpose.

1. Turn the 4mm. (dry, high-power, x 40) objective lens into position.
2. Place the hanging-drop specimen on the stage so that the droplet is clearly under the objective lens whether viewed from the back of the stage or from the side.
3. With the eye at stage-level (and viewing the stage from the side), raise the stage by the coarse adjustment until the objective lens is as near as possible to the cover-slip without actually touching it.
   (With some microscope, focus-adjustments are made by lowering the body, rather than by raising the stage of the microscope. Otherwise, the above details apply).
4. Lower the sub-stage condenser to about 1 cm. below its highest position, and open its diaphragm fully.
5. Looking down the microscope through the ocular lenses, rack the stage downwards (or raise the microscope body) by the fine adjustment until the bacteria are seen. A clearer image may now be obtained by further adjustment to the level of the sub-stage condenser and by closing the diaphragm as far as possible without reducing the brightness of the image.

   You must be careful to distinguish between true motility (cells exhibit independent movement over greater distances, particularly as opposed to the flowing movement of all bacteria within a field) and Brownian motion (all cells appear to vibrate in place).

   Confusion, in interpretation of the image, commonly results from grease or dust on the ocular lens. This may be detected by determining whether the image rotates as the ocular lens is rotated in its sleeve. If so, the surfaces of the lens should be cleaned with the soft lens-tissue provided.

Results:

Test	Positive control	Negative control	Unknown 1	Unknown 2	Unknown 3
Motility					

Streak Plate Technique
(Plating out)

Purpose

To obtain well-isolated colonies of bacteria.

Materials

Supplies

1. Microscope slides
2. Inoculating loop
3. Bunsen burner
4. Marker for plate labeling
5. Blood agar plates
6. A lead pencil for labeling the slide

Cultures

Mixed broth cultures of *Staphylococcus epidermidis* and *Escherichia coli*.

Method:

1. Label the bottom (containing the culture medium) of the Petri plate.
2. Hold the plate so that the bottom rests on the palm of the left hand (if right-handed). Left the cover (lid) of the plate and streak the inoculum (e.g. an eye swab, or a loopful of a previously grown culture) over a small segment of the agar surface (from side-to-side in parallel lines).
3. Re-sterilize the loop and allow it to cool thoroughly. Use this to spread the inoculum on the first segment over a second segment.
4. Repeat this process over a total of four or five segments, re-sterilize the loop and allowing it to cool after each segment has been inoculated.
   Flame the inoculating loop before putting it down. Incubate the plate in an inverted position.

(Flaming the loop between streaking area is not necessary for most clinical specimens)

Results:

Isolated colonies	Description
S. epidermidis	
E. coli	

Gram's staining Technique

Purpose

To distinguish between Gram-positive and Gram-negative bacteria.

Principle

Most bacteria can be differentiated by their Gram reaction due to differences in their cell wall structure. Those organism are called Gram-positive which after being stained dark purple with crystal violet are not decolorized by acetone or ethanol. Those organism are called Gram-negative which after being stained with crystal violet lose their color when treated with acetone or ethanol, and stain red with carbol fuchsin or other red counterstain.

Materials

Supplies

1. Microscope slides
2. Inoculating loop
3. Bunsen burner
4. Marker for plate labeling
5. A lead pencil for labeling the slide

Reagents

1. Crystal violet reagent
Crystal violet ---------- 20g
Ammonium oxalate---------- 9g
Ethanol or methanol, absolute---------- 95ml
Distilled water---------- 1 litter

2. Lugol's iodine
Potassium iodide---------- 20g
Iodine---------- 10g
Distilled water---------- 1 litter

3. Aceton-alcohol decolorizer
Acetone---------- 5 ml
Ethanol or methanol absolute---------- 475 ml
Distilled water---------- 2

4. Carbol Fuchsin
Basic fuchsin----------10g
Ethanol or methanol, absolute----------100 ml
Phenol ---------- 50 g
Distilled water---------- to 1 litter

5. Tab water

Cultures

- *Staphylococcus aureus* grown on blood agar
- *Escherichia coli* grown on blood agar
- Mixed broth culture of *Streptococcus pyogenes* (ATCC 19615) and *E. coli* (ATCC 25922) as positive and negative control

Methods

1. Clean a microscopic-slide, pass it through the hot portion of the flame (to remove any grease that may be present) and place it flat on the bench.
2. For liquid cultures use a sterile loop to transfer a loopful of broth to the center of the slide and allow it to remain on the smallest area possible. (Indeed, if the liquid culture is not very obviously turbid, it may be advantageous to place two or more loopfuls on the same small area of the slide).
3. For culture on solid media, use a sterile loop to transfer a loopful of saline to the center of the slide. Re-sterilize the loop and allow it to cool. With it, transfer a small portion of colony from the solid medium to the drop of saline, mix and spread it over a circular area (about 12 cm in diameter).
4. Allow the smear to air dry completely.
5. Fix the smear by rapidly passing the slide, (smear uppermost), three times through the flame of a Bunsen burner.
6. Allow the smear to cool before staining.
7. Mark the position of the smear by scratching the glass, with a lead Pencil or with the edge of another slide, opposite to the center of the smear (for easy locating and focusing the smear when it ready for microscopic examination).
8. Cover the smear with crystal violet reagent for 1 minutes.
9. Wash the slide with tap water (slowly running water).
10. Apply Lougols iodine for 1 minute.
11. Wash with tap water.
12. Decolorize rapidly (few seconds) with acetone-alcohol.
13. Wash with tap water.

Caution:
The acetone-alcohol solution is highly flammable. Use well away from an open flame.

14. Cover the smear with carbol fuchsin (counterstain) for 1 minute.
15. Wash with tap water and gently blot with paper towels (do not rub).
16. Examine the smear microscopically, first with the 40x objective to check the staining and to see the distribution of materiel, and then with the oil immersion objective to look for bacteria and cells.

Expected Results

- Gram-positive bacteria …… Dark purple
- Gram-negative bacteria …….. Pink to dark red

Student's Result

Organism	Appearance on BA	Gram stain reaction and morphology
Positive control *Streptococcus pyogenes*		
Negative control *E. coli*		
Staphylococcus aureus		
Mixed broth culture		Colony type 1: 2:

Catalase Test

Purpose
To differentiate between staphylococci (catalase +ve) and streptococci (catalase -ve) species.

Principle
Catalase is an enzyme which catalyses the rapid breakdown of hydrogen peroxides to water and oxygen. When a small amount of an organism that produces catalase (e.g. staphylococci) is emulsified in a drop of 3% hydrogen peroxide, rapid elaboration of bubbles of oxygen is produced. No bubbling is considered a negative result.

Materials
Supplies
1. Pipette 2. Wooden sticks 3. Slides

Reagent
3% (v/v) hydrogen peroxide

Cultures
Stock culture of *Staphylococcus aureus* (positive control)
Stock culture of *Streptococcus pyogenes* (negative control)
Unknown 1 and unknown 2 (colonies on blood agar).

Method
1. With a Pasteur pipettes put one drop of 3% hydrogen peroxide onto the slide.
2. With a wooden applicator stick (do not use a wire loop) quickly place one colony onto hydrogen peroxide and observe whether gas is produced as indicated by the appearance of bubbles.

Note
Catalase is present in erythrocytes and care must be taken to avoid carry over of red blood cells with the colony if it must be selected from a medium containing RBCs. Hydrogen peroxide is corrosive so do not allow contact with skin, if it splashes on skin wash immediately in lots of water.

Expected result
- Gas production : catalase positive
- No gas production : catalase negative

Student's results
Organism	Catalase Test
S. aureus	
S. pyogenes	
Unknown 1	
Unknown 2	

Coagulase Test

Purpose

To distinguish between the pathogenic *S. aureus* (coagulase +ve) and nonpathogenic coagulase negative staphylococci, such as *S. epidermidis* and *S. saprophyticus*.

Principle

Coagulase may be "free" or "bound". The slide test detects bound coagulase (clumping factor), that reacts with fibrinogen causing aggregation of the organisms. The tube test detects the free coagulase, (a thrombin-like substance present in culture filtrates), which activates prothrombin initiating clot formation in the plasma.

S. aureus produces protein A which has a specific affinity for the FC moiety of the immunoglobulin IgG. With the staphaurex kit the reaction of clumping factor and/or of protein A (present in a suspension of *S. aureus*) with latex particles which have been coated with fibrinogen and IgG against protein A causes rapid, strong agglutination of the latex particles.

Materials

Supplies

1. Card
2. Wooden sticks

Reagents

Staphaurex kit by Wellcome.

Cultures

Stock Culture of *S. aureus* (coagulase positive)
Stock Culture of *S. epidermidis* (coagulase negative)
Unknown 1 and unknown 2 (colonies on blood agar)

Method (also refer to manufacturer's package insert)

1. Shake the latex reagent to obtain an even suspension after it has reached room temperature.
2. Dispense one drop in circle on reaction card for each culture to be tested.
3. Select a colony by touching with a wooden stick, emulsify the culture in the latex drop and mix gently.
4. Rotate the card for up to 20 seconds and examine for agglutination.
5. Dispose of the card into disinfectant.

Expected results

- Clearly visible clumping of the latex particles indicates a positive result.

Student's result

Organism	Coagulase Test
S. aureus	
S. epidermidis	
Unknown 1	
Unknown 2	

Oxidase Test

Purpose

To identify bacteria that contains the respiratory enzyme cytochrome *c* (oxidase enzyme) such as *Pseudomonas*, *Neisseria* and *Campylobacter*.
Mainly used to differentiate between *Enterobacteriaceae* (oxidase –ve) and *Pseudomonas* (oxidase +ve)

Principle

The oxidase enzyme is able to oxidize the colorless reagent (tetramethyl-*p*-phenylenediamine dihydrochloride), forming an end product (indophenol) with dark purple color.

Materials

Supplies

1. Filter paper
2. Pipette
3. Wooden sticks

Cultures

Stock culture of *Pseudomonas aeruginosa* (positive control)
Stock culture of *Escherichia coli* (negative control)
Unknown 1 and unknown 2 (colonies on blood agar)

Reagent

Oxidase (Tetramethyl-*p*-phenylenediamine dihydrochloride)

Method

1. Place a few drops of 1% of the oxidase reagent on a filter paper.
2. With a wooden stick (do not use a wire loop), smear a test colony across the wet filter paper.
3. Observe any change in color within 10 seconds.
4. Discard the filter paper and stick into disinfectant.

Expected result:

- Positive: deep purple color appears within 10 seconds
- Negative: colorless

Student's results

Organism	Oxidase Test
Ps. aeruginosa	
E. coli	
Unknown 1	
Unknown 2	

Sterilization and Disinfection

Purpose

1. To evaluate the sterility of canned food, fruit juice, apple cider, fresh milk, long life milk, and laban.
2. To evaluate the sterility of skin before and after decontamination by alcohol or iodine and also to evaluate the sterility of hands before and after washing with soap.
3. To demonstrate how to deal with a blood spill.
4. To demonstrate the design of autoclaves and explain their role in sterilization.
5. To demonstrate the use of autoclave tape spore-containing indicators in assessing the performance of autoclaves
6. To demonstrate the design of class I, II, III safety cabinets
7. To explain the classification of containment facilities as P1, P2, P3, P4

Materials

Supplies

1. Microscope slides
2. Inoculating loop
3. Bunsen burner
4. Marker for plate labeling
5. A lead pencil for labeling the slide
6. Canned food, apple juice, cider, milk, laban
7. Sterile swab
8. Blood
9. Blood a gar plates
10. Antiseptic solution
11. Alcohol swab
12. Iodine
13. Autoclave tape
14. Spore strips or spore vials

Reagents

Gram stain set reagents

Method:

1**. Sterility of canned food and drinks**

Transfer a small amount of canned food, fruit juice, apple cider, milk or laban in to blood a gar plate then streak.

2**. Sterility of skin**

a. Swab a selected area of the skin, then streak it in one part of blood agar plate.
b. Decontaminate the same area with an alcohol swab or iodine and again swab the decontaminated area and streak on the other part of blood a gar plate.

3**. Sterility of hands**

a. Touch one part of a blood agar plate with your bare fingers (two or more).
b. Wash your hands with soap and water and dry them with clean tissue. Touch the other part of the same blood agar plate with the same, washed, fingers.
c. Incubate all plates at 37^{o} C for 24 hours and perform gram stain on plates showing growth.

4. **Blood spills**

Use colored liquid to imitate a blood spill on an area of the floor covered with a plastic sheet. Wearing gloves, put tissue paper, strong antiseptic over the spill and leave it for a few minutes then discard in disinfected solution. After that clean the area which the spill was on using antiseptic.

5. **Autoclave and sterility**

Examine biological and non-biological indicators that are used to evaluate the performance of autoclave. Distinguish between the colors of autoclaved and non-autoclaved spore vials containing growth indicators.

Student's results

Sample	Result (Growth or no growth)	Gram stain (if growth present)
Canned food		
Fruit juice		
Apple Cider		
Laban		
Fresh Milk		
Long-life Milk		
Skin swab with out decontamination		
Skin swab decontaminated with alcohol or iodine		
Hands before washing		

Identifications of Staphylococci

Purpose

- To recognize and describe the colonial and Gram stain morphology of Staphylococci.
- To identify *S. aureus*, *S. epidermidis* and *S. saprophyticus*.

Materials

Supplies

1. Microscope slides
2. Inoculating loop
3. Bunsen burner
4. Marker for plate labeling
5. A lead pencil for labeling the slide

Reagents

1. Gram stain set reagents
2. Catalase
3. Staphaurex kit by Wellcome
4. Novobiocin disc
5. DNase plates

Cultures

S. aureus, *S. epidermidis* and *S. saprophyticus* grown on blood agar.
Unknown 1, unknown 2, and unknown 3 grown on blood and mannitol salt agar.

Method

For each culture plate;

1. Describe the colonial morphology.
2. Perform Gram stain, catalase and slide coagulase.
3. Perform novobiocin test for coagulase –ve cultures.
4. Perform DNase test for coagulase +ve culture.

Student result

Test	Unknown 1	Unknown 2	Unknown 3
Colonial morphology on blood agar			
Gram stain morphology			
Catalase reaction			
Slide coagulase			
Novobiocin sensitivity			
DNase			

Identifications of Streptococci

Purpose

- To recognize and describe the colonial and Gram stain morphology of Streptococci.
- To identify Group A, B and D streptococci, viridans streptococci and *S. pneumoniae*.

Materials

Supplies

1. Microscope slides
2. Inoculating loop
3. Bunsen burner
4. Marker for plate labeling
5. A lead pencil for labeling the slide

Reagents

1. Gram stain set reagents
2. Catalase
3. Streptex kit (Streptococcal grouping)
4. Bacitracin disc
5. Optochin disc
6. Bile esculin plates

Cultures

Group A streptococci (*S. pyogenes*) on blood agar.
Group B streptococci (*S. agalactiae*) on blood agar.
Group D streptococci on blood agar and bile esculin.
Streptococcus pneumoniae on blood with optochin disc.
Viridans streptococci on blood agar.
Unknown 1, unknown 2, unknown 3 and unknown 4 on blood agar.

Method

1. Describe the colonial morphology for each culture plate.
2. Perform Gram stain (see page 5), catalase (see page 8) and Streptococcal Grouping (See below) for beta-haemolytic species.
3. Perform bacitracin susceptibility test for group A streptococci and bile esculin for group D streptococci.
4. Perform Gram stain, catalase and optochin test for alpha-haemolytic species.

Streptococcal Grouping (also refer to the manufacturer's package insert)

1. Dispense 0.4 ml Extraction Enzyme into an appropriately labelled tube for each culture to be grouped.
2. Make a light suspension of the streptococcus culture (by picking as few as 5 large colonies or by a single sweep of growth from an area that contains as few contaminants as possible) in the tube of the Extraction Enzyme.
3. Incubate the suspension at 37^{o}C in a water bath for a minimum of 10 min. (shake the tube after 5 min).
4. Resuspend each of the latex suspension by shaking vigorously for a few seconds and dispense one drop of each onto a separate circle on a reaction card.

5. Using a Pasteur pipette, place one drop of extract on each of the six circles on the reaction card.
6. Mix the contents in each circle (each with a separate stick) and spread to cover the complete area of the circle.
7. Rock the card gently for a maximum of one min. and examine for agglutination.
8. Dispose of the card into disinfectant.

Student result

Test	Unknown 1	Unknown 2	Unknown 3	Unknown 4	Unknown 5
Colonial morphology on blood agar					
Gram stain and morphology					
Haemolysis on blood agar					
Catalase reaction					
Streptococcal Grouping					
Bacitracin susceptibility					
Bile esculin					
Optochin					

Identifications of Enterobacteriaceae

Purpose

- To recognize and describe the colonial and Gram stain morphology for *E. coli*, *Klebsiella* spp,
  Proteus spp, *Salmonella* typhi and *Shigella* spp.
- To identify the above different species of Enterobacteriaceae.

Materials

Supplies

1. Microscope slides
2. Inoculating loop
3. Bunsen burner
4. Marker for plate labeling
5. A lead pencil for labeling the slide

Reagents

1. Gram stain set reagents
2. Oxidase
3. API 20 E analytical profile index
4. Serological

Cultures

E. coli on blood agar and MacConkey.
Klebsiella spp. on blood agar and MacConkey.
Proteus spp. on blood agar and MacConkey.
Salmonella typhi on XLD and DCA.
Shigella spp. on XLD and DCA.
Unknown 1, unknown 2, unknown 3, unknown 4 and unknown 5 on blood agar

Method

For each culture plate;

1. Describe the colonial morphology.
2. Perform Gram stain (see page 6) and oxidase test (see page 11).
3. Perform API test (see below) for oxidase –ve species.
4. Perform serological identification for *Salmonella* and *Shigella*.

API 20 E (also refer to the manufacturer's package insert)

1. Label the elongated flap of the API incubation tray with the number of the tested culture.
2. Distribute about 5 ml of water into the tray to create a humid chamber.
3. Remove the API strip from its sealed envelope and place it in the tray.
4. Aseptically prepare a suspension of the assigned culture by emulsifying a well-isolated colony in the suspension medium (or sterile distilled water).

5. Tilt the API incubation tray. Using a Pasteur pipette, fill the tube section of each compartment.
6. Fill both the tube and cupule section of the CIT, VP and GEL compartment.
7. Fill the cupules of ADH, LDC, ODC, URE and H2S tubes with sterile mineral oil to create anaerobiosis. Place the lid on the incubation tray and incubate at 37^{o}C for 18-24 hours.
8. To check for the purity of the suspension, inoculate a nonselective medium (nutrient agar) with one drop (or a loopfull) of the suspension and incubate overnight at 37C.
9. After incubation of the API, add one drop of each reagent as follows: (a) VP1 and VP2 reagents to VP; (b) TDA reagent to TDA; (c) IND reagent to IND; (d) NIT1 and NIT2 reagents to GLU.
10. Record your finding on the report sheet, using the identification table (or API chart) and identify the organism by the help of API 20E Analytical Profile Index.

Student result

Test	Unknown 1	Unknown 2	Unknown 3	Unknown 4	Unknown 5
Colonial morphology on blood agar					
Colonial morphology on MacConkey agar					
Colonial morphology on XLD agar					
Colonial morphology on DCA agar					
Gram stain and morphology					
Oxidase test					
API result					
Serotyping					

Identifications of Pseudomonas, Vibrios and Campylobacter

Purpose

- To recognize and describe the colonial and Gram stain morphology for *Pseudomonas aeruginosa*,
  Vibrio cholerae and *Campylobacter* spp.
- To identify the above different species.

Materials

Supplies

1. Microscope slides
2. Inoculating loop
3. Bunsen burner
4. Marker for plate labeling
5. A lead pencil for labeling the slide

Reagents

1. Gram stain set reagents
2. Oxidase
3. API 20 E analytical profile index
4. Serological

Cultures

Pseudomonas aeruginosa on blood a gar and MacConkey.
Vibrio cholera on blood agar and TCBS.
Campylobacter jejuni on blood agar and Campylobacter medium.
Unknown 1, unknown 2, and unknown 3 on blood agar.

Method

For each culture plate;

1. Describe the colonial morphology.
2. Perform Gram stain (see page 6) and oxidase test (see page 11).
3. Perform API test (see page 17) for oxidase –ve species.

Student result

Test	Unknown 1	Unknown 2	Unknown 3
Colonial morphology on blood agar			
Colonial morphology on Campylobacter agar			
Colonial morphology on TCBS			
Gram stain and morphology			

Identifications of Haemophilus influenzae

Purpose

- To recognize and describe the colonial and Gram stain morphology of *Haemophilus* spp.
- To identify *H. influenzae*, *H. parainfluenzae* and *H. ducreyi*.

Materials

Supplies

1. Microscope slides
2. Inoculating loop
3. Bunsen burner
4. Marker for plate labeling
5. A lead pencil for labeling the slide

Reagents

1. Gram stain set reagents
2. X, V and XV discs
3. Mueller-Hinton plates

Cultures

H. influenzae, *H. parainfluenzae* and *H. ducreyi* on chocolate agar.
H. influenzae on blood agar inoculated with haemolytic *S. aureus*.
H. influenzae on Mueller-Hinton agar with X, V and XV discs.
Unknown 1, unknown 2, and unknown 3 on chocolate agar.

Method

5. Describe the colonial morphology of *H. influenzae*.
6. Perform Gram stain (see page 6), catalase (see page 9) and oxidase test (see page 11).
7. Perform X and V discs identification.

Student's result

Test	Unknown 1	Unknown 2	Unknown 3
Colonial morphology on chocolate agar			
Gram stain and morphology			
X, V and XV results on Mueller-Hinton			

Identifications of Listeria and Corynebacterium

Purpose

- To recognize and describe the colonial and Gram stain morphology of *Listeria* and *Corynebacteria*.
- To identify *L. monocytogenes* and *Corynebacterium diphtheriae*.

Materials

Supplies

1. Microscope slides
2. Inoculating loop
3. Bunsen burner
4. Marker for plate labeling
5. A lead pencil for labeling the slide

Reagents

1. Gram stain set reagents
2. Catalase
3. Bile esculin plates

Cultures

L. monocytogenes on blood agar and bile esculin.
C. diphtheriae on blood agar.
Unknown 1, unknown 2, and unknown 3.

Method

For each culture plate;

1. Describe the colonial morphology.
2. Perform Gram stain (see page 6) and catalase test (see page 9).
3. Perform Bile esculin test.

Student's result

Test	Unknown 1	Unknown 2	Unknown 3
Colonial morphology on blood agar			
Colonial morphology on bile esculin agar			
Gram stain and morphology			
Haemolysis on blood agar			
Catalase reaction			

"Ziehl-Neelsen Staining Technique"

Purpose

To identify acid fast bacilli.

Principle

Certain bacteria, parasitic cysts and rare fungal forms, because of mycolic acid in their cell wall, retain the basic dye carbol fuchsin despite acid alcohol rinsing. This characteristic differentiates them from other bacteria and is an initial step in their identification.

Materials

Supplies

(All smears are already prepared and fixed in methanol under a biohazard hood)

- Unknown slides.
- Positive control slide.
- Negative control slide.

Reagents:

1. Carbol fuchsin

Basic fuchsin---------- 3g
Ethanol (90%-95%)---------- 10 ml
Phenol solution (5%)---------- 90 ml

2. Decolorizer

Ethanol (90%-95%)---------- 97 ml
Concentrated HCL---------- 3ml

3. Counter stain.

Methylene blue---------- 0.3 g
Distilled water---------- 100 ml

Method

1. Flood the slide with strong carbol-fuchsin and heat it from below with the Bunsen flame until you observe a slight steaming when the flame is removed. (do not boil and do not let stain dry on the slide). Allow the dye to act, with steam rising from it, for 5 minutes.
2. Wash the slide thoroughly with water.
3. Decolorize in 3% acid alcohol until no more color comes out of smear (approximately 20 min).
4. Wash the slide thoroughly with water.
5. Counterstain with methylene blue or 5% malachite green for 1 min.
6. Wash in water, allow to dry and examine using oil immersion objective.

Expected Results

- AFB: Red, straight or slightly curved rods, occurring singly or in small group.
- Other organism and cells: blue
- Background: blue

Student's Result

	Result
Positive control	
Negative control	
Unknown	

Identifications of Neisseria and Moraxella

Purpose

- To recognize and describe the colonial and Gram stain morphology of *Neisseria* and *Moraxella*.
- To identify *Neisseria gonorrhoeae* and *Moraxella catarrhalis*.

Materials

Supplies

1. Microscope slides
2. Inoculating loop
3. Bunsen burner
4. Marker for plate labeling
5. A lead pencil for labeling the slide

Reagents

1. Gram stain set reagents
2. Oxidase reagent
3. DNase plates

Cultures

N. gonorrhoeae on chocolate and Thayer-Martin agar.
M. catarrhalis on blood agar and chocolate.
Unknown 1 and unknown 2.

Method

For each culture plate;

1. Describe the colonial morphology.
2. Perform Gram stain (see page 6) and oxidase test (see page 11).
3. Perform carbohydrate utilization test.
4. Perform DNase test.

Student's result

Test	Unknown 1	Unknown 2	Unknown 3	Unknown 4	Unknown 5
Colonial morphology on blood agar					
Gram stain and morphology					
Hemolysis on blood agar					
Oxidase reaction					

Susceptibility test Using Disc Diffusion Method

Purpose

To determine antibiotic sensitivity patterns of pathogenic organisms.

Materials

Supplies

1. Sterile swab
2. Saline
3. Diagnostic sensitivity test agar plate
4. Rotator
5. A Macfarland standard turbidity tube of 0.5

Reagents

Cultures

S. aureus on blood agar (control for gram positive bacteria).
E. coli on blood agar (control for gram negative bacteria).
Unknown 1 on blood agar (test organism).

Method

1. Emulsify several colonies for the test organism; control for gram positive and control for gram negative.
2. Adjust the turbidity of the emulsion using Macfarland standard turbidity tube of 0.5.
3. Dip sterile cotton swab into the suspension. Run swab against inside of saline bottle to remove excess fluid.
4. Place the D.S.T plate into the rotator and switch on.
6. Place out the test organism in the center and control around the outer edge leaving gap between them the width of the line on the rotator.
7. Place the appropriate antibiotic sensitivity discs around the gap, between test and control organisms and incubate aerobically at 37 C for 24 hours.
7. Using a ruler measure the clear zone around each disc for the test and control organisms.

Expected results

- Sensitive: Same size or ≤ 3mm difference between control and test.
- Moderate: control zone - test zone ≥ 3mm.
- Resistant: the test zone size < 3

Student's result

Antibiotic	Result
Oxacillin	
Pencillin	
Erythromycin	
Clindamycin	
Vancomycin	
Fucidic acid	
Tetracyclin	
Gentamicin	
Rifampicin	

Virology Practical

Techniques of Tissue Culture
Virus Isolation in Tissue Culture

1. Draw the structure of an enveloped RNA virus with helical capsid symmetry.

Label all components.

2. Draw the structure of a naked DNA virus with icosahedral symmetry.

Label all components.

3. Under which families are the following viruses classified?

a. Influenza viruses: ----------

b. Rotaviruses: ----------

c. Measles virus: ----------

d. Rebella virus: ----------

e. Dengue virus: ----------

4. How many serotypes are recognized for the following viruses?

a. Parainfluenzaviruses: ----------

b. Polioviruses: ----------

c. Adenoviruses: ----------

d. Papillomaviruses: ----------

5. From electron microscopy photographs, determine the structure of the following viruses (capsid symmetry, envelope):

a. Herpesviruses

b. Adenovirus

6. Draw and label the steps of virus multiplication cycle for a typical RNA virus with positive polarity.

7. How does the multiplication of a DNA virus differ from the above?

8. How does the multiplication of an RNA virus with negative polarity differ from No.6?

9. What are the main constituents of tissue culture medium?

a.
b.
c.
d.
e.

10. What are types of cell lines used in virus isolation? Specifying the main advantages and disadvantages for each one?

11. What are the various steps of cell culture propagation (cell passage)?

12. What is the name of the safety cabinet used in routine virology laboratories?

How does it work?

13. What is meant by the following classification of safety levels?

BSL1. ----------

BSL2. ----------

BSL3. ----------

BSL4. ----------

14. What are the four different routes of inoculation of Embryonated eggs for virus isolation?

a. _____

b. _____

c. _____

d. _____

Demonstration of different cytopathic effects (CPE) caused by various viruses in tissue culture

1. Herpes Simplex Virus (HSV)

- Draw the CPE of (HSV) in Vero cell line. (Compare with uninfected cell-control)

2. Cytomegalovirus

- Draw the CPE of (HSV) in Vero cell line. (Compare with uninfected cell-control)

3. Measles virus

- Draw the CPE of (HSV) in Vero cell line. (Compare with uninfected cell-control)

4- Enterovirus

- Draw the CPE of (HSV) in Vero cell line. (Compare with uninfected cell-control)

Mycology Practical

Germ Tube Test

Purpose

To differentiate between *Candida albicans* (produce germ tube) and other *Candida* species.

Principle

A germ tube is a filamentous extension from a yeast cell that is about half the width and three to four times the length of the cell. The germ tube of *Candida albicans* has been described as having no constriction at the point of origin in contrast to those of *Candida tropicalis*, which characteristically are constricted.

Materials

Supplies

1. Microscope slides and cover slips
2. Test tubes and rack
3. Sterile Pasteur pipettes

Reagents

- Horse serum or Calf serum

Cultures

- Control organisms: *C. albicans* (*ATCC 60193) & *C. tropicalis* (ATCC 66029) maintained on Sabouraud dextrose agar at 30°C.
- Unknown organisms.

Method

1. To a labelled test tube, add 1 ml of horse serum.
2. Emulsify single colony of yeast from Sabouraud agar.
3. Incubate at 37°C for 2.5 to 3 hours.
4. Place a drop of the suspension on a microscope slide.
5. Place a cover slip over the suspension and examine under high power (x40) for the presence of a germ tube (observe a minimum of five germ tubes before calling the isolate positive).

Results

The presence of yeast cells (a minimum of five) with a lateral (tail-like) extension that have no constriction (septum) at point of attachment confirms the presence of *C. albicans*.

	Results
Positive control	
Negative control	
Unknown Sample	

Lactophenol Cotton Blue Mounts

I. Tease Mount Procedure

A tease mount is the most common and quickest technique used to mount fungi for microscopic examination. Since the mould's growth is teased apart with dissecting needles, conidia or spores may be dislodged from the conidiogenous or sporogenous cells. It may be necessary to use the slide culture technique if the identification cannot be made from the tease mount.

Procedure:

(1) Place a drop of lactophenol just off center on a clean microscope slide.
(2) With a long-handled inoculating needle, gently remove a small portion of growth midway between the colony center and edge. Place the material in the lactophenol.
(3) With two dissecting needles, gently tease the fungus apart so that it is thinly spread out in the lactophenol.
(4) Place a clean coverslip at the edge of the lactophenol and slowly lower it with a sharp pointed object.
(5) Avoid trapping air bubbles under the coverslip. Remove excess lactophenol from the edges of the coverslip by blotting with a paper towel or filter paper.
(6) Examine the slide under the microscope.

II. Scotch Tape Lactophenol Mount Procedure

The Scotch tape mount is an easy and fast procedure that is used for the identification of filamentous fungi since most structures will be intact for observation thank to the gummed side of the tape. As with the Lactophenol cotton blue mount, the organism will be immersed in the solution, rendering the organism safe for handling outside of the biological safety hood. Limitations include: the tape will dissolve eventually so that it is not to be used for permanent mounts; the procedure can only be performed on moulds growing from agar plates.

Procedure:

(1) Cut a strip of Scotch transparent tape and place one end between wood stick, gummed side out.
(2) Open Petri-dish plate with opposite hand and press tape against the fungal colony to identify.
(3) Place a drop of lactophenol on a labeled clean microscopic slide.

(4) Press tape against slide with Lactophenol and remove the wood stick.
(5) Smooth the tape back on the slide. (You can place another drop of lactophenol on the top of the tape and place a clean coverslip on top of slide).
(6) Examine the slide under the microscope.

	Results
Aspergillus spp.	
Penicillium spp	
Unknown Sample	

Study Questions

Laboratory Conduct - Safety

1. List the major routes of infection?
2. Describe the correct procedure to follow in case of spilling or dropping a live culture?
3. What is the universal precaution in working in the clinical la?
4. Name at least four personal protective equipments?

Sampling & transfer of bacterial cultures

1. Describe the importance of the following items in microbiology laboratory?
a) Wire-loop, b) Pipettes, c) Bijou, d) Petri-dish, e) Bunsen/Electric burner
2. Why you should flame the inoculating loop prior and after each inoculation?
3. Why you should cool the inoculating loop prior to obtain the bacterial inoculum?
4. Why you should flame the neck of the universal tubes immediately after uncapping and before recapping?

Motility test

1. What is "swarming"?
2. How does "true motility" differ from "Brownian movement"?
3. What morphological structure is responsible for bacterial motility?
4. Draw the following morphological structures which are responsible for bacterial motility:
a) Monotrichous, b) Peritrichous
5. What is the value of a hanging-drop preparation?
6. Why are living, unstained bacterial preparations more difficult to observe microscopically than stained preparations?

Streak plate technique

1. Define the following:
a) culture medium b) pure culture c) mixed culture d) bacterial colony
2. At what temperature does agar solidify? At what temperature does agar melt?
3. Why are culture media sterilized before use?
4. Why should a Petri dish not be left open for any extended period?
5. Why it is necessary to isolate individual colonies from a mixed growth?
6. Observation of a streak-plate culture shows more growth in Quadrant 4 than in Quadrant 3, explain?

Gram's staining technique

1. State the four reagents used in the Gram stain procedure? What is the purpose of each?
2. What are the advantages of the Gram stain?
3. Describe at least two conditions in which an organism might stain gram variable?
4. Why are thick or dense smears less likely to provide a good smear preparation for microscopic evaluation?
5. What are the advantages of differential staining procedures over the simple staining technique?
6. What is the most crucial step in the performance of the Gram staining procedure?

Catalase test

1. What is catalase?
2. What is the substrate of the catalase reaction? Why are bubbles produced in a positive catalase test?
3. Why will a false-positive catalase test result if the organism are tested on a medium containing blood?
4. Name a bacterial species which produce catalase enzyme?

Coagulase test

1. Why is coagulase test important?
2. Define the following: a) coagulase slide test b) coagulase tube test
3. Name a bacterial species which produce coagulase enzyme?
4. "Coagulase enzyme is a virulent factor", explain?

Oxidase test

1. What is the function of cytochrome oxidase?
2. What is the name of the reagent in the oxidase test?
3. Name a bacterial species which produce oxidase enzyme?

Sterilization & disinfection

1. Define the following:
   a) Sterilization b) Disinfection c) Antisepsis d) Fungicidal
2. How are microorganisms destroyed by moist heat? By dry heat?
3. Are some microorganisms more resistant to heat than others? Why?
4. Is moist heat more effective than dry heat? Why?
5. Define the principle of sterilization with an autoclave?
6. It is necessary to use biological and/or non-biological controls to monitor heat-sterilization techniques, explain?
7. Name the correct method to sterilize the following items:
   a) Surgical instruments b) Clean lab. Glassware c) Plastic syringes
   d) Plastic Petri-dishes e) Agar medium f) Metal loop
8. Name three types of disinfectants?
9. Explain why milk is subjected to pasteurization rather than sterilization?

Identification of Staphylococci

1. Describe the morphological appearance of *Staphylococci* by Gram stain?
2. What properties of *S. aureus* distinguish it from *S. epidermidis* and *S. saprophyticus*?
3. How *S. epidermidis* distinguished from *S. saprophyticus?*
4. From what specimen type would *S. saprophyticus* most likely be isolated?
5. Describe the purpose and the principle of the following:
   a) Mannitol salt agar b) DNase agar c) Coagulase latex agglutination
6. What is MRSA? Why it is significant?
7. Explain the purpose of adding blood to blood agar medium?
8. Explain the purpose of high salt concentration in the MSA?
9. How can the genera *Staphylococcus* and *Micrococcus* be differentiated from each other?

Identification of Streptococci

1. Differentiate the microscopic morphology of streptococci and pneumococci as seen by Gram stain?

2. What type of hemolysis is produced by the following streptococci? Why?
a) S. pneumoniae *b) Streptococcus pyogenes*
3. How is *S. pneumoniae* distinguished from other streptococci with the same haemolytic properties?
4. What kind of culture media and atmospheric and incubation conditions are best for cultivation of *S. pyogenes* and *S. pneumoniae*?
5. Explain why is blood agar considered a differential medium?
6. Name the chemical compound present in the optochin disc?
7. Why it is important to differentiate between Group D Enterococcus and non-Enterococcus?
8. Describe the purpose and the principle of the following:
a) Sodium hippurate test b) Bile esculin test c) Lancefield grouping
d) CAMP test e) PYR test?

Identification of Enterobacteriaceae

1. List at least five common characteristics shared by all members of the family Enterobacteriaceae?
2. What is the value of serological identification of a microorganism as compared with culture identification?
3. Provide a flowchart indicating how you would make the laboratory diagnosis of Enterobacteriaceae?
4. Describe the purpose and the principle of the following:
a) MacConkey agar b) XLD agar c) DCA agar d) API 20E
e) Oxidation/Fermentation test f) Urease test g) Indol test
h) Salmonella serology
5. Describe the colonial morphology of *Salmonella* spp on XLD agar? Explain the chemical reaction behind your description?
6. Describe the colonial morphology of *E. coli* on MacConkey agar? Explain the chemical reaction behind your description?
7. Explain the purpose of adding crystal violet in the MacConkey agar?
8. Compare between the MacConkey agar and deoxycholate citrate agar?
9. Name indicators added to MacConkey , DCA and XLD agar media?
10. What kind of culture media and atmospheric and incubation conditions are best for cultivation of *E. coli, Klebsiella spp., Salmonella spp. and Shigella spp.*?
11. How does *Shigella sonnei* differ from other *Shigella* spp.?

Identification of Pseudomonas, Vibrios and Campylobacter

1. Describe the purpose and the principle of the following:
a) TCBS agar b) Alkaline peptone water c) Oxidase test
2. How would you identify *Pseudomonas aeruginosa* isolated from UTI?
3. What kind of culture medium and atmospheric and incubation conditions are best for cultivation of *Campylobacter jejuni*?
4. What is the purpose of antimicrobial agents present in selective medium used for isolation of *Campylobacter*?
5. Explain the purpose of adding thiosulphate and sucrose into the TCBS agar medium?
6. What kind of culture media and atmospheric and incubation conditions are best for cultivation of *Pseudomonas aeruginosa*?
7. What characteristics exclude *Pseudomonas* spp. from the family Enterobacteriaceae?
8. What is pyocyanin?

Identification of Haemophilus influenzae

1. What is chocolate agar?
2. Define X and V factors?
3. What is the "satellite" phenomenon?
4. Name three species of *Haemophilus* and how would you distinguish these species in the lab?
5. What kind of culture media and atmospheric and incubation conditions are best for cultivation of *Haemophilus influenzae*?

Identification of Listeria and Corynebacterium

1. How is *Listeria monocytogenes* distinguished from *Corynebacteriun diphtheriae* in the lab?
2. What kind of culture media and atmospheric and incubation conditions are best for cultivation of *Listeria monocytogenes and Corynebcaterium diphtheriae*?

Identification of Neisseria and Moraxella

1. Explain how would you distinguish between *N. gonorrhoeae, N. meningitidis, and Moraxella catarrhalis* in the lab?
2. What are intracellular gram-negative diplococci?
3. Why are selective media used for primary culture of specimen from female urogenital tract? How the media would be selective?
4. What diseases do *N. gonorrhoeae, N. meningitidis, and Moraxella catarrhalis* cause?

Identification of Mycobacterium

1. Explain why sputum is "digested" and concentrated before culture?
2. Why are tubercle bacilli acid-fast?
3. Name the different chemical reagents used in the Ziehl-Neelsen stain? What is the purpose of each?
4. What kind of culture medium and atmospheric and incubation conditions are best for cultivation of *Mycobacterium tuberculosis*?
5. Describe what you would see microscopically for a positive acid-fast sputum sample?

Mycology Practical

1. How do fungi (including yeasts) differ from bacteria?
2. What different safety precautions you should flow in the mycology lab?
3. Explain the advantages and disadvantages of the following mycology tests:
a) KOH preparation b) India ink c) Lactophenol Cotton Blue stain
4. What fungus can be identified reliably by using the germ tube test?
5. What is the main advantage of using the slide culture technique for identifying fungi?
6. Define and draw the following fungal structures:
a) Hypha b) Mycelium c) Blastoconidia Arthrospores
7. List four general rules for good collection of fungal specimens?
8. List three different media used for cultivation of fungi? What is the purpose of each?