Faculty of **M**edicine and

Surgery (**FOM**)

Department of

Microbiology and

Immunology

Medical **M**icrobiology

Laboratory **M**anuals

Contents

Subjects and objectives:

Clinical skills will be gained in conventional techniques related to the identification, classification and characterisation of some important pathogens. Techniques for processing clinical samples in the routine microbiology laboratory will be brought out.

Safety Safeguards in Microbiology Laboratories

Lab Safety

- Bacteria are transmissible in the laboratory.
- Caution must be taken at all times to evade infecting yourself or others.

Routes of Entry of Microbes

Organisms can enter the body by:

- Breathing aerosols.
- Swallowing infectious substances.
- Skin scratches or abrasions.

Use of some reagents also carries certain hazards.

Safety Procedures

- Always wear A Lab Coat. Always keep it buttoned up. This keeps your outdoor clothing free from germs.
- Wear rubber gloves for handling bacterial cultures and doing staining.
- Dispose of waste from the practical sessions in the yellow clinical waste bags provide, not the 'household' waste bins.
- Do not put personal properties on the lab benches.
- Do not sit on benches.
- Never eat, drink or smoke in the lab.
- Never put labels or pipette infectious material in your mouth.
- Do not place loops, pipettes or spreader on the bench tops. Use the racks provided.
- Always wash your hands before exiting the lab.
- Accidents: breaking, spillage or personal injury, must be reported instantly to the demonstrator or laboratory supervisor.

Bacterial Staining

- process in which bacteria are stained and visualised
  - Simple staining:
    - Only one dye is used. Not very useful in bacteriology.
  - Differential staining:
    - more than one dye is used to Differentiate between bacteria. Gram's staining, ZN staining
  - Special staining:
    - more than one dye used to visualize Special bacterial structures . E.g. Capsule staining, Spore staining.

Gram Stain

Overview

Gram staining is a method used to categorise bacteria into Gram positive & Gram negative based on:

- Cell wall Structure.
- Cell wall Chemical composition.
- Cell wall Thickness.

Procedure: **Figure-2-3**

- Take a glass slide and label it with a marker or a pencil.
- Use a sterile disposable loop to place a small drop of saline or water in the centre of the slide.
- Touch a sterile loop alongside the chosen colony you wish to examine. Pick up a very small and detectable portion of the colony.
- Emulsify this in the drop, spreading the drop into a circle about 1 cm in diameter.
- Leave the slide on the bench to dry and dispose of the loop.
- When the slide is dry 'fix' the bacteria by passing it, inoculated side upward, briefly through a Bunsen burner.
- Put the slide(s) on a staining rack and proceed as follows: Figure-3

- Flood with **Crystal violet** and leave for about half a minute
- Wash with tap water
- Flood with **Iodine** and leave for about half a minute
- Tip the iodine from the slide, DO NOT WASH, and add **Alcohol-acetone** solution. Leave for 10 seconds. **MAKE SURE TO PUT ALCOHOL AWAY FROM BUNSEN BURNER**.
- Wash with tap water and blot dry
- Flood with **Safranin O** and leave for at least half a minute
- Wash with tap water and blot dry

Examination of Gram-stained slides (figure-4-5)

- Locate the smear with the x 10 (low power) objective of the microscope and adjust focus and light source as necessary.
- Place a drop of immersion oil on the surface of the smear and changer over to the x 100 (oil immersion) objective. Adjust focus and light intensity as necessary.
- Look for an area of the slide where the bacteria are well separated from each other and note whether the culture is Gram-positive (blue-black) or Gram-negative (red). Figure-1
- Note shape and characteristic arrangement of the organisms, e.g. clumps, pairs, chains, tetrads etc.

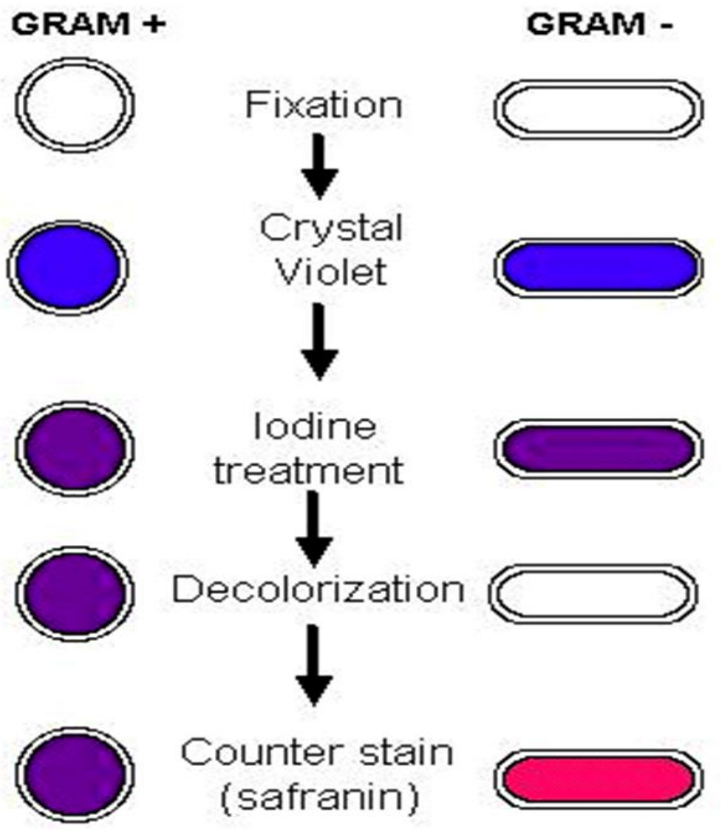


Figure 1: outlines of Gram staining principle

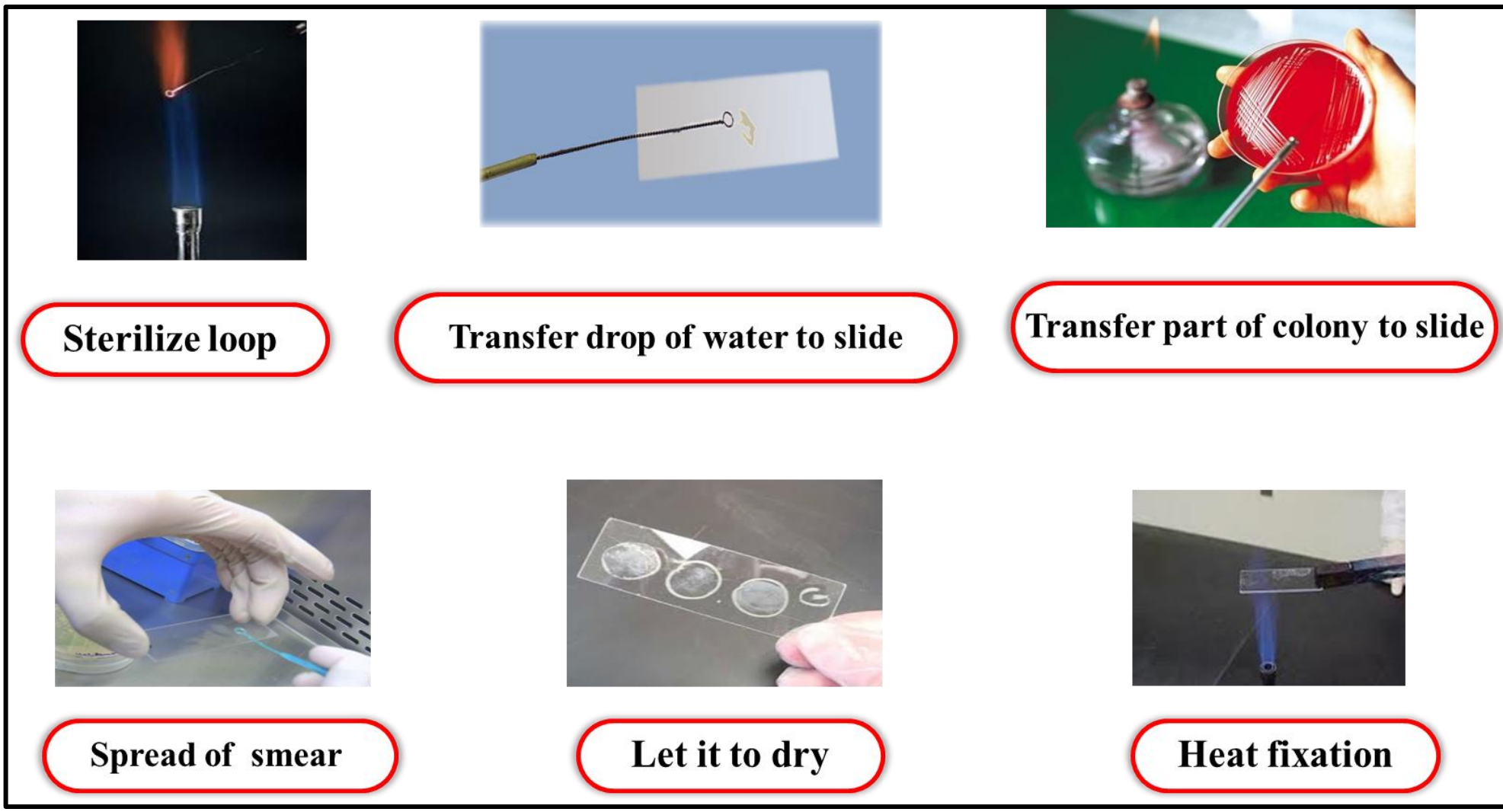


Figure 2: steps to prepare smears

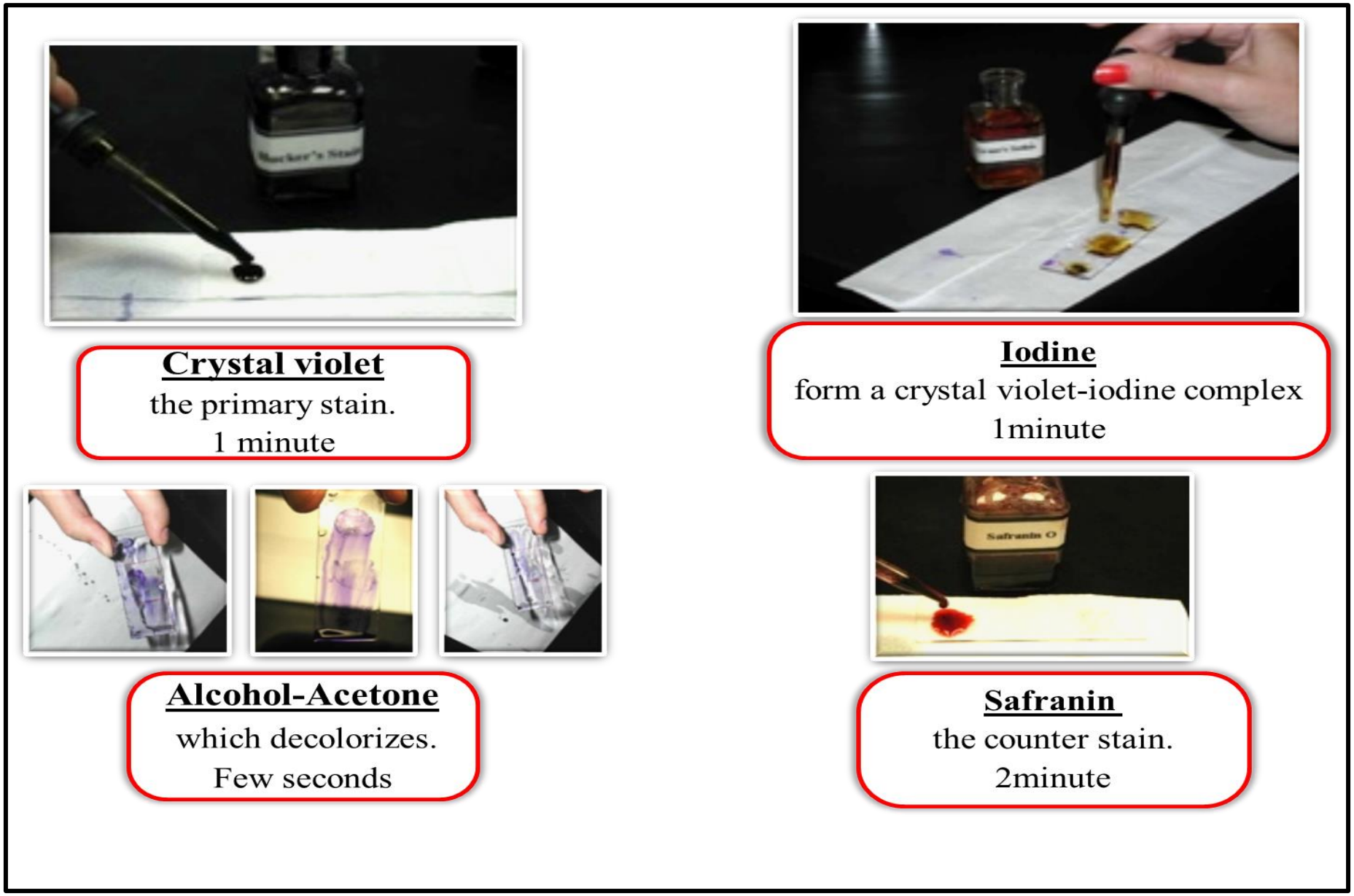


Figure 3: Steps of staining procedure

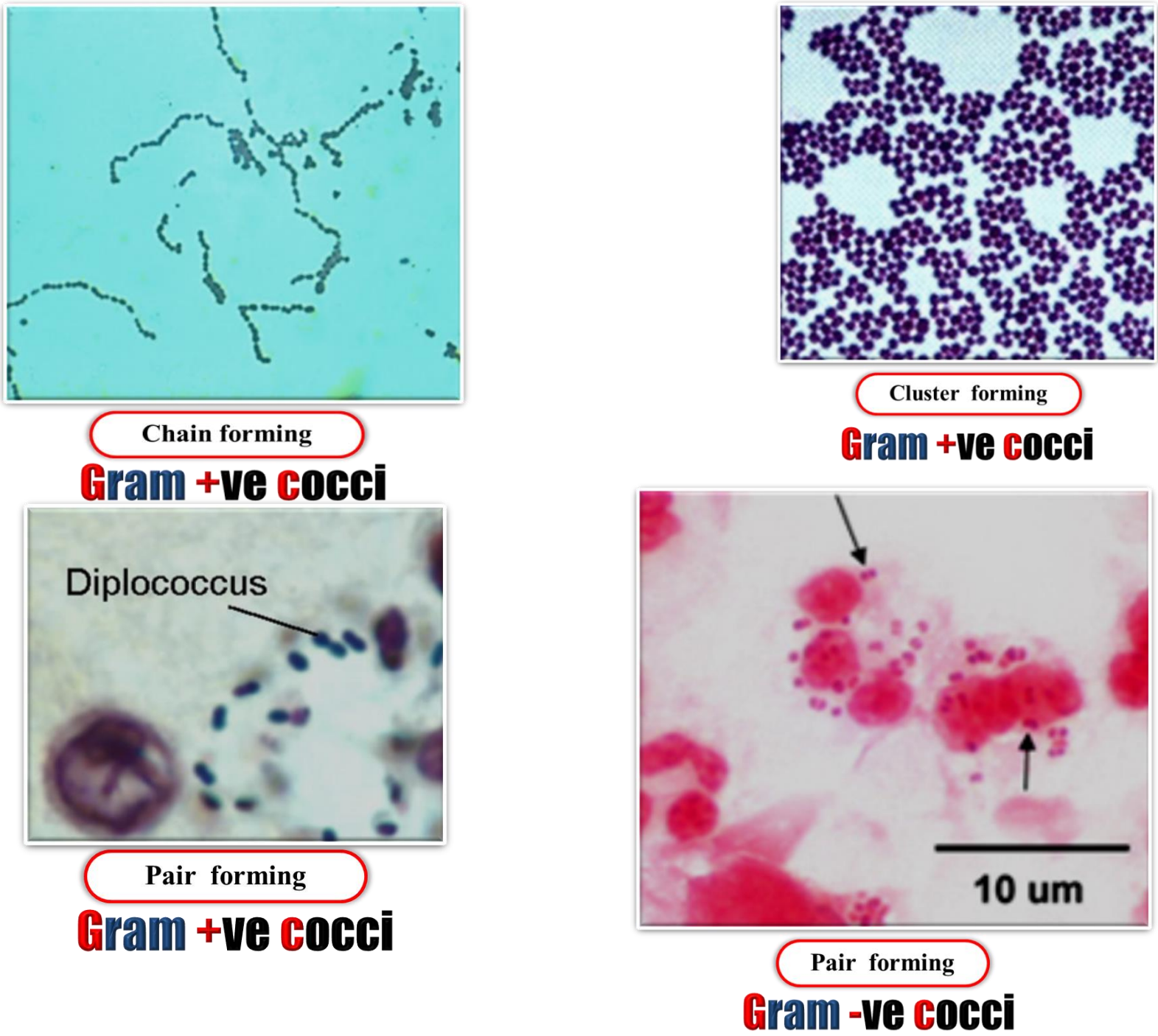
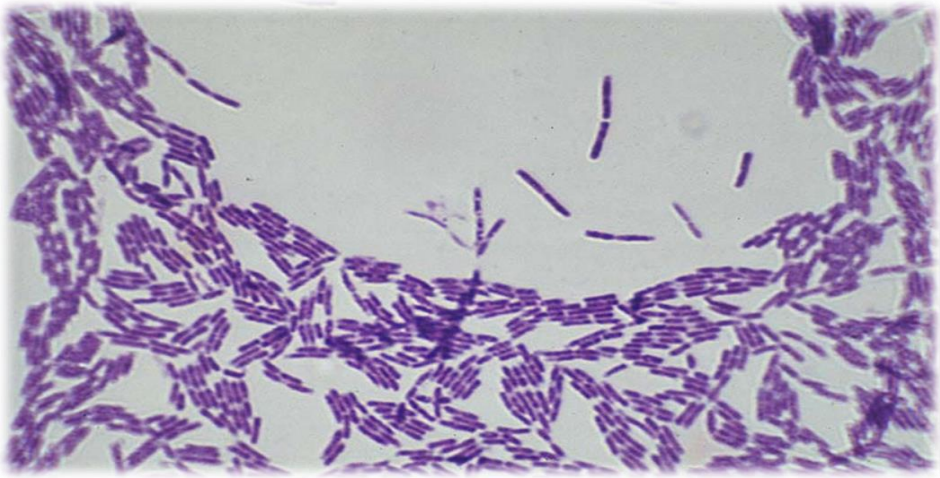
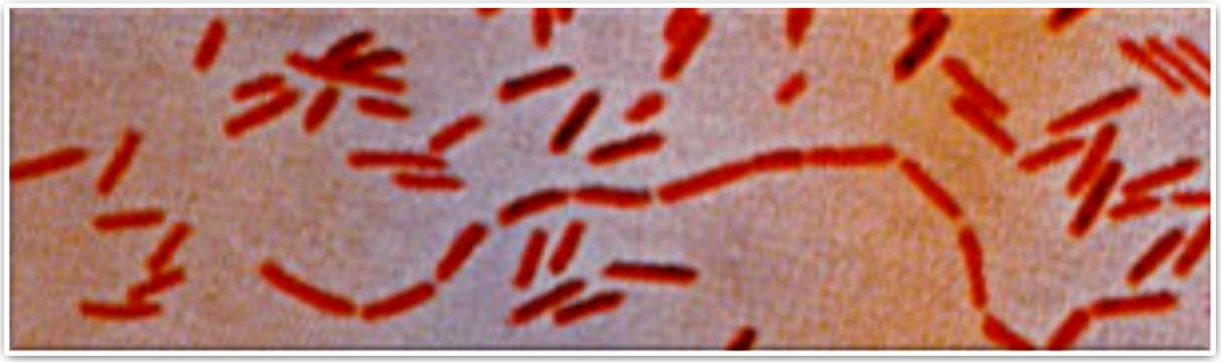


Figure 4: Examples of Gram Stain results of Coccus-shaped Bacteria



Chain Forming

Gram +ve Bacilli



Chain Forming

Gram -ve Bacilli

Figure 5: Examples of Gram Stain results of Bacillary-Shaped Bacteria

Acidfast Stain: Background and Introduction

Mycobacterium and many *Nocardia* species are called **acid-fast** because during an acid-fast staining procedure they retain the primary dye carbol fuchsin despite decolorization with the powerful solvent **acid-alcohol**. Nearly all other genera of bacteria are **nonacid-fast**. The acid-fast genera have lipoidal **mycolic acid** in their cell walls. It is assumed that mycolic acid prevents acid-alcohol from decolorizing protoplasm. The acid-fast stain is a **differential stain.**

Ziehl Neelsen Acid-fast stain

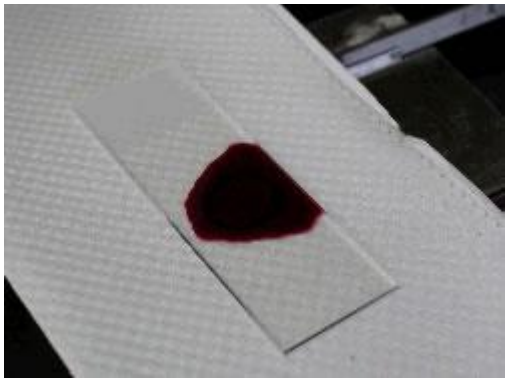
ACID-FAST STAIN		**Cell Color**	**Cell Color**
Procedure	**Reagent**	**Acid-fast Bacteria**	**Nonacid-fast Bacteria**
Primary dye	Carbolfuchsin	RED	RED
Decolorizer	Acid-alcohol	RED	COLORLESS
Counterstain	Methylene blue	RED	BLUE

Step 2: Smear Preparation (Review)

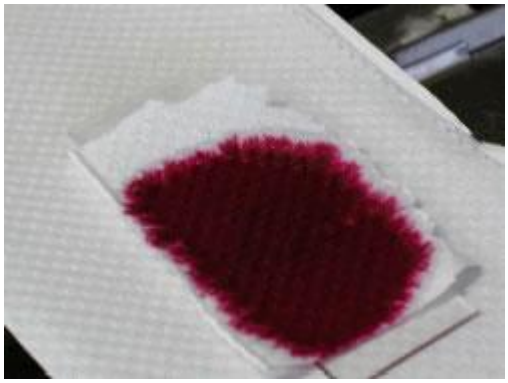
1. Add one loopful of sterile water to a microscope slide.
2. Make a heavy smear of *Mycobacterium smegmatis*. Mix thoroughly with your loop. Then transfer a small amount of *Staphylococcus epidermidis* to the same drop of water.
   You will now have a mixture of *M. smegmatis* and *S. epidermidis*.
3. Air dry and heat fix.

Step 3:

Cover the smear with carbolfuchsin dye. Carbolfuchsin a potential carcinogen. Please wear gloves and work with the stain in the hood.



Place a piece of paper towel on top of the dye. Be sure the paper towel is saturated with the dye.

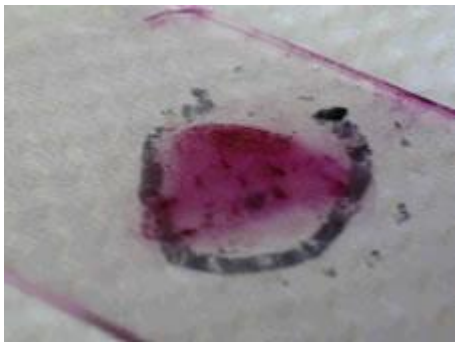


Step 4:

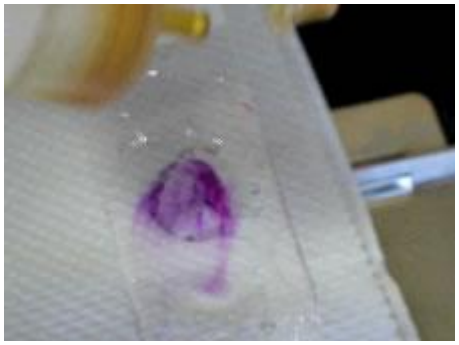
Dry heat for 2 minutes.

Step 5:

Cool and rinse with water.

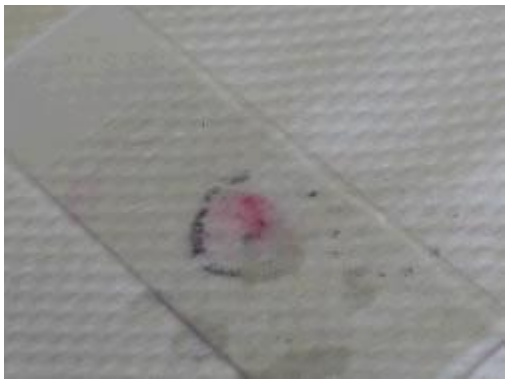


Decolorize with acid-alcohol for 15-20 seconds.



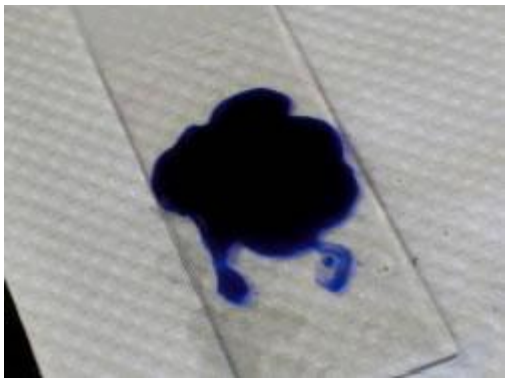
Step 6:

Wash the top and bottom of slide with water and clean the slide bottom well.

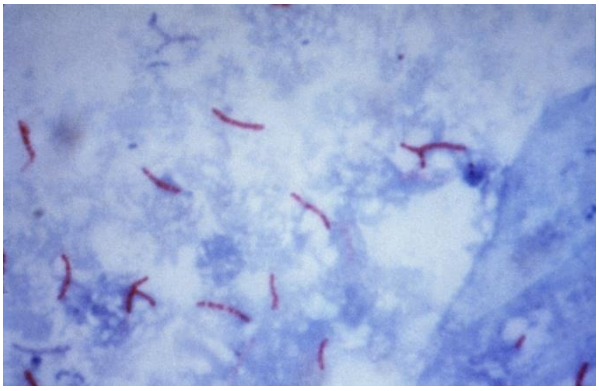


Step 7:

Counterstain with Methylene Blue for 30 seconds to 1 minute.



Wash and blot the slide with bibulous paper.



The Cultivation And Identification Of Bacteria

A Morphology and Staining

Bacteria are identified and classified on the basis of many different characteristics. Amongst these are the microscopic appearance of the bacterial cells and their reaction to Gram's stain (Gram-positive or Gram-negative) and the macroscopic appearance of colonies of bacteria growing on solid culture media.

B Cultivation

For growth microorganisms require:

(a) a source of energy

(b) carbon, nitrogen, mineral salts and vitamins

(c) certain 'accessory' growth factors

(d) water

These may be supplied either in the form of a **defined culture medium** of known chemical composition or, more commonly, a complex medium containing protein hydrolysates or digests or meat. The various types of culture media employed in the clinical bacteriology laboratory may be classified as follows: -

Nutrient media: (Nutrient both, nutrient agar, peptone agar etc) which are protein hydrolysates or watery extracts of meat able to support the growth of non-fastidious organisms.

Enriched media: (Blood agar, serum agar, chocolate agar etc) in which 'accessory' growth factors are supplied to the more nutritionally demanding bacteria.

Indicator media: (MacConkey agar etc) are nutrient media containing carbohydrates or other suitable substrates along with an indicator to show whether these have been utilized. These served to differentiate between colonies of certain bacterial species.

Selective media:

Many of these are also indicator media. For example, gut bacteria are able to grow in the presence of concentrations of bile salts inhibitory to most other bacteria. This useful differential characteristic is exploited in **MacConkey's medium**, which contains sodium taurocholate. As well as bile, MacConkey's medium contains lactose, its main carbon and energy source, and a pH indicator,

neutral red. Bacteria able to utilise lactose, form acid and thus produce bright red colonies. Intestinal pathogens, such as salmonellae and shigellae, which do not metabolise the lactose, use the peptones in the medium as an alternative energy source and raise the pH, turning the indicator yellow. This medium is therefore also an **indicator medium.**

Describing colonial appearances

During the course, you will be asked to describe the appearance of bacterial colonies growing on agar plates since this may be of considerable help in identification. The following points may help:

(a) First note the name of the organism and the medium on which it is growing. Colonial appearances of bacteria may vary greatly, and characteristically, on different media.

(b) Your plates will usually show areas in which numerous colonies are crowded together and others in which they are well separated from each other. **Describe individual, well-separated colonies of bacteria.**

(i) the size of the colony – tiny; diameter <0.5mm; small; diameter 1-2mm etc

(ii) the shape, edge and elevation – see diagrams

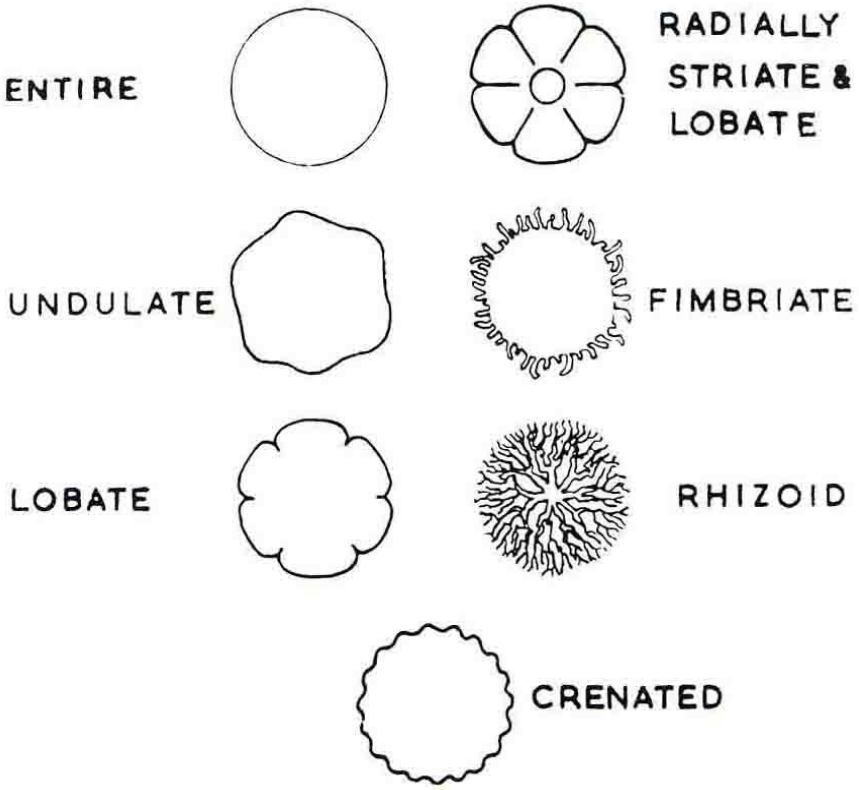
(iii) colour and whether opaque or translucent

(iv) any change in the medium surrounding the colony, especially presence or absence of haemolysis on blood agar.

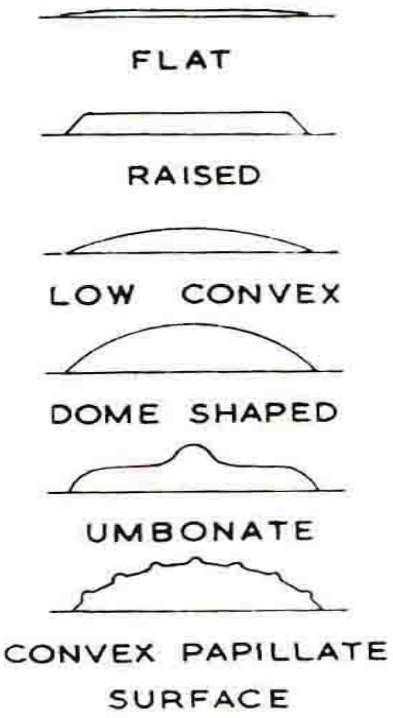
Example

Staphylococcus aureus, blood agar, aerobic. Opaque, white, slightly domed colonies, circular, 1 to 2mm in diameter with an entire edge and a smooth surface. There is a narrow zone of haemolysis.

Elevations and edges of common types of bacteria



Edges (above) and elevations (below) of bacterial colonies



Blood Agar

An improved Blood Agar Base possessing enhanced nutritional properties suitable for the cultivation of fastidious pathogens and other microorganisms.

CLED Medium (CLED)

The medium supports the growth of all urinary potential pathogens giving good colonial differentiation and clear diagnostic characteristics.

A dehydrated Cystine-Lactose-Electrolyte Deficient (CLED) medium made to the formula described by Mackey and Sandys as a modification for urinary bacteriology of the Electrolyte Deficient Medium developed by Sandys.

This medium is recommended for urinary bacteriology, supporting the growth of all urinary pathogens and giving good colonial differentiations and clear diagnostic characteristics. The presence of important contaminants such as diphtheroids, lactobacilli and micrococci is also clearly elicited, giving an indication of the degree of contamination.

In the laboratory, CLED Medium provides a valuable non-inhibitory diagnostic agar for plate culture of urinary organisms. It is electrolyte deficient to prevent the swarming of *Proteus* species.

MacConkey Agar

A differential medium for the detection, isolation and enumeration of coliforms and intestinal pathogens in water, dairy products and biological specimens.

Although principally used for coliforms, this medium may also be employed for the differentiation of other enteric bacteria (including pathogens) and is suitable for the differentiation of Pasteurella species[4].

Agar Chocolated Horse Blood

Chocolate agar (enriched) is a non-selective medium for the primary isolation of fastidious bacteria such as *Neisseria meningitidis* and *Haemophilus spp*. It is recommended as a primary plating medium for spinal fluids, eye cultures, gonococcal cultures, and any other specimen, which may contain fastidious

organisms. It is also used for maintenance and shipping of Neisseria and *Haemophilus spp*.

Chocolate agar contains haemoglobin solution and supplemental ingredients that are required by some fastidious organisms. Both factors X (hemin) and V (nicotinamide adenine dinucleotide [NAD]) are present, supplying the special growth factor requirements of Haemophilus influenzae and, when incubated in CO_2, of *Neisseria meningitidis* and *Neisseria gonorrhoeae*. Whole sheep erythrocytes release NADase which inactivates any NAD in the medium. Blood from rabbits and horses does not release the NADase enzyme. Chocolate agar (enriched) contains GCHI (gonococcal/*Haemophilus influenzae*) enrichment which is a supplement that provides the V factor (NAD), vitamins, amino acids, coenzymes, dextrose and ferric ions which promote growth of *Neisseria*.

Bile Aesculin Agar

A differential medium for the isolation and presumptive identification of enterococci / Group D streptococci.

The major use of Bile Aesculin Agar is to differentiate between enterococci / Group D streptococci and non Group D streptococci. It may also be used for the presumptive identification of other groups of organisms. Enterococci / Group D streptococci hydrolyse aesculin to form aesculetin and dextrose. Aesculetin combines with ferric citrate in the medium to form a dark brown or black complex, which is indicative of a positive result. Bile salts will inhibit Gram-positive bacteria other than enterococci / Group D streptococci. The value of bile tolerance together with hydrolysis of aesculin as a means of presumptively identifying enterococci / Group D streptococci is widely recognised.

XLD medium

A selective medium for the isolation of salmonellae and shigellae from clinical specimens and foods. Xylose-Lysine-Desoxycholate Agar relies on xylose fermentation, lysine decarboxylation and production of hydrogen sulphide for the primary differentiation of shigellae and salmonellae from non-pathogenic bacteria. Rapid xylose fermentation is almost universal amongst enteric bacteria, except for members of the *Shigella, Providencia* and *Edwardsiella* genera[1]. Xylose is thus

included in the medium so that *Shigella* spp. may be identified by a negative reaction.

Salmonella spp. are differentiated from non-pathogenic xylose fermenters by the incorporation of lysine in the medium. *Salmonellae* exhaust the xylose and decarboxylate the lysine, thus altering the pH to alkaline and mimicking the *Shigella* reaction. However, the presence of *Salmonella* and *Edwardsiella* spp. is differentiated from that of shigellae by a hydrogen sulphide indicator.

The high acid level produced by fermentation of lactose and sucrose, prevents lysine-positive coliforms from reverting the pH to an alkaline value, and non-pathogenic hydrogen sulphide producers do not decarboxylate lysine. The acid level also prevents blackening by these micro-organisms until after the 18-24 hour examination for pathogens. Sodium desoxycholate is incorporated as an inhibitor in the medium. The concentration used allows for the inhibition of coliforms without decreasing the ability to support shigellae and salmonellae.

DCA

This modification of Leifson's D.C.A. medium was introduced in 1942. The medium was designed to be more inhibitory to commensal flora whilst allowing for adequate growth of Salmonella spp and Shigella spp. The citrate and desoxycholate levels are significantly increased. To keep the desoxycholate in solution the pH also had to be increased. The medium still uses lactose fermentation and hydrogen sulphide production as differential indicators.

TCBS Cholera Media (TCBS)

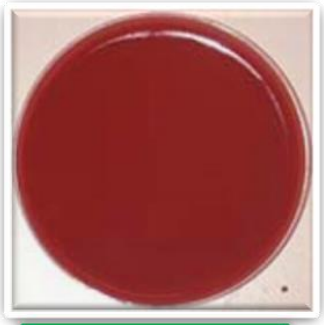
The medium is suitable for the growth of *Vibrio cholerae*, *Vibrio parahaemolyticus*, and most other *Vibrios*[9]. Most of the Enterobacteriaceae encountered in faeces are totally suppressed for at least 24 hours. Slight growth of *Proteus* species and *Enterococcus faecalis* may occur but the colonies are easily distinguished from vibrio colonies.

Camplylobacter Selective Agar Skirrow

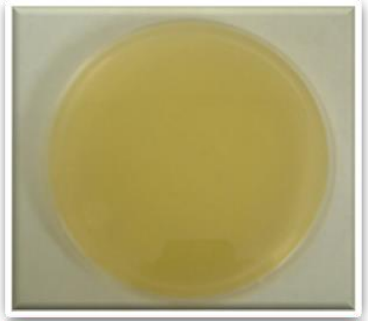
Campylobacter Skirrow Agar is a selective solid medium used for the cultivation and isolation of Campylobacter jejuni from fecal specimens. Addition of antibiotics refrains normal stool flora overgrowth and enhances C. jejuni recovery.

Solid media

Simple media.



CBA



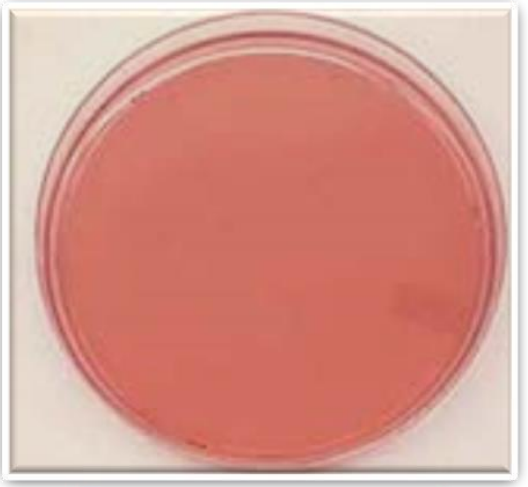
Nutrient Agar

Enriched media.

Blood agar

TCBS

Selective & differential media.



Mconckey agar

Biochemical Tests in Bacteriology

Catalase and Oxidase Tests

Catalase is an enzyme which catalyses the dissociation of hydrogen peroxide to oxygen and water.

Almost all medically important Gram-negative bacteria are catalase positive but the test is of useful discriminatory value in the Gram-positive group

Catalase + ve	**Catalase – ve**
Cornyebacterium	Lactobacilli
Bacillus	Clostridium

Procedure: Place one drop of hydrogen peroxide on a convenient group of colonies of bacteria on a nutrient agar plate, with a Pasteur pipette. Alternatively, as you might want to use the same culture for other tests, we recommend that you pick off a colony or group of colonies with a sterile loop onto a microscope slide and add the hydrogen peroxide to that bacterial sample.

Result: Catalase positive organisms will fizz but you should see no bubbles if the bacteria are catalase negative.

The oxidase test: Detects the enzyme cytochrome C oxidase and is more discriminatory for Gram-negative than for Gram-positive. This test is particularly useful for distinguishing species *of Pseudomonas* and *Vibrio* from members of the Enterobacteriaceae (*Escherichia coli, Proteus* spp. *Klebsiella* spp etc).

Procedure: Use a sterile loop to remove a representative colony from the plate and smear lightly on a strip of filter paper impregnated with oxidase reagent (tetramethyl-para-phenylenediamine).

Result: A positive oxidase reaction, denoted by the development of a deep purple colour, will occur within 15 seconds. Colour developing after a long period should be ignored.

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.

Glucose broth with Durham tubes

This is a differential medium. It tests an organism's ability to ferment the sugar glucose as well as its ability to convert the end product of glycolysis, pyruvic acid into gaseous byproducts. This is a test commonly used when trying to identify Gram-negative enteric bacteria, all of which are glucose fermenters but only some of which produce gas.Like MSA, this medium also contains the pH indicator, phenol red. If an organism is capable of fermenting the sugar glucose, then acidic byproducts are formed and the pH indicator turns yellow. Escherichia coli is capable of fermenting glucose as are Proteus mirabilis (far right) and Shigella dysenteriae (far left). Pseudomonas aeruginosa (center) is a nonfermenter. The end product of glycolysis is pyruvate. Organisms that are capable of converting pyruvate to formic acid and formic acid to H2 (g) and CO2 (g), via the action of the enzyme formic hydrogen lyase, emit gas. This gas is trapped in the Durham tube and appears as a bubble at the top of the tube.

Escherichia coli and Proteus mirabilis (far right) are both gas producers. Notice that Shigella dysenteriae (far left) ferments glucose but does not produce gas. *Note - broth tubes can be made containing sugars other than glucose (e.g. lactose and mannitol). Because the same pH indicator (phenol red) is also used in these fermentation tubes, the same results are considered positive (e.g. a lactose broth tube that turns yellow after incubation has been inoculated with an organism that can ferment lactose).

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 CO_2. If CO_2 is produced, it reacts with components of the medium to produce an alkaline compound (e.g. Na_2CO_3). 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 dysenteria*e are citrate negative.

Methyl Red / Voges-Proskauer (MR/VP)

This test is used to determine which fermentation pathway is used to utilize glucose. In the mixed acid fermentation pathway, glucose is fermented and produces several organic acids (lactic, acetic, succinic, and formic acids). The stable production of enough acid to overcome the phosphate buffer will result in a pH of below 4.4. If the pH indicator (methyl red) is added to an aliquot of the culture broth and the pH is below 4.4, a red color will appear (first picture, tube on the left). If the MR turns yellow, the pH is above 6.0 and the mixed acid fermentation pathway has not been utilized (first picture, tube on the right). The 2,3 butanediol fermentation pathway will ferment glucose and produce a 2,3 butanediol end product instead of organic acids. In order to test this pathway, an aliquot of the MR/VP culture is removed and α-naphthol and KOH are added. They are shaken together vigorously and set aside for about one hour until the results can be read. The Voges-Proskauer test detects the presence of acetoin, a

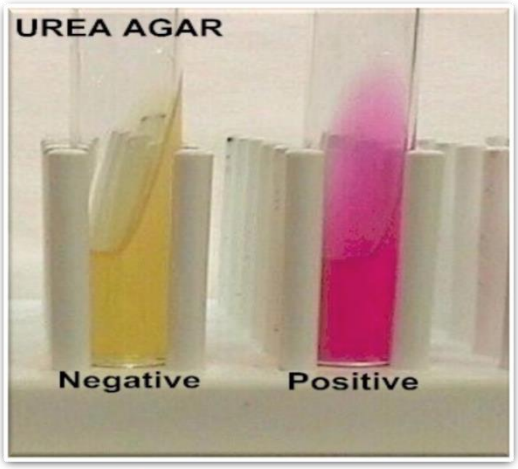
precursor of 2,3 butanediol. If the culture is positive for acetoin, it will turn “brownish-red to pink” (tube on the left in the second picture). If the culture is negative for acetoin, it will turn “brownish-green to yellow” (tube on the left in the second picture). Note: A culture will usually only be positive for one pathway: either MR+ or VP+. *Escherichia coli* is MR+ and VP-. In contrast, *Enterobacter aerogenes* and *Klebsiella pneumoniae* are MR- and VP+. *Pseudomonas aeruginosa* is a glucose nonfermenter and is thus MR- and VP-.

Urease test

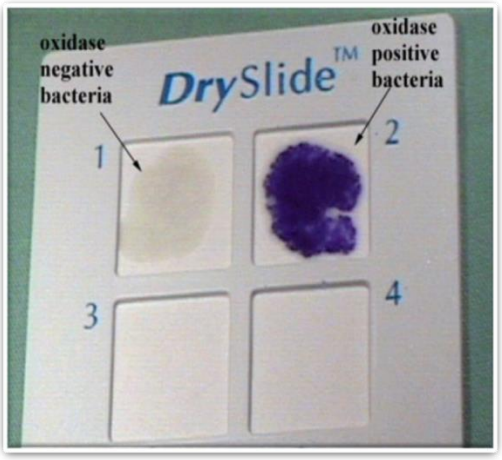
This test is used to identify bacteria capable of hydrolyzing urea using the enzyme urease. It is commonly used to distinguish the genus *Proteus* from other enteric bacteria. The hydrolysis of urea forms the weak base, ammonia, as one of its products. This weak base raises the pH of the media above 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).

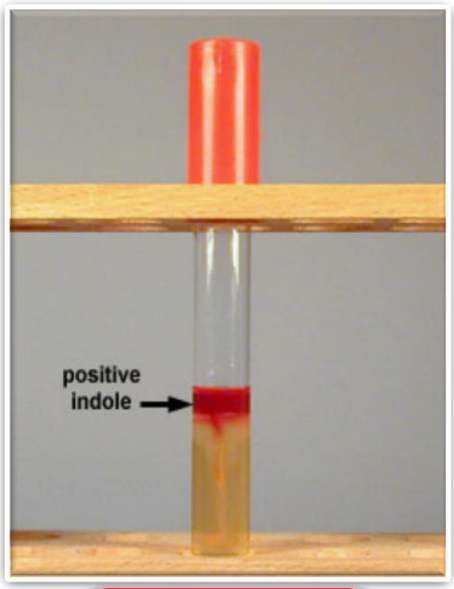


Urease test.



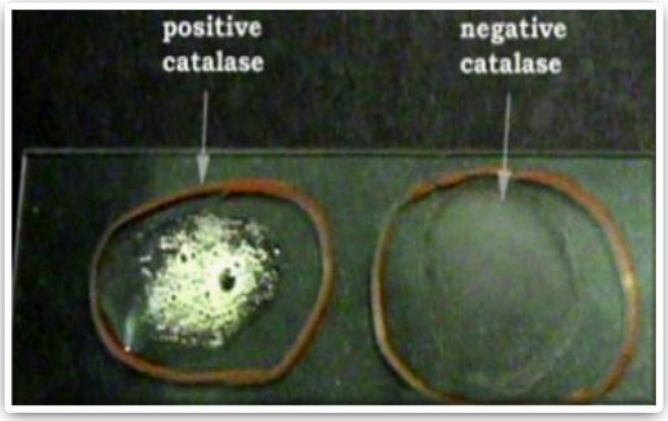
Oxidase test

Sugar fermentation test



Indole test.

Tubes coagulase



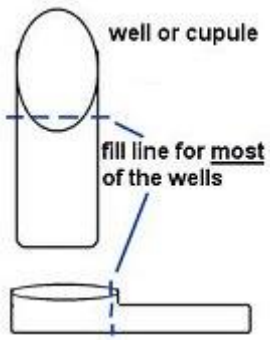
API

Procedure:

I. Label the incubation tray provided with your name, date and organism, then remove the lid and API strip and dispense approximately 5 ml of distilled water into the tray segment finally replacing the API strip.

II. Using aseptic technique, inoculate a tube of the appropriate media with the organism to make a bacterial suspension at the correct turbidity. Gently homogenise the solution with a sterile Pasteur pipette (i.e. try to break up and visible clumps).

III. Holding the strip at a slight angle up from the table top, inoculate the bacterial suspension prepared in step (i) into each well with the sterile pipette used to homogenise above. Touch the end of the pipette to the side of the cupule, allowing capillary action to draw the fluid into the well as you slowly squeeze the bulb. This should eliminate any bubbles forming in the wells. Each well should be filled up to the neck (see diagram below).



IV. Those test wells with boxes around their names will be filled all the way up to the top of the well e.g. CIT

V. Upon completion of all the inoculations, the cupules of those test wells, which are underlined, are completely covered with mineral oil, having been filled to the level indicated in step (iii). E.g. ADH

VI. Re-cover the tray with the lid and incubate the strip is at 37° C for the recommended time (usually 18-24 hours).

VII. Add the appropriate reagents to the compartments (see a demonstrator who will dispense them with a dropper bottle)

VIII. Interpret the result of your API test with the corresponding interpretation tables provided below and enter them into the apiweb for identification.

Table 1: Interpretation Table for API 20E Identification Strip

Tests	Active ingredients	Quantity (mg/cup)	Reactions or Enzymes	Results	
				NEGATIVE	POSITIVE
ONPG	2-nitrophenyl-ßD-galactopyranoside	0.223	ß-galactosidease	colourless	yellow (1)
ADH	L-arginine	1.9	arginine diydrolase	yellow	red / orange (2)
LDC	L-lysine	1.9	lysine decarboxylase	yellow	red / orange (2)
ODC	L-ornithine	1.9	ornithine decarboxylase	yellow	red / orange (2)
CIT	trisodium citrate	0.756	citrate utilization	pale green / yellow	blue-green / blue (3)
H_2S	sodium thiosulfate	0.075	H_2S production	colourless / greyish	black deposit / thin line
URE	urea	0.76	urease	yellow	red / orange (2)
TDA	L-tryptophane	0.38	tryptophane deaminase	TDA / immediate	
				yellow	reddish brown
IND	L-tryptophane	0.19	indole production	JAMES /immediate	
				colourless pale green / yellow	pink
VP	sodium pyruvate	1.9	acetoin production (Vogues Proskauer)	VP 1 + VP 2 / 10 min.	
				colourless	pink / red (5)
GEL	gelatin (bovine origin)	0.6	gelatinase	no diffusion	diffusion of black pigment
GLU	D-glucose	1.9	fermentation or oxidation (4)	blue / blue-green	yellow / greyish yellow
MAN	D-mannose	1.9	fermentation or oxidation (4)	blue / blue-green	yellow
INO	Inositol	1.9	fermentation or oxidation (4)	blue / blue-green	yellow
SOR	D-sorbitol	1.9	fermentation or oxidation (4)	blue / blue-green	yellow
RHA	L-rhamnose	1.9	fermentation or oxidation (4)	blue / blue-green	yellow
SAC	S-sucrose	1.9	fermentation or oxidation (4)	blue / blue-green	yellow
MEL	D-melibiose	1.9	fermentation or oxidation (4)	blue / blue-green	yellow
AMY	Amygdalin	0.57	fermentation or oxidation (4)	blue / blue-green	yellow
ARA	L-arabinose	1.9	fermentation or oxidation (4)	blue / blue-green	yellow
OX	oxidase test -- See test package insert		cytochrome oxidase	oxidase test -- See test package insert	

(1) A very pale yellow should also be considered positive.
(2) An orange colour after 36-48 hours incubation must be considered negative.
(3) Reading made in the cupule (aerobic).
(4) Fermentation begins in the lower portion of the tube; oxidation begins in the cupule.
(5) A slightly pink colour after 10 minutes should be considered positive.

API 20 E after incubation...Positive results for all tests :

Microbiology Info.com

API 20 E after incubation...Negative results for all tests :

Antibiotic Sensitivity Testing

Preparation of a Standard Inoculum

Standard inocula for test and control organisms have been prepared for you using the following method:

- Touch at least four morphologically similar colonies with a sterile loop.
- Transfer growth to 5ml of Iso-Sensitest broth and incubate with shaking until visible turbidity is equal or greater than 0.5 McFarland standard.

Now…

1. Adjust the density of the suspension to 0.5 McFarland standard by adding distilled water. Comparison is aided by using a white background with contrasting black line.
2. Use the suspension within 10 minutes.

2. Inoculation of sensitivity plates

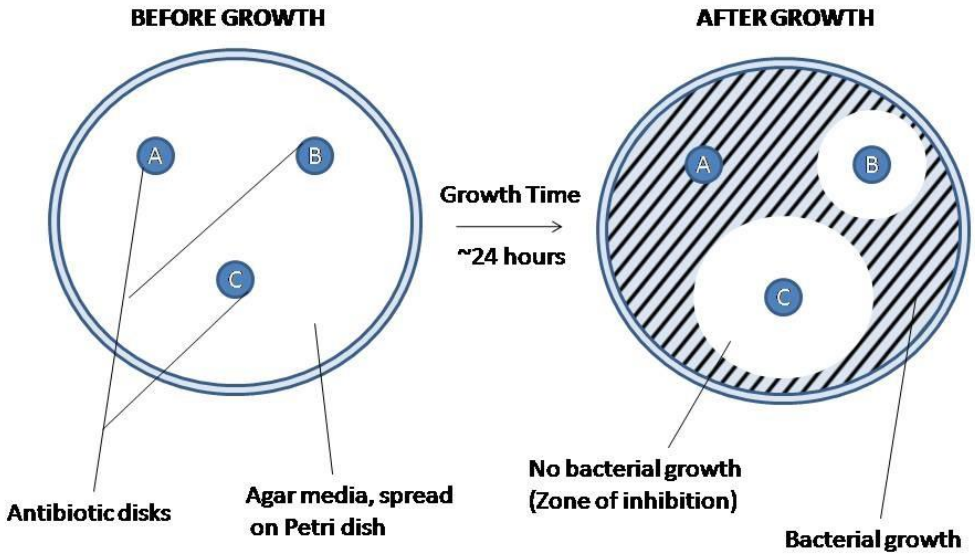
Disk Diffusion Test

1. Using the suspensions prepared use a sterile swab to spread the inoculum over the entire surface of one Iso-Sensitest plate **for both test and control organisms (2 plates in total)**.
2. Leave to dry for 5 minutes
3. Apply the six antibiotic discs (Cefoxitin 10µg, Teicoplanin 30µg, Fusidic acid 10µg, Gentamycin 10µg, Vancomycin 5µg, and Erythromycin 5µg) to the Iso-Sensitest plates.
4. Incubate the Iso-Sensitest plate at 37^0C overnight.

3. Reading Test and Control plates

1. You are provided with 3 sets of incubated sensitivity plates.
2. Using a ruler measure and record the zone sizes on each set of plates.
3. Record the zone sizes for the control organism on the white board and mark on the expected zone sizes from the table provided.
4. Compare the zone sizes for the test organism against those in the table. Analyse your findings and interpret the test results.

Test and Control Organisms	Cefoxitin	Teicoplanin	Fusidic Acid	Gentamicin	Vancomycin	Erythromycin
Control						
Test A						
Test B						


BEFORE GROWTH
AFTER GROWTH
A
B
C
Growth Time
~24 hours
Antibiotic disks
Agar media, spread on Petri dish
No bacterial growth (Zone of inhibition)
Bacterial growth


IPM 10
TZP 110
GM 10
MEM 10
AN 30
CAZ 30
SXT
NN 10
AM 10
CRO 30
CZ 30
CIP 5

Antibiotic Codes

Amoxycillin	**AML**
Amoxycillin/Clavulanic acid	**AMC**
Ampicillin	**AMP**
Bacitracin	**BC**
Benzylpenicillin	**P**
Cefoxitin	**FOX**
Ceftazidime	**CAZ**
Ceftriaxone	**CRO**
Cefuroxime	**CXM**
Cephradine	**CEP**
Cephtazadime	**CET**
Chloramphenicol	**CHL**
Ciprofloxacin	**CIP**
Clindomycin	**DA**
Colistin	**CT**
Erythromycin	**E**
Fusidic acid	**FD**
Gentamycin	**CN**
Imipenem	**IPM**
Levofloxacin	**LEV**
Methicillin	**MET**
Metronidazole	**MTZ**
Nalidixic Acid	**NA**
Neomycin	**NEO**
Novobiocin	**NV**
Optochin	**OP**
Oxacillin	**OX**
Piperacillin	**PRL**
Rifampicin	**RD**
Sulfamethoxazole	**RL**
Teicoplanin	**TEC**
Tetracycline	**TE**
Trimethoprim	**W**
Vancomycin	**VA**

Sterilisation and Disinfection

- **Sterilization:**
  - is complete killing, or removal, of all living organisms (including spores) from a particular location or material.
- **Disinfection:**
  - is the killing of many, but not all microorganisms , or it is the destruction of pathogenic microorganisms by processes that fail to meet the criteria for sterilization.
- The methods of Sterilization & Disinfection include the use of physical methods, chemical methods, filtration & radiation.

a) Dry heat

Many objects are best sterilized in the absence of water by dry heat sterilization; killing by dry heat is due to

protein denaturation, oxidative damage and toxic effect of elevated levels of electrolytes.

Methods of Sterilization by Dry Heat

1. Flaming

Inoculating loops and points of forceps may be heated in the Bunsen flame, till they are red-hot. Articles such as mouth of the culture tubes, cotton wool plugs, glass slides etc. are passed over the flame without allowing it to become red hot.

2. Incineration

This is an excellent method for rapidly destroying, animal carcasses, pathological material and disposables.

3. Hot Air Oven

This is the most widely adopted method of sterilization by dry heat. The hot air oven utilizes radiating dry heat for sterilization. This type of energy does not penetrate materials easily and thus, long periods ofexposure to high temperature are necessary. For example, at a temperature of 160°C, a period of two hours is required for the destruction of bacterial spores. Hot air oven is used to sterilize glassware, forceps, scissors, scalpels, glass syringes, liquid paraffin, dusting powder etc. A holding period of 160°C for an hour is used. The oven is usually heated by electricity, with heating elements in the wall of the chamber and it must be filled

with a fan to ensure even distribution of hot air and elimination of air pockets. The materials should be arranged in a manner which allows free circulation of hot air in between the objects. It should not be over-loaded.

b) Moist heat

Moist heat kills microorganisms by coagulating their proteins and is much more rapid and effective than dry heat because water molecules conduct heat better than air. Lower temperature and less time of exposure are therefore required than for dry heat. Moist heat readily kills viruses, bacteria, fungi etc.

a) Temperature below 100°C

i.Pasteurization of milk

For pasteurization of milk, there are two methods

• Holding Method or Low Temperature Holding Method (LTH)

In this method, the milk is exposed to a temperature of 63°C (145°F) for 30 minutes in an appropriately designed equipment. This is followed by sudden cooling to 13°C or below.

Flash Process or High Temperature Short Time (HTST)

In this method, the milk is exposed to a temperature of 72°C for 15 seconds in the equipment. This is followed by sudden cooling to 13°C or below. The finished product should be stored at a low temperature to retard growth of microorganisms and pasteurization removes the pathogenic bacteria in milk. By these processes all non-sporing pathogens such as mycobacteria, salmonellae and brucella are destroyed 'Coxiella bumetic' is relatively heat resistant and may survive the holder method.

b) Temperature at 100°C

i. Boiling

Most of the vegetative forms of bacteria, fungi etc. are killed almost immediately at 90-100°C, but sporing bacteria required considerable periods of boiling. Boiling water is not considered as a sterilizing agent because destruction of bacterial spores and inactivation of viruses cannot always be assured. Under ordinary circumstances, most species of microbes can be killed within 10 minutes. However, spores of bacteria and fungi, protozoa cysts and large concentrations of Hepatitis A viruses requires up to 30 minutes exposure or more because inadequate information exists on the heat tolerance of many microorganisms, boiling water is not reliable for sterilization purpose especially the sterilization of instruments and

for surgical procedures.In cases where boiling is considered adequate, the material should be immersed in water and boiled for a period of 10-30 minutes. The lid of the sterilizer should not be opened during that period. Addition of little acid, alkali or washing soda will increase the efficiency of the process.

ii. Steam under atmospheric pressure (100°C)

Steam under atmospheric pressure is used to sterilize culture media which may decompose if subjected to higher temperature. A Koch or Arnold sterilizer is an instrument that generates free floating steam.The container and the medium are simultaneously sterilized and evaporation from the medium is prevented one exposure of 90 minutes usually ensures complete sterilization of the medium. This is an inexpensive method.

iii.Sterilization above 100°C (steam under pressure)

Heat in the form of saturated steam under pressure is the most practical and dependable agent for sterilization. Steam under pressure provides temperature above those obtainable by boiling. Moreover, it has advantages of rapid heating, penetration and moisture in abundance, which facilitates the coagulation of the protein of microorganisms, resulting in complete destruction of all forms of microbial life, including bacterial endospores. It is important to note that the sterilizing agent is the moist heat not the pressure. The laboratory apparatus designed to use steam under regulated pressure is called an autoclave. It is essentially a double jacketed steam chamber equipped with devices which permit the chamber to be filled with saturated steam and maintained at a designed temperature and pressure for any period of time The articles to be sterilized are placed in the sterilizing chamber and steam is maintained in the steam jacket into the sterilizing chamber, cool air is forced out and a special valve increases the pressure to 15 pounds/square inch above normal atmospheric pressure. The temperature rises to 121.5°C and the superheated water molecules rapidly conduct heat into microorganisms and will be killed. The time for destruction of the most resistant bacterial spore is reduced to 15 minutes. For denser objects, up to 30 minutes of exposure may be required.

Filtration

is the process of removal of microorganisms from liquid or gases using a mechanical device known as filter. This is an excellent way to reduce the microbial population in solution of thermo labile materials such as sera, antibiotic solutions, intravenous solutions, carbohydrates solutions used in the preparation of culture media etc. As fluid passes through the filter, microorganisms are trapped in the pores of the filtering material. The solution that drips through the filter is collected in a previously sterilized container. Porosity, electric charges of the filter, electric charge carried by the organisms, nature of the fluid being filtered etc. can influence efficiency of filtration.

Irradiation

is an effective method of sterilization. Two types of radiations are used for sterilization.

a) Non ionizing radiation

- **Infrared radiation**

b) Ionizing radiation

- **X rays**
- **Gamma rays**

Chemical Methods

Characteristics of a desirable disinfectant

The disinfectant must be effective against a wide variety of infections/agents, at high dilutions and in the presence of organic matter. The chemical must be toxic for infection agents but it should not be toxic to people or corrosive for common upon storage, colourless with a pleasant odors, soluble in water and lipids for penetration into microbes and have a low surface tension so that it can cracks in surface. If possible, thedisinfectant should be relatively inexpensive.

The factor influencing the effectiveness of chemical disinfectants:

1. Size of the microbial population

2. Nature of microbes present

3. Concentration and nature of the disinfectant

4. Duration of exposure

5. Temperature

6. Local environment

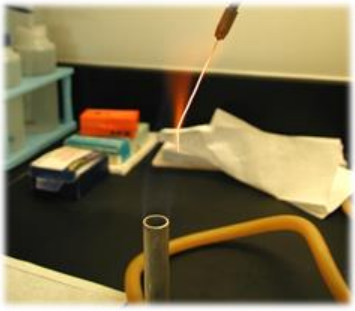
The main modes of action of disinfectant:

1. Protein coagulation

2. Disruption of cell membrane, thus resulting in exposure of the contents of the cell to the adverse

Sterilization by physical methods

- **Dry heat:**
  - Red heat (flaming):



Sterilize the metal objects like bacteriologic loops

Sterilization by physical methods

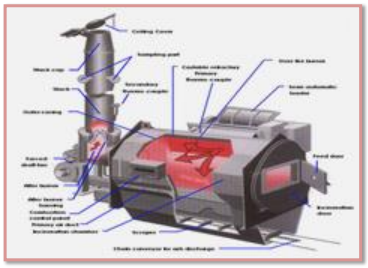
- **Dry heat:**
  - Hot air oven:

Temperature used 170° C for 1 hour.

Sterilize the glass objects

Sterilization by physical methods

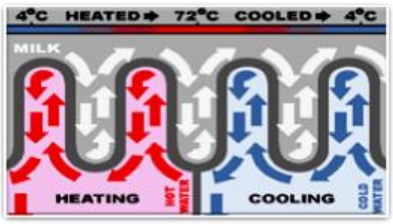
- **Dry heat:**
  - **Incineration**:



Get rid of all disposal products in hospitals or factories

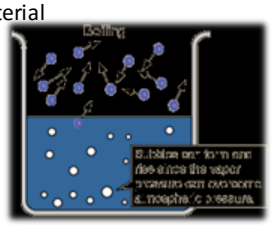
Sterilization by physical methods

- **Moist heat:**
  - Below 100°C is called **Pasteurization** used to get rid of organisms that transmitted by milk like brucella sp ,coxiella sp ,mycobacterium bovis.



Sterilization by physical methods

- **Moist heat:**
  - At 100°C is called **Boiling** not efficient because it can't kill the endospores & some bacterial toxins..

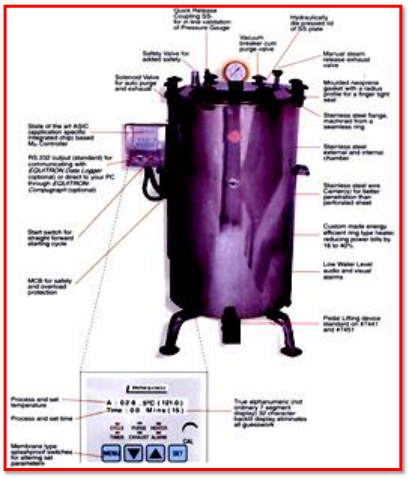


Sterilization by physical methods

- **Moist heat:**
  - **Autoclave**:
    - steam under pressure to raise the temperature to 121 C at 15-17 psi for at least 15 minutes.
    - At this elevated temperature all living cells, including endospores and viruses, are killed.

Sterilization by physical methods

- **Moist heat:**
  - **Autoclave:**

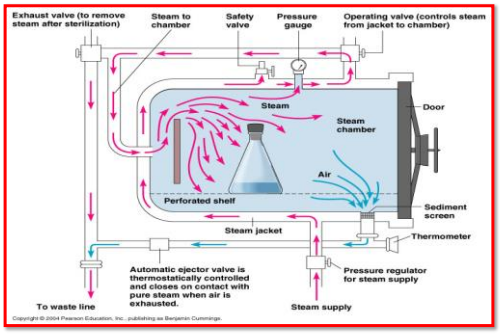


Sterilization by physical methods

- **Moist heat:**
  - **Autoclave:**

Sterilization by physical methods

- **Moist heat:**
  - **Autoclave:**



Sterilization by physical methods

- **Factors affecting the method:**
  - Pressure steam.
  - Temperature.
  - Time.
  - Type of media.
  - The organism

Sterilization by physical methods

- **Autoclave: Tests for efficiency**

Chemical

Chemical tapes
Bowie Dick tapes

oPrinciple:

oNotice the change of the colour of the tape

Sterilization by physical methods

- **Autoclave: Tests for efficiency**

Biological

Spores of Bacillus sp

oPrinciple:

oPrepare spore in tubes which cntains (sugar & indicator).

oAfter autoclaving the tubes were incubated for 48 hrs.

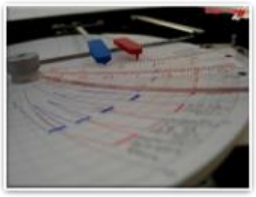
oThe change in colour of the indicator means that the spore had germinate and utilize the sugar so the process is failed

Sterilization by physical methods

- **Autoclave: Tests for efficiency**

Physical

Digital

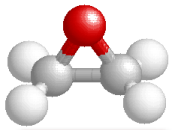


Graphs

Slide 16

Sterilization by Chemical methods

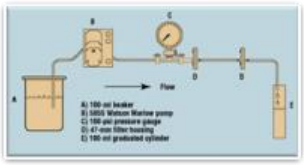
- **Low Temperature Steam formaldehyde & ethylene oxide (gaseus sterilization):**
  - Very low amount of ethyline oxide used to sterilize medical equipments



Ethylene oxide

Sterilization by physical methods

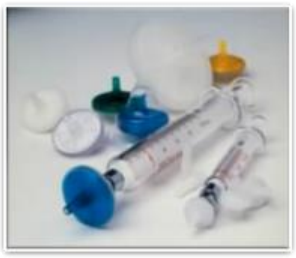
- **Filtration:**



Membrane filter

Sterilization by physical methods

- **Filtration:**



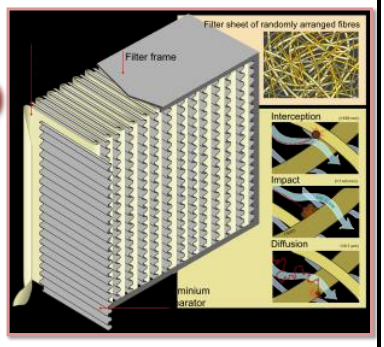
Syringe filters

Sterilization by physical methods

- **Filtration:**

HEPA filter

High Efficient Particulate Air filter

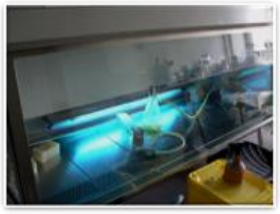


Sterilization by physical methods

- **Radiation**:
  - Ionizing Radiation (γ rays):
  - Used to sterilize plastic equipments like syringe & catheters

Sterilization by physical methods

- **Radiation:**
  - Ultra-Violet radiation:
  - Sources:
    - Natural: The Sun.
    - Artificial: UV lamps (Mercury-Vapor lamps)
    - For sterilization of operating theatre and theatre equipments.



Disinfectants

- The aim is to decrease the chance of the micro-organisms to cause an infection.

Disinfectants

- **Factors affecting the disinfectants:**
  - Concentration of the disinfectant.
  - Time of exposure.
  - The Type of the organism.
  - The Amount of the organism.
  - The temperature.
  - The pH.
  - Presence of organic material that enhance the growth of the organism (like blood)

Disinfectants

- **Groups of disinfectants:**

Phenolics disinfectants

Disinfectants

- **Groups of disinfectants:**

Hypochlorites

- They are active against Gram+ve & Gram–ve bacteria & viruses.

Disinfectants

- **Groups of disinfectants:**

Glutaraldehyde

Disinfectants

- **Groups of disinfectants:**

Ethyle alcohole 70%

–)Isopropyl alcohol) highly active against Gram+ve & Gram–ve bacteria & acid fast bacilli.

Disinfectants

- **Groups of disinfectants:**

Formaldehyde

Disinfectants

- **Groups of disinfectants:**

Antiseptics

Chlorhexidine Gluconate, Cetrimide

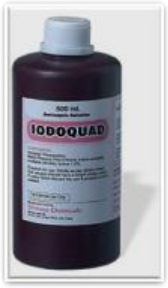
Disinfectants

- **Groups of disinfectants:**

Antiseptics

Parachlorometaxylenol
PCMX

Propyl alcohol



Mycology

CULTURE MEDIA FOR FUNGI

Clinical specimens are processed promptly and plated on isolation media to recover fungi that may be causing disease. Media and incubation temperatures are selected to allow for the growth of pathogenic and opportunistic yeasts and fungi.

NB. If Histoplasma, Coccidioides, Paracoccidioides, Blastomyces or Penicillium marneffei are suspected, Category 3 Containment is required.

ISOLATION MEDIA

A variety of media are available for the primary inoculation and recovery of fungi from clinical specimens. No one specific medium or combination of media is adequate for all specimens. Media must be carefully selected based on specimen type and fungal suspected agents. Media is dispensed into containers such as screw cap tubes or Petri dishes. Petri plates offer the advantage of a large surface area for isolation and dilution of inhibitory substances in the specimens, but must be poured thick with at least 25 ml of medium to resist dehydration during incubation. Because plates are vented, they are more likely to become contaminated during incubation.

All inoculated media should be read every 2 days following incubation and twice weekly thereafter. Plates must be opened only within a biological safety cabinet to prevent contamination of the plate and exposure of personnel to potentially dangerous fungi.

Media in tubes have a smaller surface area but offer maximum safety and resistance to dehydration and contamination. If the specimen is from a contaminated site, it is important to include media that contain inhibitory substances such as chloramphenicol, gentamycin, or cycloheximide. Chloramphenicol or gentamycin will inhibit most bacterial contaminants, while cycloheximide inhibits most saprobic moulds. It is important to remember that cycloheximide may also inhibit opportunistic fungi such as some species of Aspergillus, Fusarium, Scopulariopsis, Pseudallescheria, zygomycetes, some dematiacious fungi, and yeasts such as Cryptococcus neoformans and some Candida species. Antibacterial agents may inhibit the growth of aerobic

actinomycetes like Nocardia sp. It is important to use media with and without inhibitory agents. Specimens from normally sterile sites can be inoculated to media without inhibitory substances.

A. Sabouraud Dextrose Agar (SAB)

2% glucose and is slightly acidic (pH 6.5). It is the standard medium for recovery and maintenance of a wide variety of fungi commonly isolated in the clinical laboratory. The original SAB formulation specifies 4% glucose. Emmons' modification with less glucose is preferred as an isolation medium because some isolates, notably Blastomyces dermatitidis may not be recovered using the original Sabouraud formulation.

B. Brain-Heart Infusion Agar (BHI)

BHI is an enriched medium that enhances the recovery of Cryptococcus neoformans from sterile specimens such as CSF. BHI is also used in yeast-mould conversions for Sporothrix and Paracoccidioides.

C. Brain-Heart Infusion Agar + Gentamycin + Chloramphenicol (16 µg/ml) + 10% sheep's blood

BHI + GC + 10% bl is an enriched medium useful for the recovery of fungi such as C.

neoformans from contaminated specimens.

F. Blood Culture Media

Two plated media are commonly used in conjunction with Isolator Lysis centrifugation:

1. Inhibitory Mould Agar with chloramphenicol and gentamycin for the isolation of fungi and the inhibition of bacteria.

2. Brain-Heart Infusion Agar for fungi and bacteria, which may be substituted for Chocolate Agar.

G. Brain-Heart Infusion Broth

BHI broth with penicillin is added to the normal battery when zygomycetes are suspected. These fungi can be very difficult to recover. The use of broth

provides optimal medium/specimen contact. The aseptic addition of sterile penicillin discs will inhibit bacteria. Malt extract agar is an effective alternative to broth media for the isolation of zygomycetes.

H. Sterile Bread

Sterile bread without preservatives is recommended for the recovery of zygomycetes from clinical specimens. Bread is often superior to other media for recovering this group of opportunistic pathogens. A piece of bread is sterilized in a humidified Petri dish. Specimens from non-contaminated sites can be directly inoculated. Contaminated specimens should be treated with antibacterial agents before inoculation. Zygomycetes will grow rapidly, often filling the entire Petri dish within a few days.

I. Malt Extract Agar

Malt agar is a useful alternative to bread for recovery of zygomycetes and is excellent for environmental cultures.

J. Yeast Extract-Phosphate Medium (YEP)

YEP medium was developed for the enhanced recovery of Blastomyces dermatitidis and Histoplasma capsulatum from contaminated specimens. The incorporation of chloramphenicol inhibits bacteria and the addition of a drop of concentrated ammonium hydroxide (approximately 58%) inhibits bacteria and yeasts.

1. Place 0.05-0.1 ml of specimen on surface of yeast extract-phosphate agar plate and streak.

2. To one side of the plate, opposite from the heavily streaked area, immediately add 1 drop (approximately 0.05 ml) of concentrated ammonium hydroxide (NH_4OH) using a sterile pipette. Do not streak, but allow NH_4OH to diffuse through the agar.

3. Wait 24 hours; seal the plates with Sellotape.

4. Incubate at room temperature (25-30°C) for 6 weeks before discarding as negative.

K. Dermatophyte Test Medium (DTM)

DTM is used to recover dermatophytes from heavily contaminated clinical specimens and to presumptively indicate the presence of a dermatophyte. Dermatophytes, as well as a few other fungi and bacteria turn the medium from pink to red.

Needle or Sellotape Lactophenol Mount

The Needle or Sellotape 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 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 plates.

Procedure

(1) Cut a strip of transparent Sellotape and place ends between thumb and index finger, gummed side out.

(2) Making a loop by closing fingers, open plate with opposite hand and press tape against the colony to be identified.

(3) Place a drop of lactophenol on a labeled slide.

(4) Press tape against slide with lactophenol.

(5) Smooth the tape back on the slide by opening fingers and using gauze.

(6) Place another drop of lactophenol on top of the tape.

(7) Place a large 20 x 40 mm coverslip on top of slide.

(8) Examine the slide under the microscope.

Germ tube test:

Inoculate a small amount of each yeast into 0.5 ml of horse serum. Incubate them in a water bath at 37°C for 1.5-2.0 hr. Remove a drop of the serum onto a microscope slide, add a coverslip and examine microscopically for germ tube production

Dermatophytes

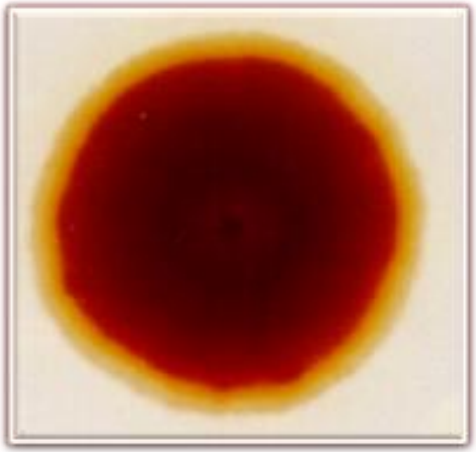
Microsporum gypseum

- flat, spreading, granular, with a deep cream to tawny-buff to pale cinnamon coloured

Sabouraud's agar

1

Dermatophytes

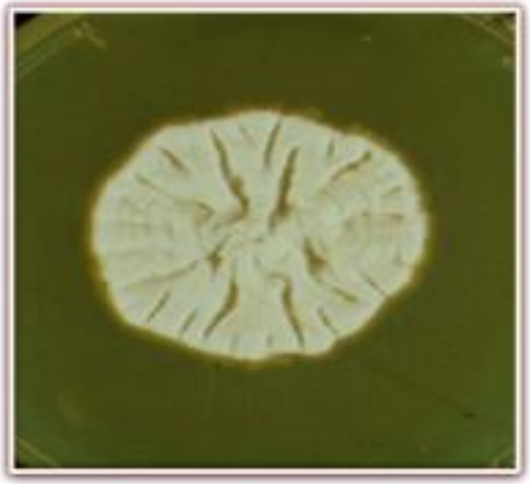


Trichophyton rubrum

- Brown to wine-red

Sabouraud's agar

Dermatophytes



Trichophyton verrucosum.

- slow growing, small, button-or-disk-shaped, white to cream coloured

Sabouraud's agar

3

Dermatophytes

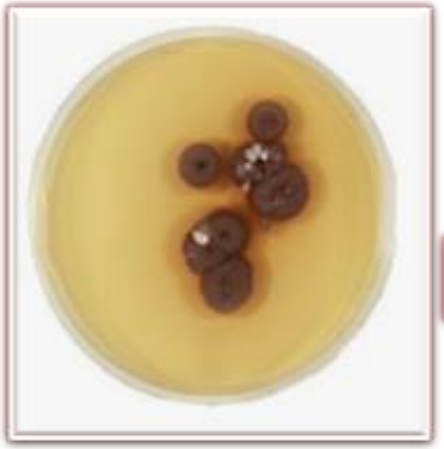
Trichophyton schoenleinii

- waxy yellow with a deeply folded honey-comb-like

Sabouraud's agar

4

Dermatophytes



Trichophyton violaceum

- heaped and folded and a deep violet in colour

Sabouraud's agar

5

Dermatophytes

Trichophyton tonsurans.

- colour may vary from pale-buff to yellow, to dark-brown

Sabouraud's agar

6

Dermatophytes

Trichophyton soudanense

- characteristically it is a deep apricot-yellow (orange) in colour

Sabouraud's agar

Dermatophytes

Epidermophyton floccosum

- greenish-brown or khaki coloured

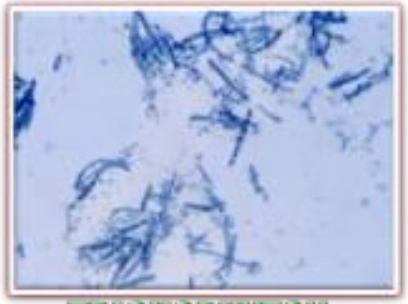
Sabouraud's agar

8

Dermatophytes

MICROSPORUM

- Spindled shape macroconidia & few microconidia



TRICHOPHYTON

- Many microconidia & scanty penciled-shaped macroconidia

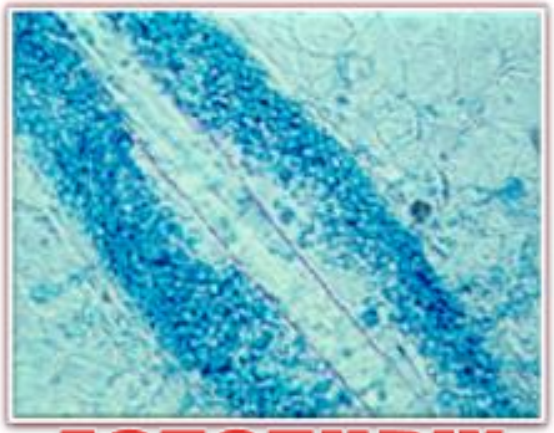
EPIDERMATOPHYTON

- Finger shaped macroconidia and no microconidia

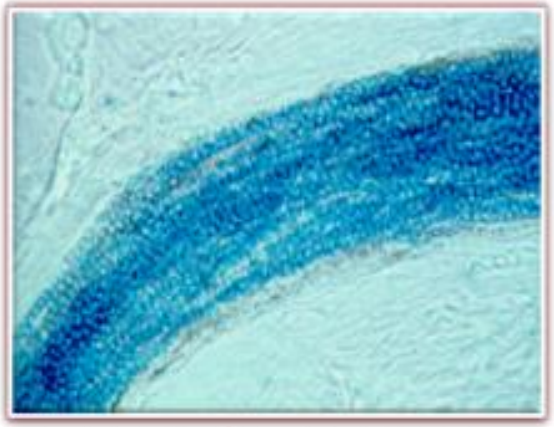
Needle mounts (Lacto-phenol – Cotton blue staining)

9

Dermatophytes



ECTOTHRIX

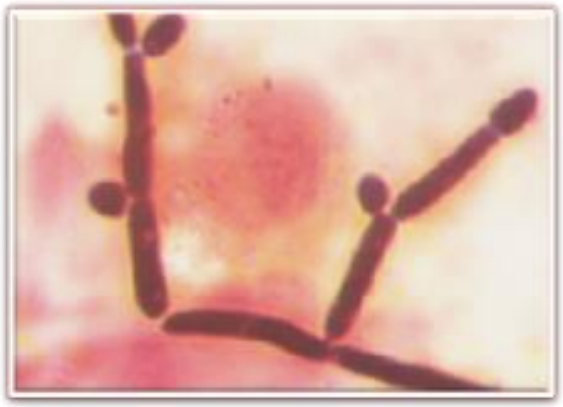


ENDOTHRIX

Spore formation in the hair follicle

11

Candida

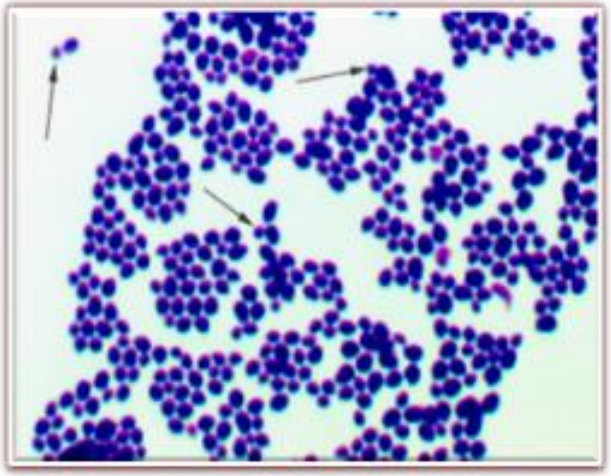


Yeast with Pseudohyphae,
Constrictions at ends,
budding yeast along hyphae.

Direct Microscopy

12

Candida

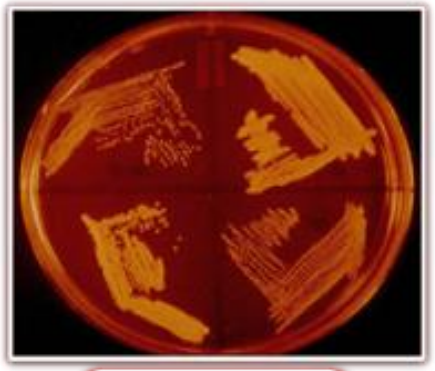


- Large G+ve budding yeast

Gram stain

13

Candida



Blood agar

Sabouraud''s agar

- Large white-creamy colonies

Culture

14

Candida

- Incubation in horse serum only Candida albicans can form these tube like structure

Germ Tube Test (G.T.T)

15

Candida

Corn Meal Agar

Chlamydospores

Chlamydospores formation

Candida

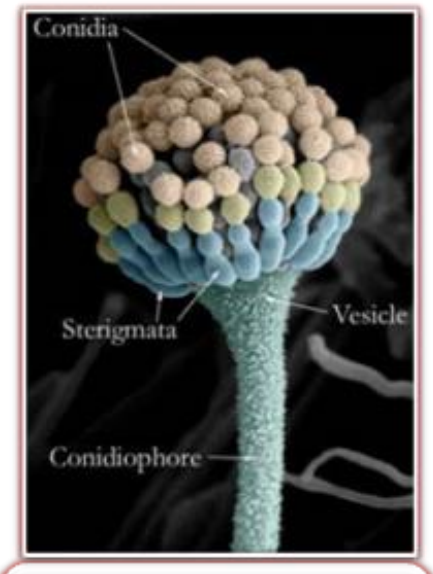
Candida albicans requires both nitrogen & carbon as essential growth factors.

Opacity will appear around both nitrogen & carbon disks

Auxanogram (Sugars & Nitrogen Assimilation)

17

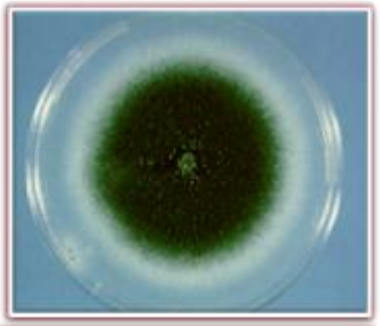
- Aspergillus



Aspergillus head

1

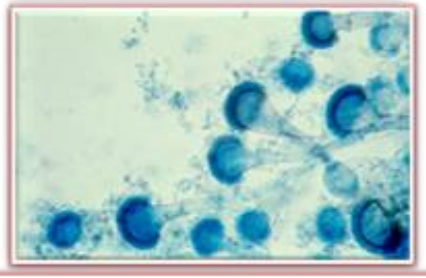
Aspergillus



- colonies show typical blue-green surface pigmentation

Culture

aspergillus fumigatus

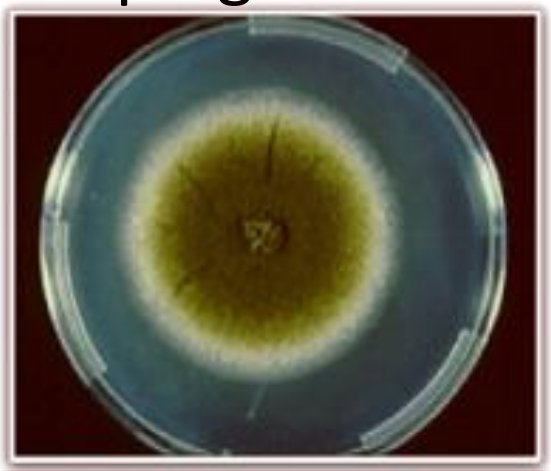


- Conidial heads are columnar short.
- Conidiophores are short, smooth-walled vesicle and have conical or globose shaped.
- 1 raw strigmata.

(Lacto-phenol – Cotton blue staining)

2

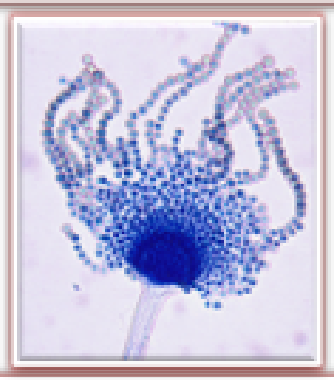
Aspergillus



- colonies show typical bright to dark yellow-green

Culture

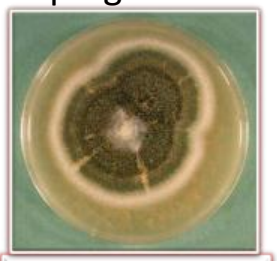
aspergillus flavus



- Conidial heads are typically radiate, later splitting to form loose columns,
- Conidiophores are hyaline and coarsely roughened.
- Vesicles are oval.
- 1or 2 raws strigmata.

(Lacto-phenol – Cotton blue staining)

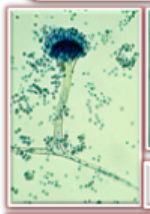
Aspergillus

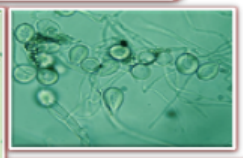


- Colonies are typically plain green in color with dark red-brown periphery

Culture

aspergillus nidulans





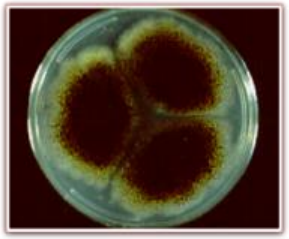
Thick-walled horse-shoe Hülle cells of aspergillus nidulans.

- Conidial heads are short, parallel .
- Conidiophores are usually short & smooth-walled .
- Vesicles are round and rough-walled.

(Lacto-phenol – Cotton blue staining)

4

Aspergillus



- colonies are typically plain brown-black

Culture

aspergillus niger

- Conidial heads are large globose,
- Conidiophores are smooth-walled, with large rounded vesicle
- Conidia are irregular

(Lacto-phenol – Cotton blue staining)

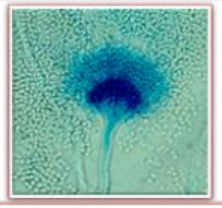
5

Aspergillus

- Colonies are typically plain brown in color with a yellow to deep dirty brown reverse

Culture

aspergillus terreus

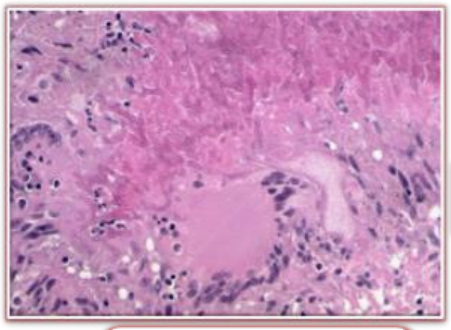


- Conidial heads are large, columnar. Conidiophores are hyaline and smooth-walled with small rounded vesicle..
- Conidia are irregular.

(Lacto-phenol – Cotton blue staining)

6

Aspergillus



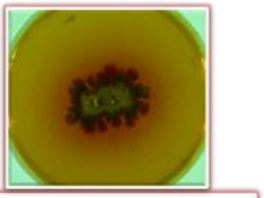
- Dense granuloma surrounding fungal hyphae

Histopathology

7

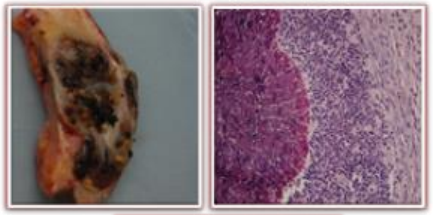
Mycetoma

Madurella mycetomatis



- Colonies are brown-black

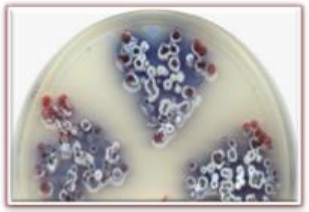
Culture



- Black grains (H/E)

Grains

Mycetoma



- Colonies colour start as dirty yellow then changes to be gray

Culture

Stremtomyces somaliensis

- Hard Yellow grains

Grains

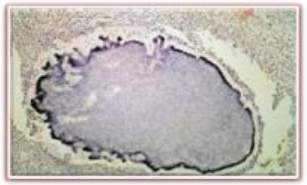
10

Mycetoma

actinomadura madurae

- Colonies are white cerebriform

Culture

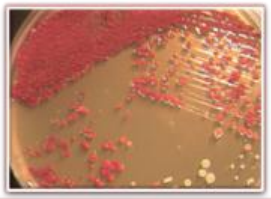


- White soft grains (H/E)

Grains

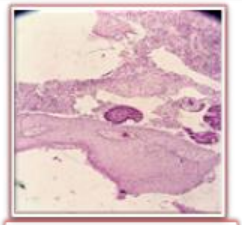
Mycetoma

actinomadura pelliterii



- Colonies are dry red wrinkled

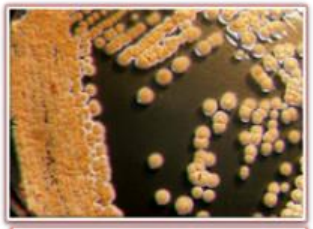
Culture



- White soft grains

Grains

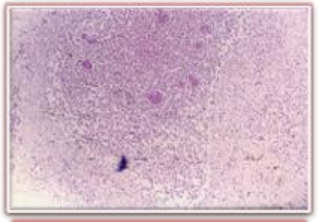
Mycetoma



- Colonies are white creamy then change to orange

Culture

Nocardia brasiliensis



- small White hard grains

Grains

Parasitology

Intestinal parasites

Faecal samples are samples gotten from faeces and sent to the laboratory in a suitable container for examination. It is a specimen commonly used for the detection of intestinal parasites.

1. Collect faecal samples
2. Number faecal samples
3. Macroscopic and Microscopic examination of faecal samples
4. Technique/Method of preparing slides for microscopic examination MAIN BODY

Laboratory Techniques For Selected Intestinal Parasites

The most commonly used specimen for the detection of intestinal parasites is faeces . Faecal specimens are examined for the presence of trophozoites and cysts of protozoa, eggs or ovae and larvae of helminths as shown below. Whole adult worms or segments of some worms may also be seen.

Trophozoites , cysts, eggs or ovae and larvae can be seen only with the microscope , but the adult worms or segments of tapeworms can be seen with the naked eye.. Samples of faeces must be properly collected, processed and examined for the detection of parasites.

Number Of Specimens

The number of specimens required to detect intestinal parasites will depend on the quality of the specimen, the severity of infection and the accuracy of the examination performed. For a routine examination for parasites before treatment, examination of three consecutive specimens is recommended. However, examination of specimens collected on alternate days shows a higher percentage of positive findings. A maximum of six specimens, collected

Visual Or Macroscopic Examination Of Faeces

Before processing the specimen of faeces, it should be visually examined. Its colour, consistency and presence of blood, pus, mucus or parasites should be reported.COLOUR: Normally, stool or faeces is brown in colour. Variation from this colour may occur under certain conditions. Reddish colour may be due to bleeding from the lower gastrointestinal tract. Consumption of beet-root may also give a red colour to the stool.

Preparation of wet mounts reagents:

(i) Saline, physiological (0.85%)

(ii) Lugol's iodine

Stock (5%) solution

Iodine 5g

Potassium iodide 10g

Distilled water 100ml

Dissolve the potassium iodide in about 30ml of water and add the iodine. Mix

until dissolved and make up the volume with distilled water store in a brown

bottle.

Technique/method:

(i) Place a drop of saline in the centre of the left of the slide and place a drop of

iodine solution in the centre of the right half of the slide.

(ii) With an applicator stick, pick up a small portion of faeces from an appropriate site

and mix it with saline on the slide to form a uniform suspension. In the same way,

prepare a suspension of faeces in the iodine on the slide.

(iii) Cover the drop of saline suspension with a cover slip by holding it at an angle and

lowering it gently on to the slide to reduce formation of air bubbles. Cover the PREPARATION OF WET MOUNTS

REAGENTS:

(i) Saline, physiological (0.85%)

(ii) Lugol's iodine

Stock (5%) solution

Iodine 5g

Potassium iodide 10g

Distilled water 100ml

Dissolve the potassium iodide in about 30ml of water and add the iodine. Mix

until dissolved and make up the volume with distilled water store in a brown

bottle.

Examination

a. Focus on the wet mount using a lower power (x10) objective.

b. Regulate the light with the sub stage condenser, the diaphragm and the light

source. Since most of the parasites are pale or colourless, too much or too little

light may not be useful. Lowering the condenser and closing the diaphragm

partially can give adequate light.

c. Examine the entire cover slip in a systemic order. Focus the objective on the

top left hand corner and move slide slowly up and down or backwards and forwards. When any parasite or suspicious material is

observed, change to the high-dry objective (x40). Increase the light by opening the sub stage

Concentration methods

It may be necessary to use concentration methods for the detection of faecal parasites for the

following reasons:

(i) The parasites are not detected in the direct microscopic examination but the

symptoms of intestinal parasitic infection still persist.

(ii) The eggs of parasites such as Taenia species or Schistosoma species are usually

few in number, and therefore, may have been missed out in the direct wet mount.

(iii) After treatment, it is necessary to check if it has been effective

(iv) To investigate the prevalence and incidence of parasitic infection for

epidemiological purposes.

There are two types of concentration methods, floatation techniques and sedimentation

techniques. These methods employ the use of specific gravity to separate most of the faecal

debris from the parasites.

NOTE: A floatation technique uses a liquid with high specific gravity for the separation of

protozoan cysts and certain helminthic eggs and larvae from the faecal debris. The parasites

Urine sample

Parasites which can be detected in Urine are:

(i) Eggs of Schistosoma haematobium

(ii) Microflariae of Wuchereria bancrofti

(iii) Trophozoites of Trichomonas vaginalis

If present in sufficient numbers, these parasites can be detected while examining centrifuged

deposits of urine. In areas where schistosomiasis is endemic, the first indication of infection is haematuria which can be detected either by chemical test or microscopically. A heavy infection with Schistosoma may lead to gross haematuria which is seen visually. A milky urine may show microfilariae of Wuchereria bancrofti.

Collection of urine:

A special care is needed for the collection of urine for suspected schistosomiasis because the number of ova excreted in urine varies throughout the day. It is highest in the terminal portion of the urine between 10.00am to 2.00pm. The last few drops of urine contain the maximum number of eggs. Therefore, the specimen should be collected between these times and should be terminal urine at least 10ml in volume.

Alternatively, a 24 hour specimen may be collected with formalin (1 ml per 100ml of urine) as a preservative for the eggs. If it is not possible to examine the fresh specimen within one hour of collection, this too, should be preserved with formalin using the same proportion. It is advisable to examine large volume of urine because the ova are very scanty and can be easily missed. If not preserved with formalin, the eggs may hatch to release miracidia.

Examination Of Urine For The Eggs Of Schistosoma Haematobium

There are two methods in use for the detection of Schistosoma eggs in Urine.

(i) The sedimentation technique is less sensitive, but cheaper and simpler to perform.

(ii) The filtration technique is used when quanttitive information is needed, eg in

public health laboratory.

NOTE: ONLY SEDIMENTATION TECHNIQUE IS DISCUSSED IN THIS MANUAL

Sedimentation technique/ method

(i) Collect a 24 hour or terminal urine sample as explained above, and check for

haematuria.

(ii) Shake the urine well and allow it to sediment for 1 hour.

(iii) At the end of 1 hour, carefully withdraw the supernatant without disturbing the

sediment.

(iv) Transfer the sediment to a centrifuge tube and centrifuge at 500g for 5 minutes

(v) Discard the supernatant and examine the sediment as a wet preparation, using the

10x objective to search for the ova of Schistosoma haematobium.

Blood sample

The parasites which can be detected in blood are:

(i) Plasmodia

(ii) Trypanosomes

(iii) Leishmania

(iv) Filarial worms

Some parasites such as microfilariae and trypanosomes can be detected in the direct wet mount of fresh blood by their characteristic shape and motility. However, specific identification of the parasite requires a permanent stain. For permanent staining, two types of blood films can be prepared. Thick films allow a larger volume of blood to be examined, thus making it easier to detect light infections with fewer parasites, while species identification is difficult. Thin films are necessary to see the morphological characteristics of the parasites and to identify them.

Collection of specimens for blood films:

CAUTION: Careful attention to safety technique is necessary at the time of collection of blood samples and preparation of blood films. A number of parasitological, bacterial and viral diseases can be transmitted through blood.

Blood films should be prepared before the commencement of any treatment. It is preferable to prepare blood films with fresh blood without anti-coagulant. If it is not possible, blood anti-coagulanted with EDTA (10mg/5ml blood) should be used; and blood films should be prepared as soon as possible, preferably, within one hour of collection.

Preparation of blood films

For accurate examination of blood films, it is necessary to use absolutely clean, grease-free slides. Well washed slides cleaned with 70% alcohol are recommended.

Thick blood films

To make a thick film, place two or three small drops of fresh blood without anticoagulant on a clean slide. With a corner of another slide, mix the drops in a circular motion over an area about two cm in diameter. Continue mixing for about 30 seconds to prevent formation of fibrin strands that may obscure the parasites after staining. Allow the film to dry in air at room temperature. Before staining, the thick films are laked to lyse the red blood cells and to remove haemoglobin so that the parasites can be easily detected. To lake the films, they are either placed in buffer solution before staining or placed directly into an aqueous stain like Giemsa stain.

Thin blood films

In thin films, the number of parasites is much less than in the thick films, but it permits specific identification of parasites.

The thin film is prepared in exactly the same way as the one used for a peripheral blood smear examination. Allow the thin blood film to air-dry. The need for fixation before staining depends on the type of stain used.

Staining blood films

Blood films should be stained as soon as possible as delay may result in stain retention. Romanowsky stains such as Giemsa, Leishman or Field, can be used for staining of parasites in blood film. Leishman's stain has the fixative combined with the staining solution, so that fixation and staining both occur at the same time. Therefore, the thick film must be laked before staining by Leishman stain. In

Giemsa and Field stains, on the other hand, the fixative and the stain are separate. Thus the thin film must be fixed in methanol before staining. After staining, the smears should be air-dried.

Giemsa staining technique/method

Giemsa stain is a Romanowsky stain that requires dilution in buffered water or buffered saline before use. The stain is available commercially either as a concentrated stock solution or in a powdered form.

GIEMSA STAIN (stock solution)

Giemsa stain powder 0.6g

Methanol, absolute (acetone-free) 50ml

Glycerol 50ml

GIEMSA STAIN (working solution)

The stock should be diluted 1:10 with buffer for thin films and 1:50 for thick films.

Phosphate buffer used for the dilution of the stain should be neutral or slightly alkaline (pH7.0 to 7.2). If the pH of the Tap water in the laboratory is satisfactory, it may be used for

the entire staining procedure, evening for the final rinsing.

Technique /method for thin films

(i) Fix the thin blood film in absolute methanol for 1 minute. If both the thick and the thin films are on the same slide, carefully dip only the thin smear in methanol. Allow the smear to dry in air.

(ii) Prepare an appropriate dilution of the stock Giemsa stain using the phosphate buffer or tap water.

(iii) At the end of the staining period, gently flush the stain off the slide with water. Do not tip off the stain before washing, as this will leave stain deposits over the smear.

(iv) Dip the slide briefly in the buffer or rinse under gently running tap water.

(v) Wipe the under-surface of the slide to remove excess stain

(vi) Allow it to air-dry in a vertical position.

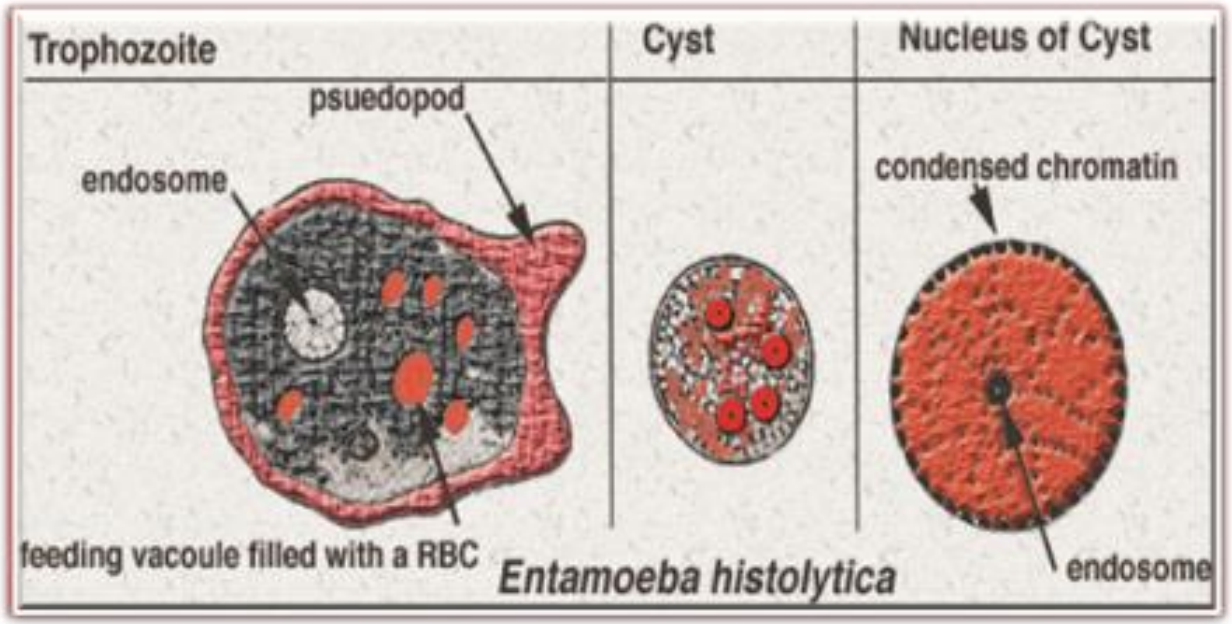
Technique/method for thick film

The procedure to be followed for thick films is the same as that for thin films except that the thick film should not be fixed in methanol, but directly stained in diluted Giemsa.

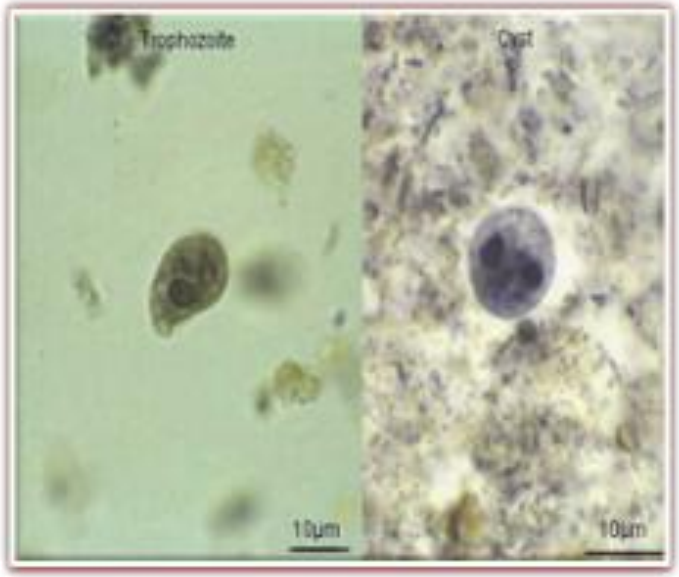
RESULTS: Malarial parasites have dark red chromatins, blue cytoplasm, red Schuffner's

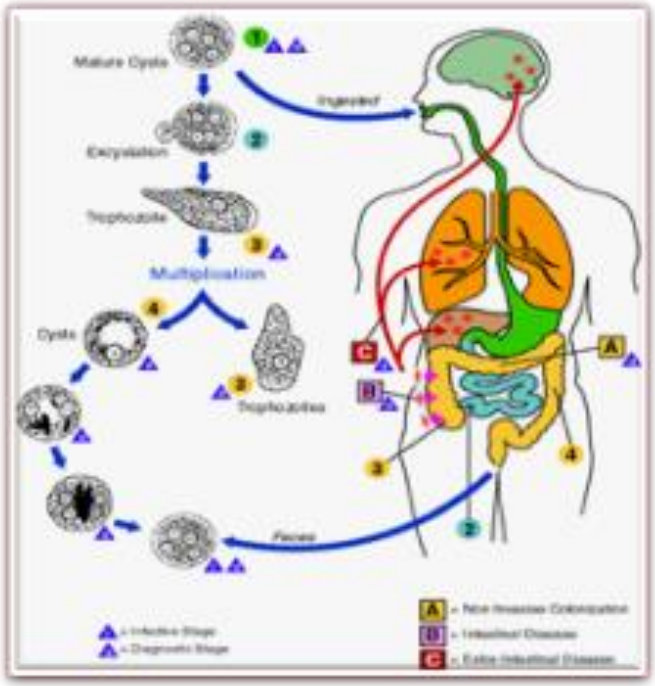
dots and red-mauve Maurer's dots.

Entamoeba histolytica



Entamoeba histolytica





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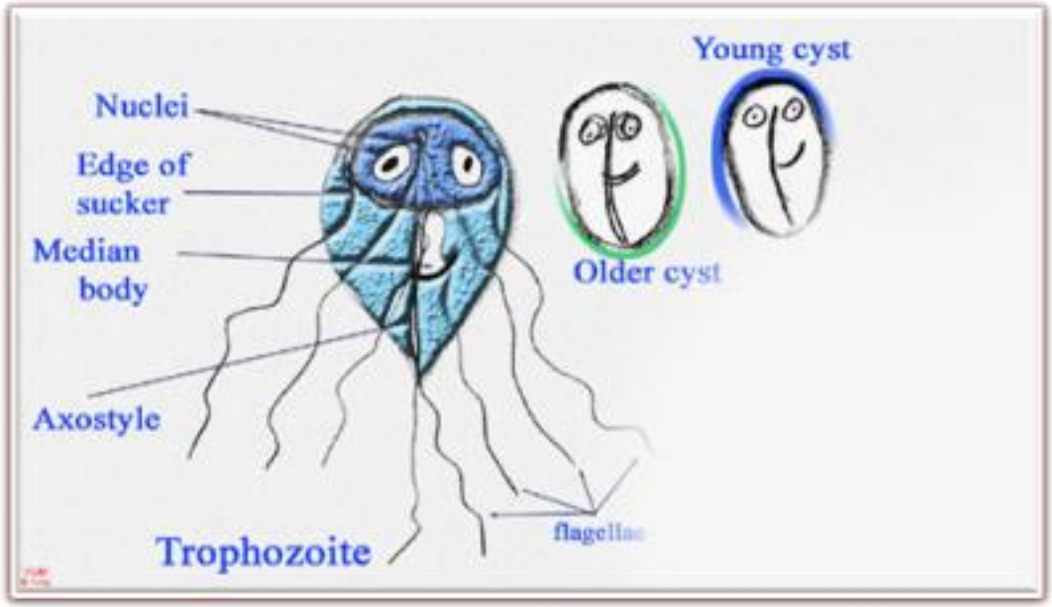
Giardia Lamblia



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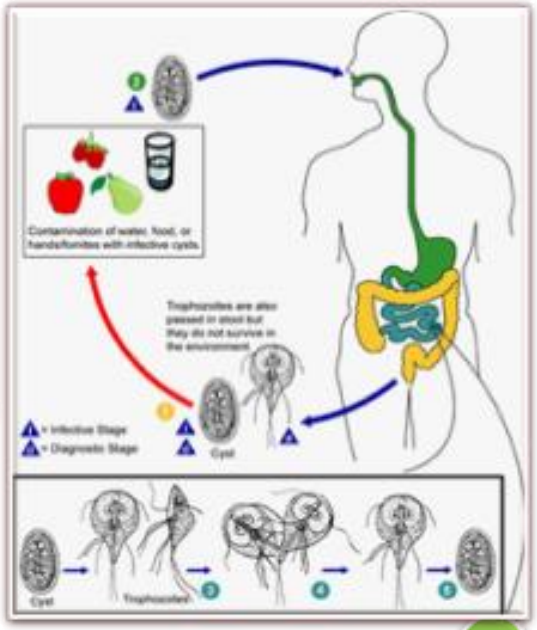
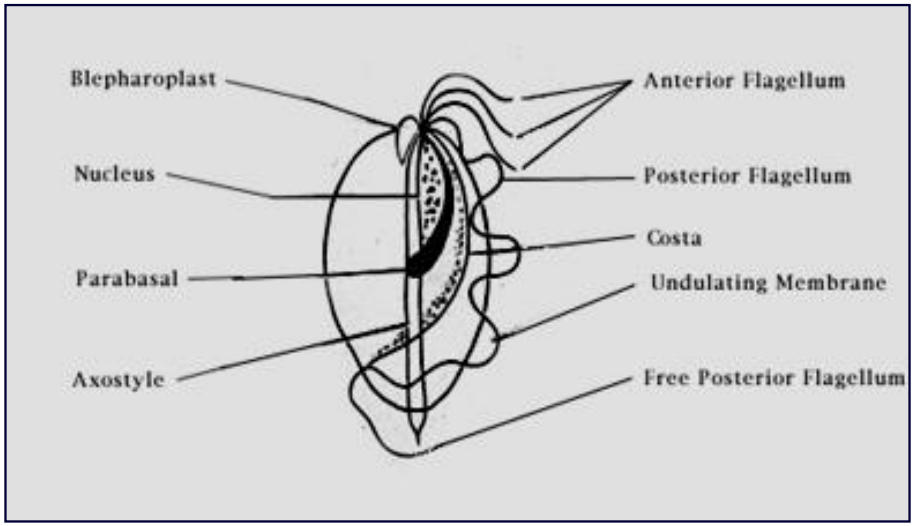
Giardia Lamblia



Trichomonas vaginalis



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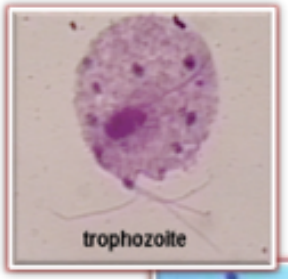
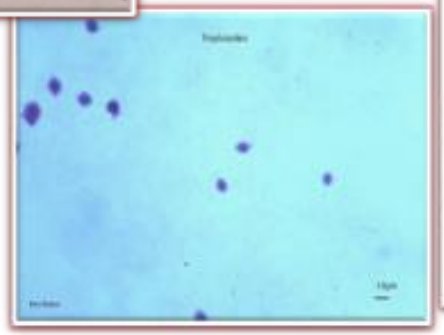
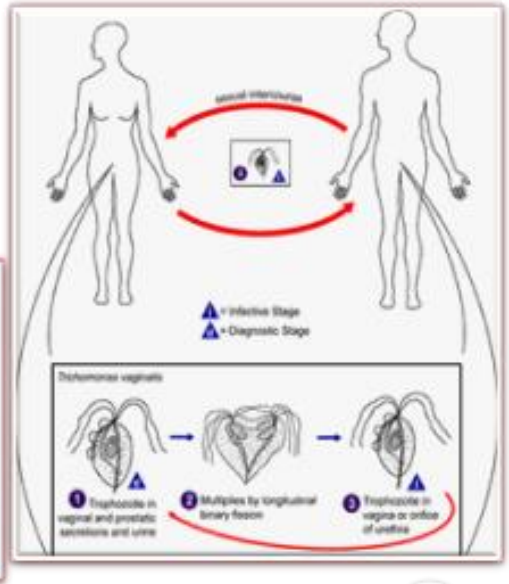
Trichomonas vaginalis

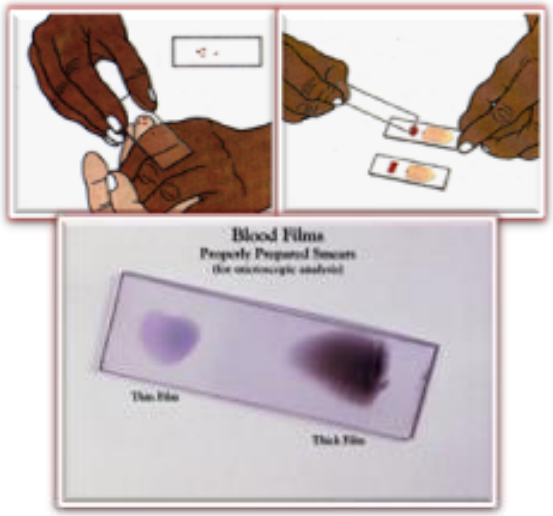






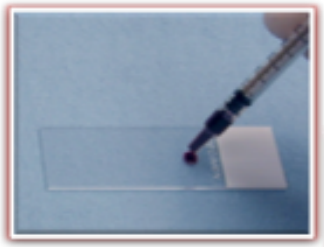
6

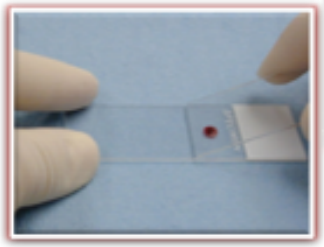
Blood films for Malaria

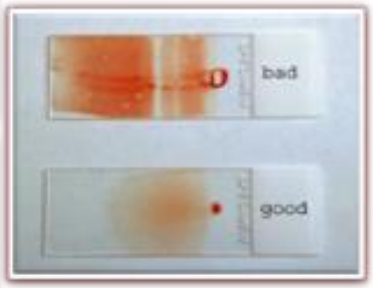


- Thin films
  - allow species identification, because the parasite's appearance is best preserved in this preparation.
- Thick films
  - allow the microscopist to screen a larger volume of blood and are about eleven times more sensitive than the thin film.
  - picking up low levels of infection is easier on the thick film.
  - but the appearance of the parasite is much more distorted and therefore distinguishing between the different species can be much more difficult.

Thick blood film

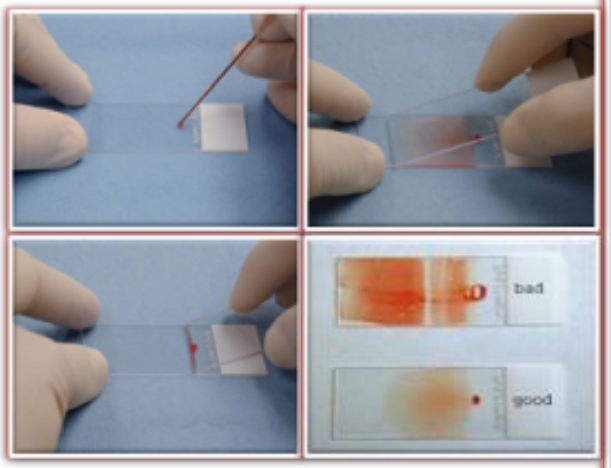






- Three drops of blood (0.05) are put close together on the slide.
- Spread by the corner of other slide to cover an area of about 1 cm in diameter.

Thin blood film



- Place a small drop of blood near one end of a slide & immediately
- The end of a second slide is applied to the first slide at angle of 45°.
- the end of the second slide is then drawn back to contact the drop then spread along the first slide.
- The spreader should have both corners cut off so that the resultant film is narrower than the slide.

3

Field's method to stain thick blood films:

Field's Stain: is composed of

1. Solution A: Methylene blue + buffered water
2. Solution B: Eosin + buffered water.

1. Dip the slide in solution A for 5 seconds.
2. Immediately wash by waving gently in clean tap water for seconds.
3. Dip the slide in solution B for 2-3 seconds.
4. Wash again with tap water for 5 seconds.
5. Put the slide upright to dry.

4

Leishman's method to stain thick blood films:

Leishman's stain is composed of:

1. Leishman's powder.
2. Methanol

1. Place the slide absolutely on staining rack
2. Cover the film with some drops of Leishman's powder and leave for minute or half.
3. Add equal number of drops of buffered distilled water of pH 7.2. mix well with water pipette and allow to stain for 10 mints.
4. Flood off quickly with water (never tilt the slide) to wash and put the slide upright to dry.

5

Giemsa's method to stain blood films:

Giemsa's stain is composed of:

1. Giemsa's powder.
2. Glycerine
3. Methanol

for thin blood films:

1. Fix film in methyl alcohol for 3 mints.
2. Cover the film with diluted stain (1:10).
3. Leave for 25-30 mnts.
4. Wash with tap water.
5. Blot to dry.

for thick blood films:

1. To the dry film pour the diluted stain (3%).
2. Leave for 30 mnts.
3. Wash with tap water.
4. Blot & allow to dry in air.

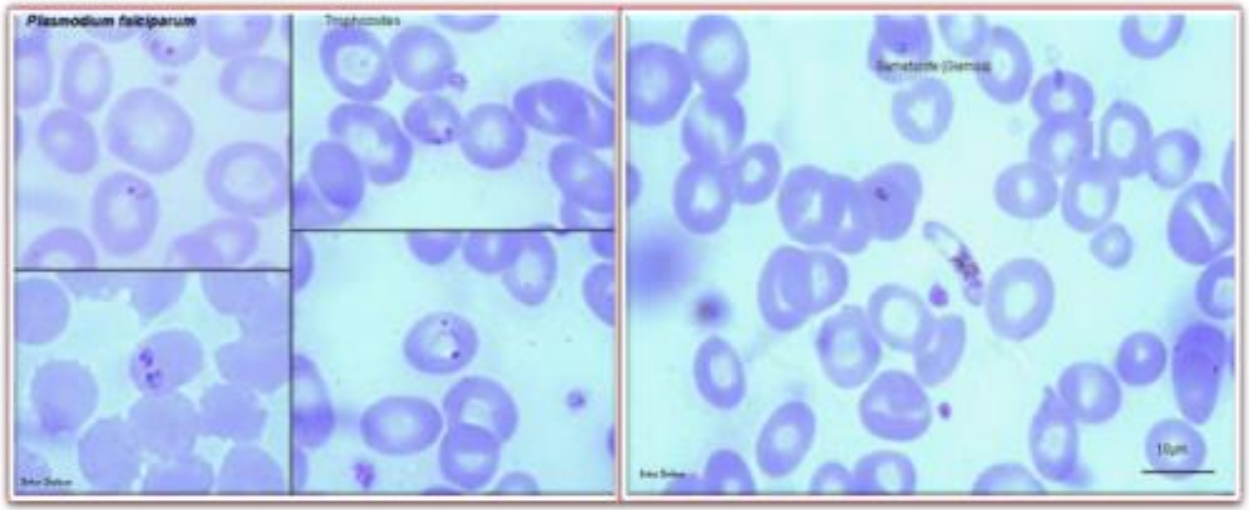
6

Plasmodium falciparum

1. Red Cells are not enlarged.
2. Rings appear fine and delicate and there may be several in one cell.
3. Some rings may have two chromatin dots.
4. It is unusual to see developing forms in peripheral blood films.
5. Gametocytes have a characteristic crescent shape appearance.
6. Maurer's dots may be present.

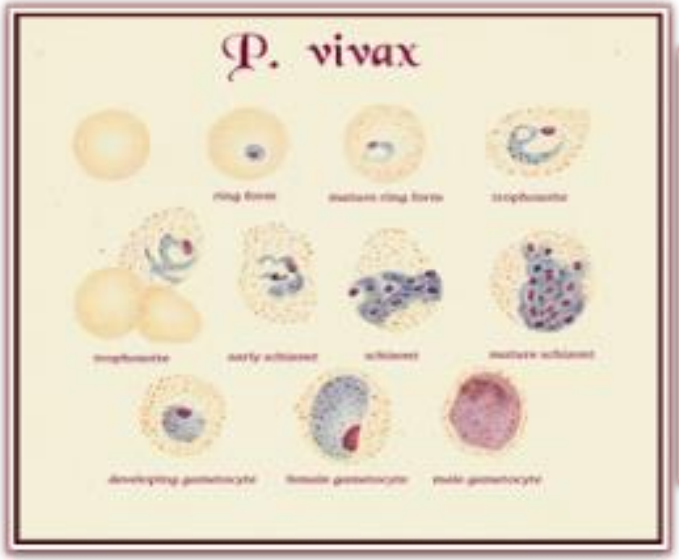
7

Plasmodium falciparum



8

Plasmodium vivax



1. Red cells containing parasites are usuallyenlarged.

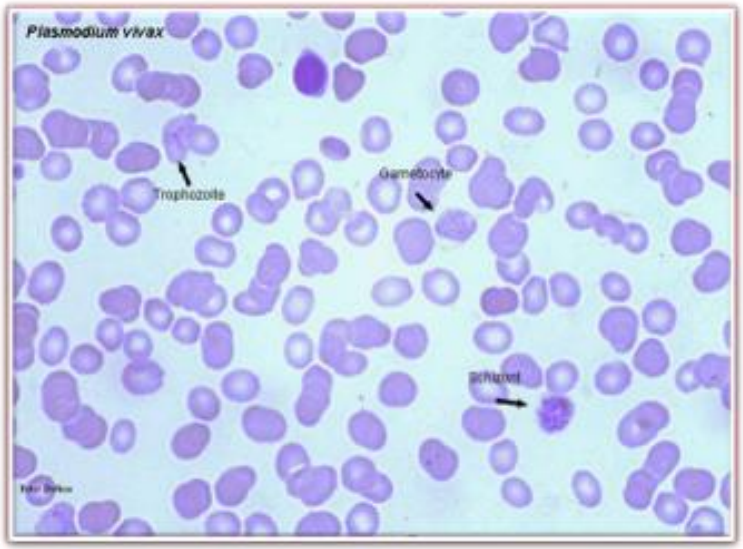
2. Schuffner's dots are frequently present in the red cells as shown above.

3. The mature ring forms tend to be large and coarse.

4. Developing forms are frequently present.

9

Plasmodium vivax



Plasmodium malarae

1. Ring forms mayhave a squarish appearance.

2. Band forms are a characteristic of this species.

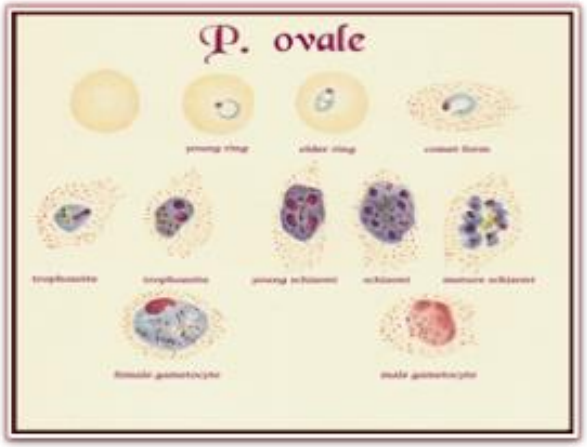
3. Mature schizonts mayhave a typical daisyhead appearance with up to ten merozoites.

4. Red cells are not enlarged.

5. Chromatin dot may be on the inner surface of the ring.

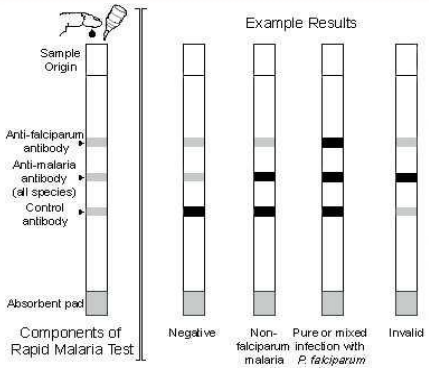
11

Plasmodium ovale



1. Red cells enlarged.
2. Comet forms common (top right).
3. Rings large and coarse.
4. Schuffner's dots, when present, may be prominent.
5. Mature schizonts similar to those of P. malariae but larger and more coarse.

ICT Malaria



- capture of the parasite antigens from the peripheral blood using either monoclonal or polyclonal antibodies against the parasite antigen targets.

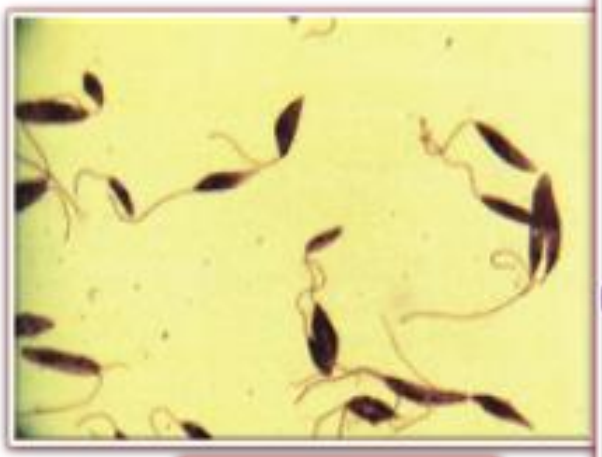
1. Histidine-rich protein 2 of P. falciparum (PfHRP2) is a water soluble protein that is produced by the asexual stages and gametocytes of P. falciparum, expressed on the red cell membrane surface, and shown to remain in the blood for at least 28 days after the initiation of antimalarial therapy.

2. Plasmodium aldolase is an enzyme of the parasite glycolytic pathway expressed by the blood stages of P. falciparum as well as the non-falciparum malaria parasites.

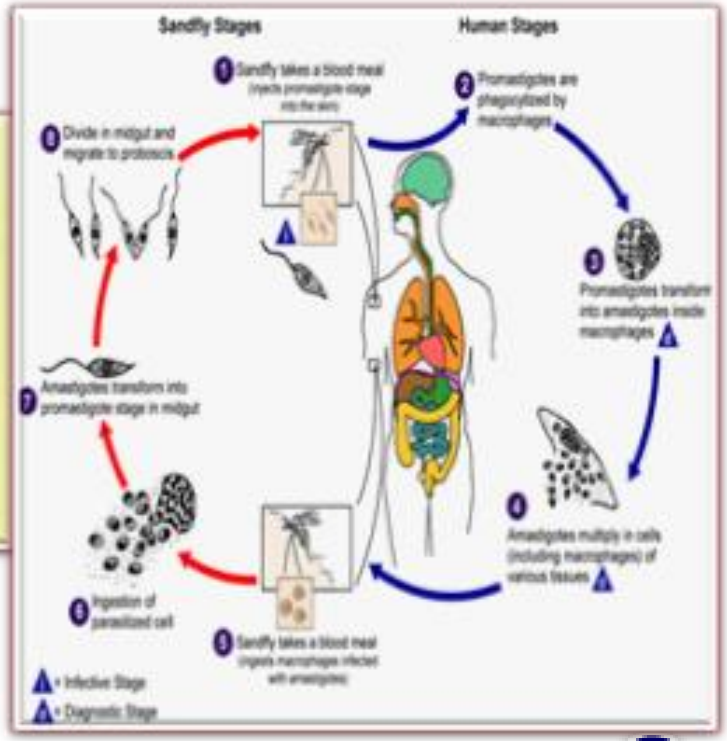
3. Parasite lactate dehydrogenase (pLDH) is a soluble glycolytic enzyme produced by the asexual and sexual stages of the live parasites and it is present in and released from the parasite infected erythrocytes.

13

Leishmania

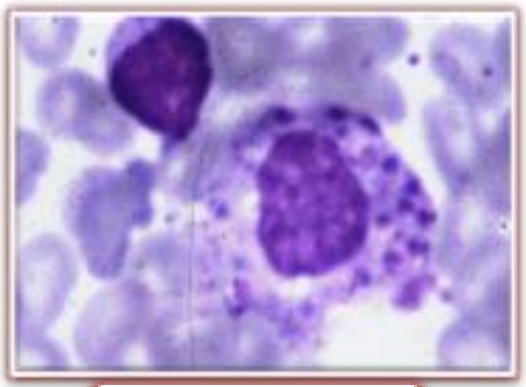


Promastigotes

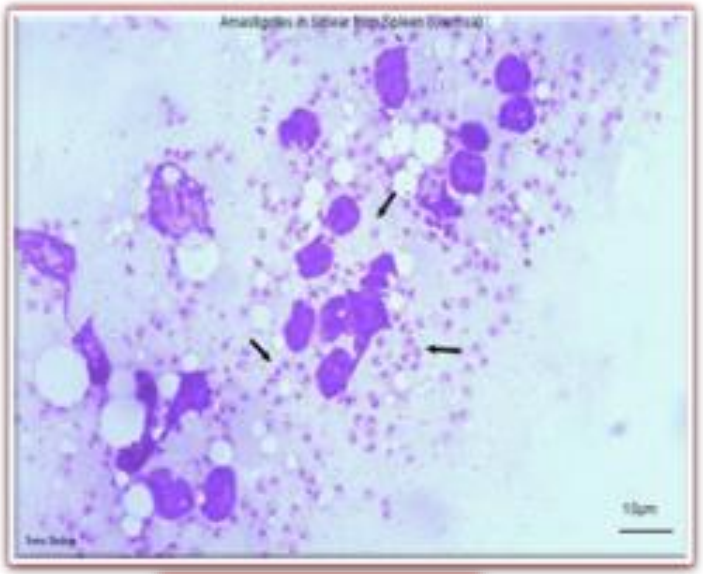


1

Leishmania



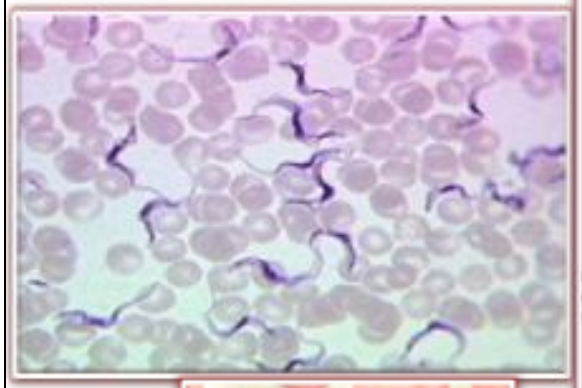
Bone Marrow

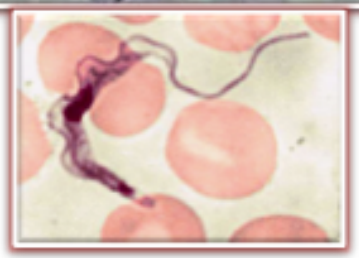


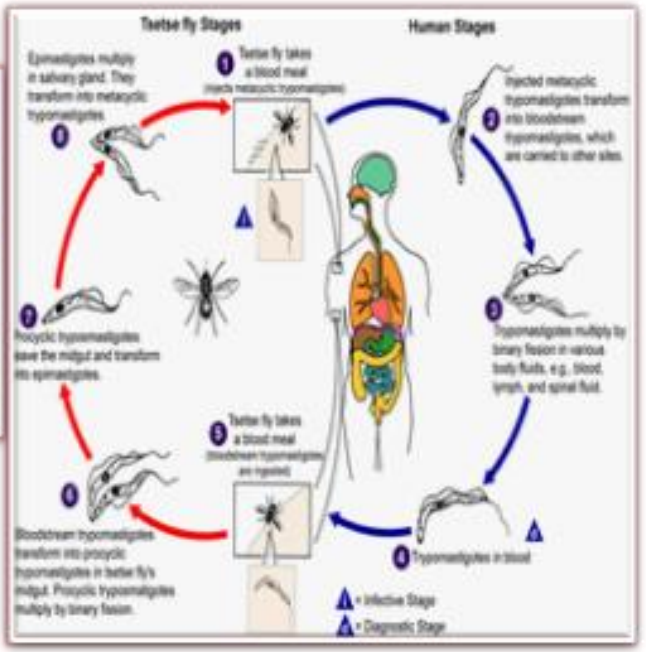
Spleen

Amastigotes

Trypanosomes

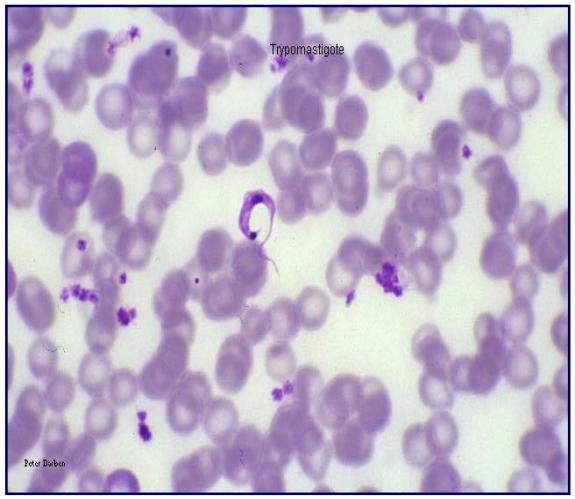


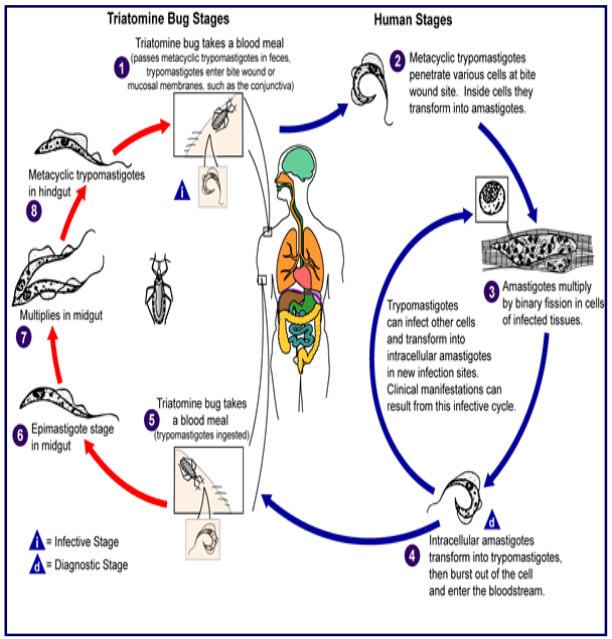




Trypanosoma brucei

Trypanosomes

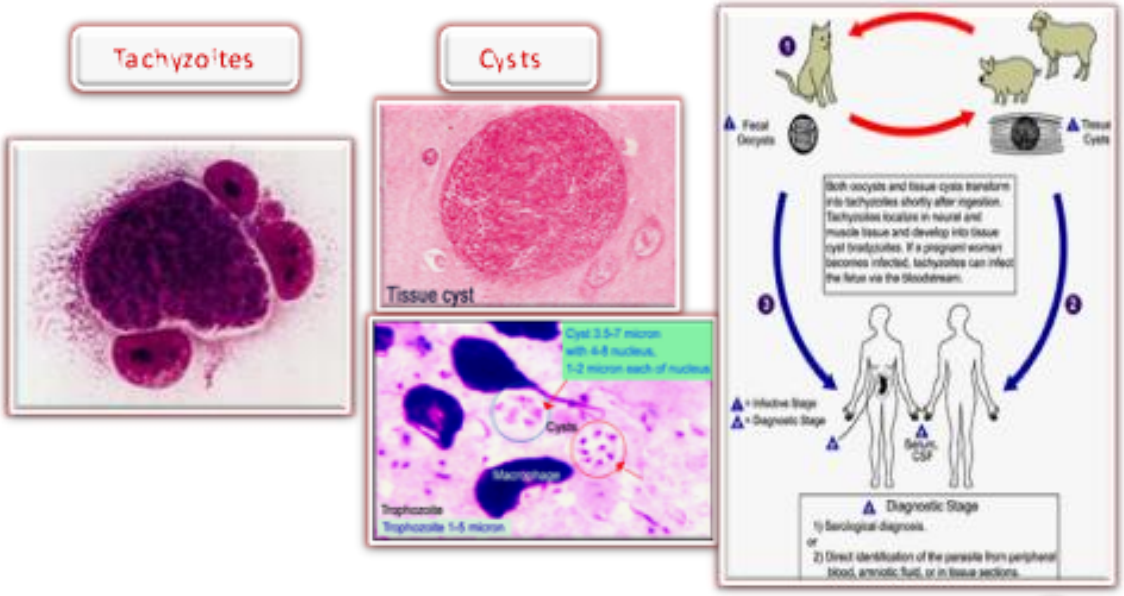




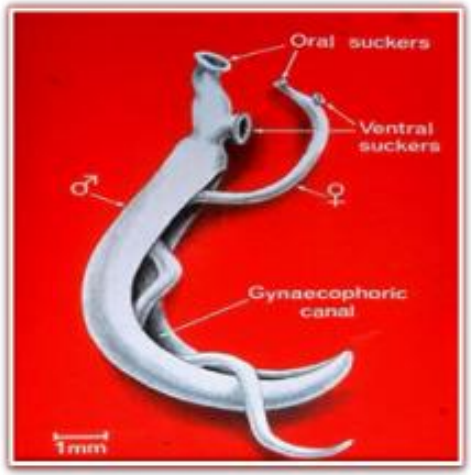
Trypanosoma cruzi

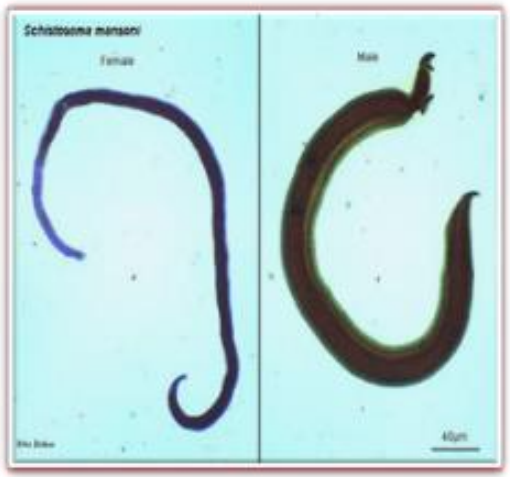
5

Toxoplasma gondii



Schistosoma





Schistosoma

SCH. HEMATOBIUM
OVA

2

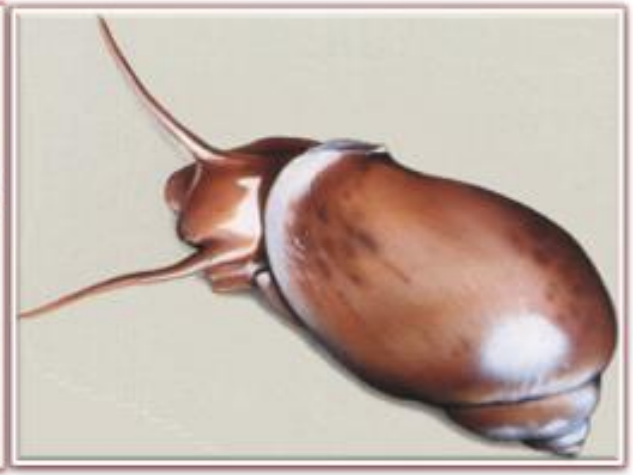
- Schistosoma

SCH. MANSONI OVA

3

Schistosoma

BIOMPHALARIA sp



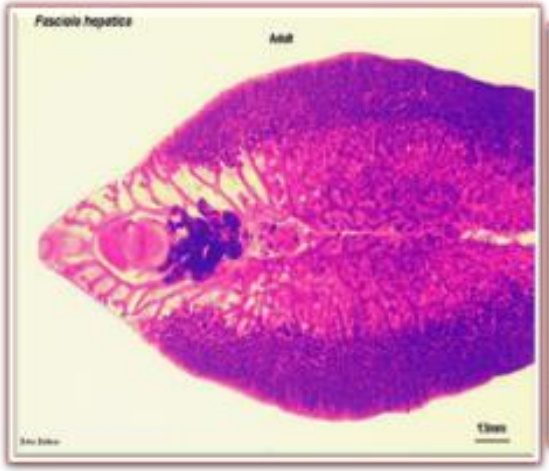
BULINUS sp

Schistosoma

CECARIA

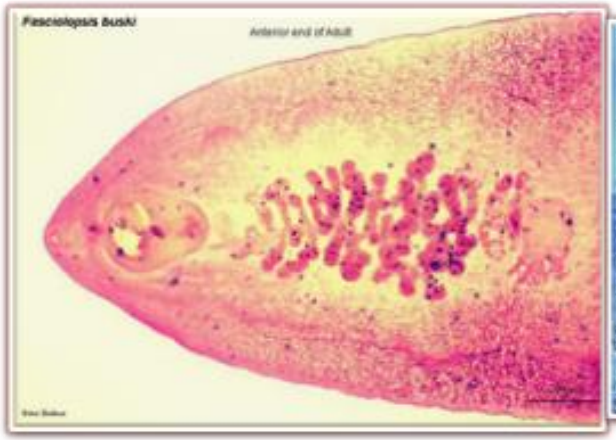
5

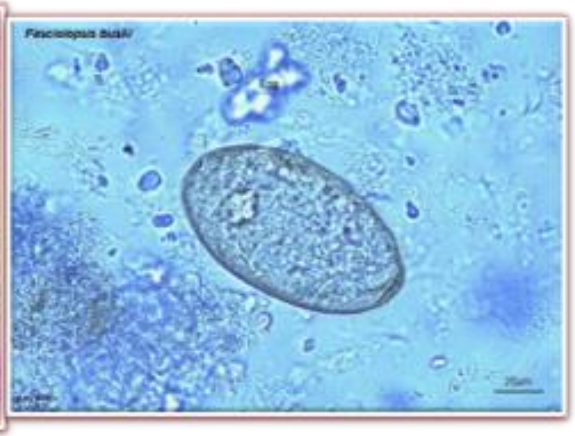
Flukes



7

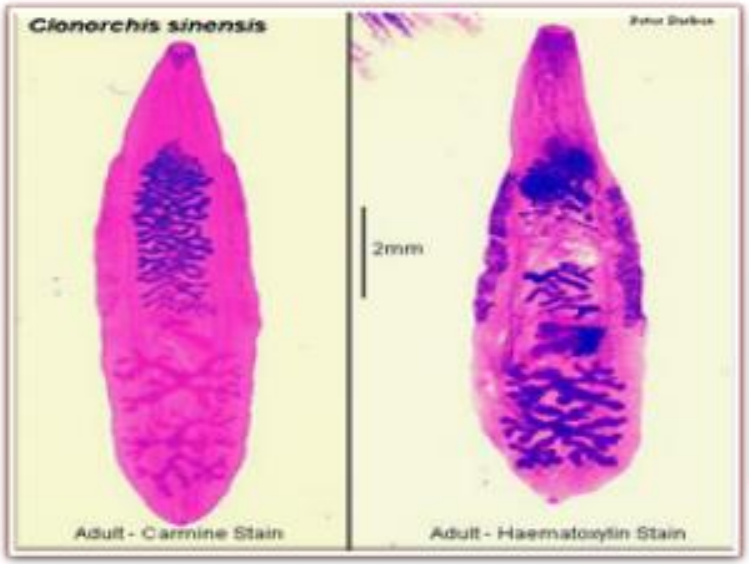
Flukes

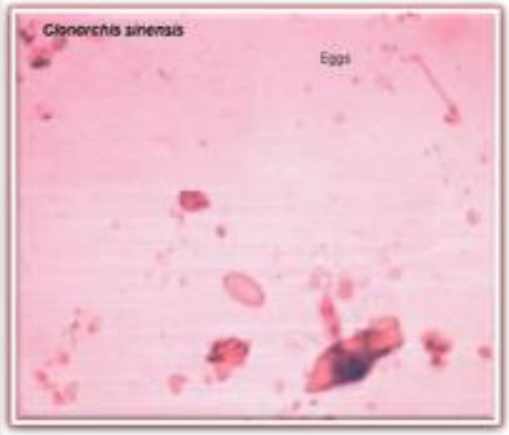




8

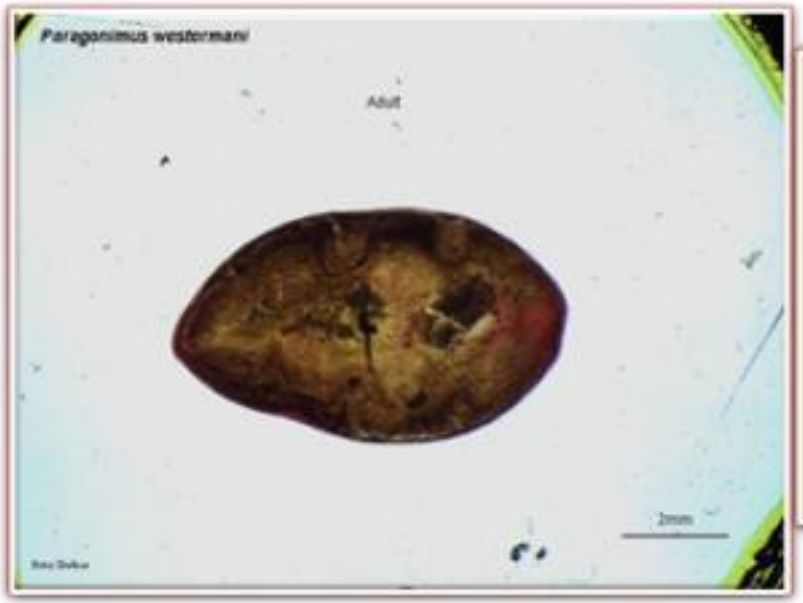
Flukes

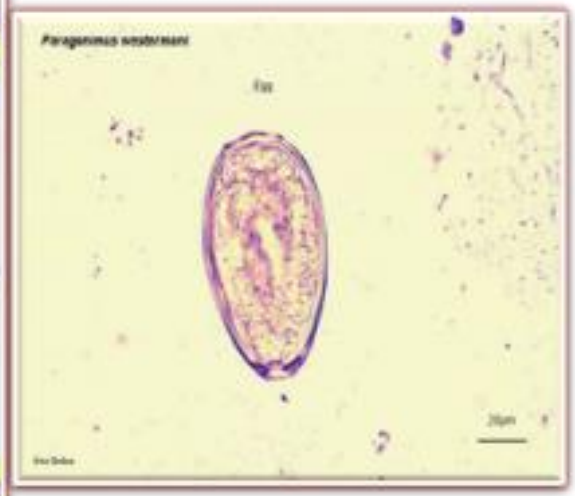

Clonorchis sinensis
2mm
Adult - Carmine Stain
Adult - Haematoxylin Stain


Clonorchis sinensis
Eggs

9

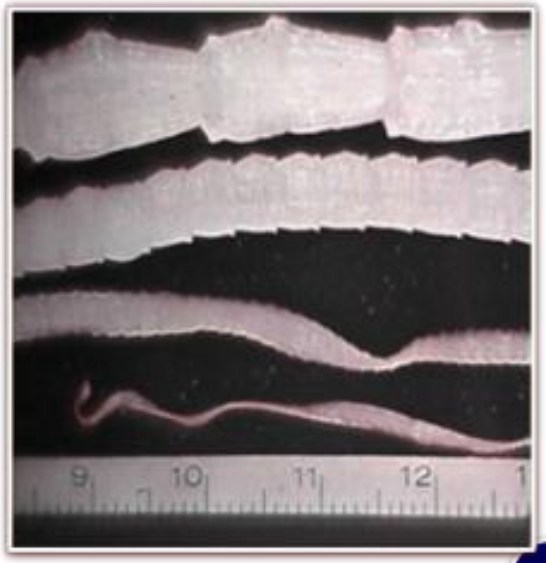
Flukes


Paragonimus westermani
Adult
2mm


Paragonimus westermani
Egg

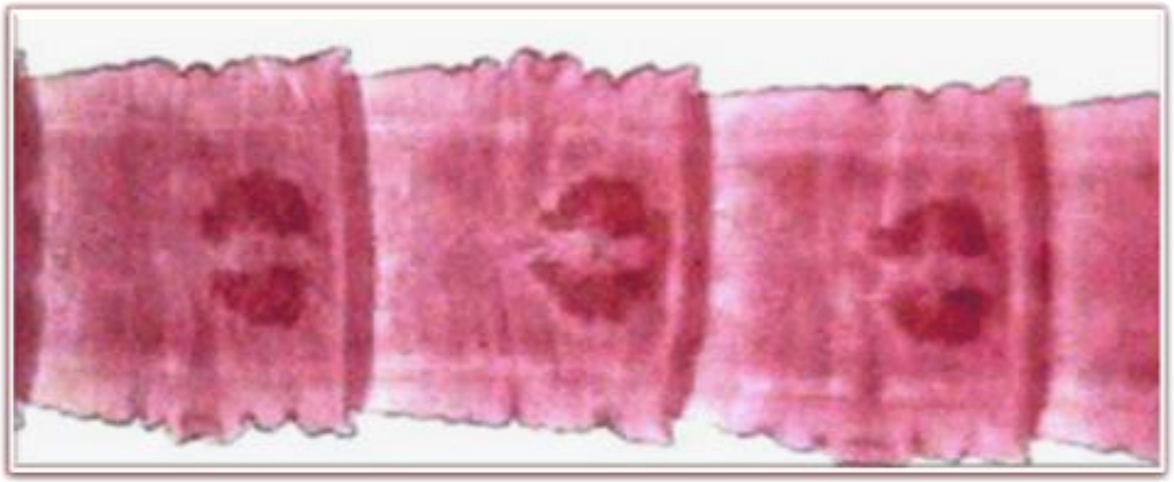
10

Taenia



11

Taenia



BROGLOTIDS

Taenia

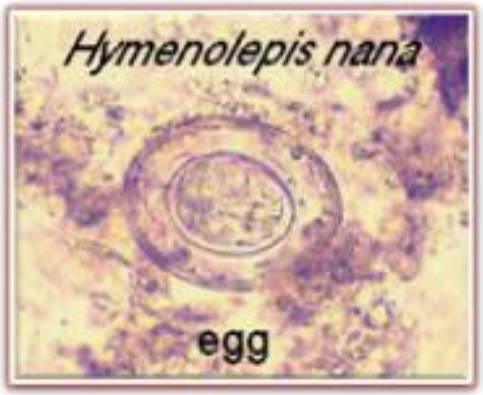
Taenia solium

Taenia saginata

Uterine segments

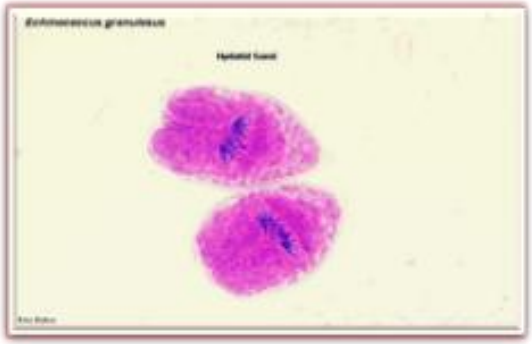
13

h.Nana



15

Dog tape worm



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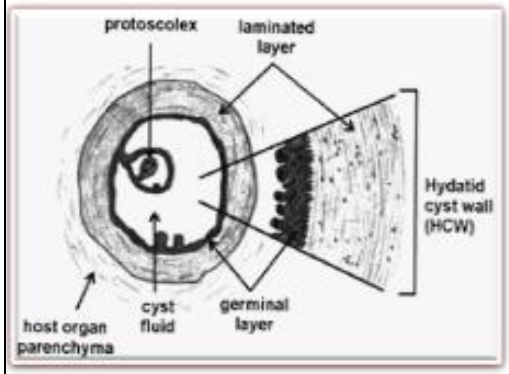
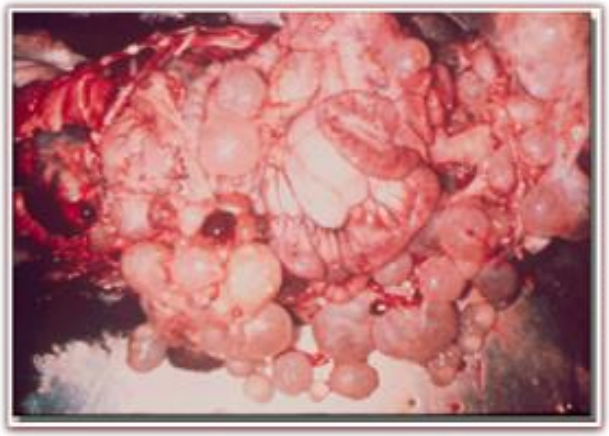
Dog tape worm

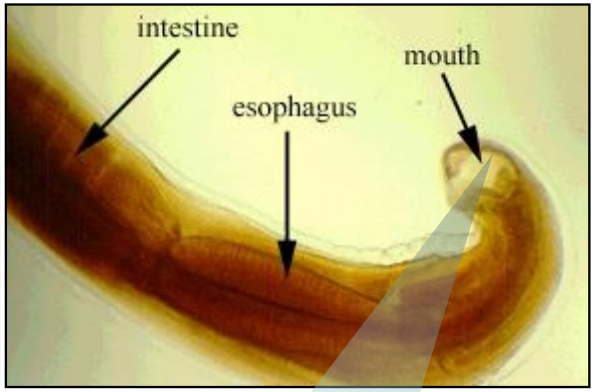


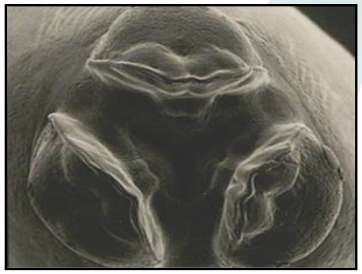


Hydatid cyst

18

Ascaris

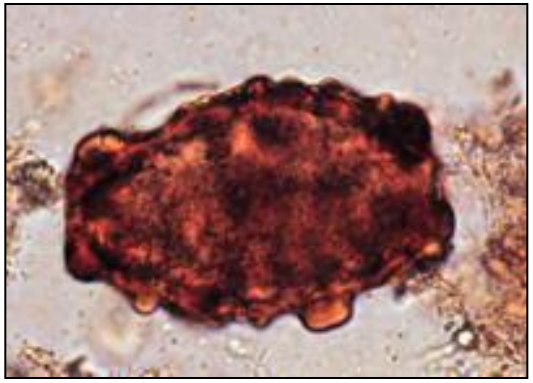




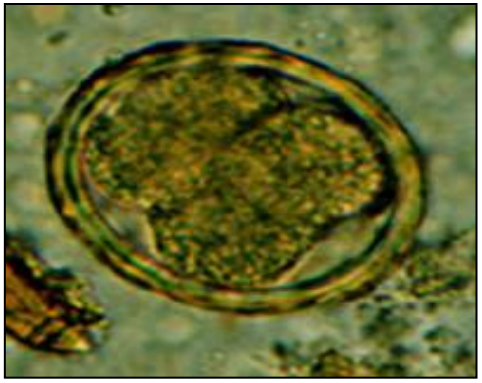
Triple lip

Ascaris

Ova

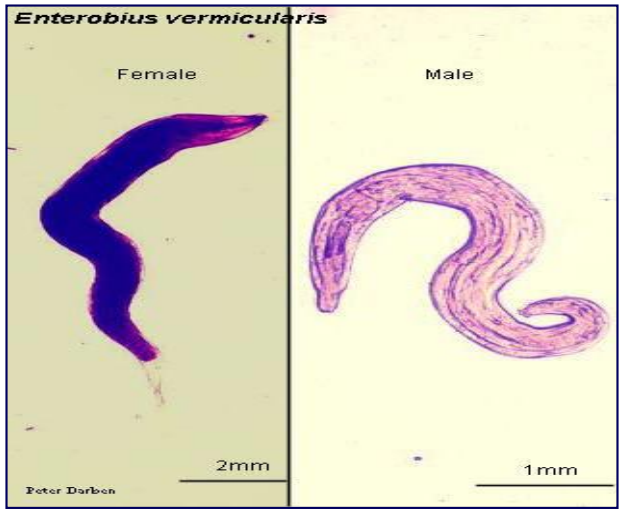


Unfertilized



Fertilized

Pin worms



Pin worms

Ova

D

D shaped

Whip worms

Whip worms

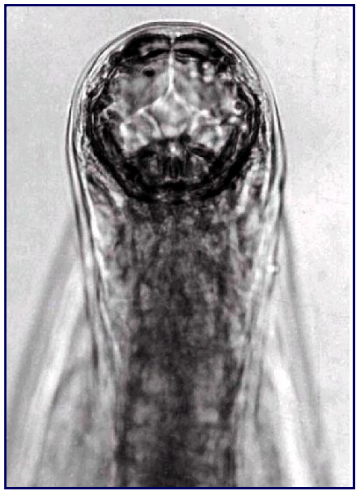
Ova

Barreled shaped

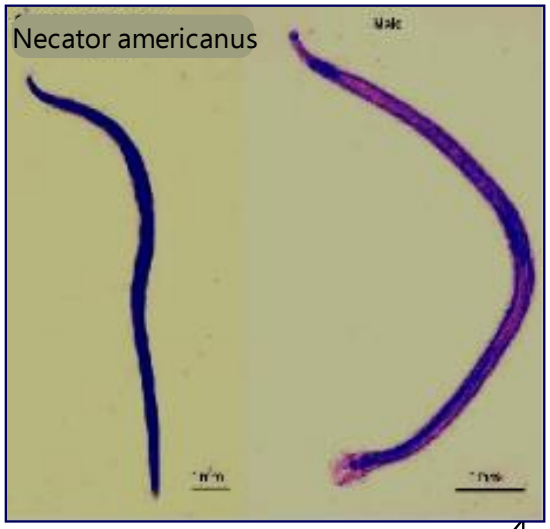
Hooked worms

Teeth

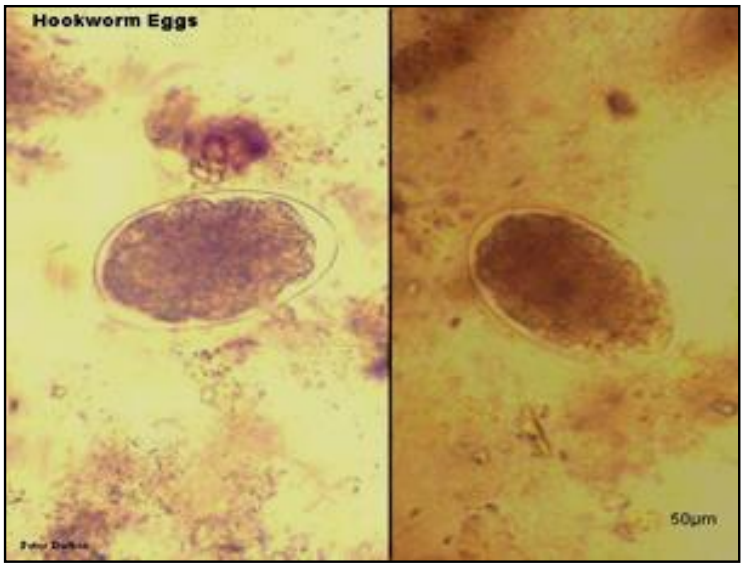
Hooked worms



Cutting plates



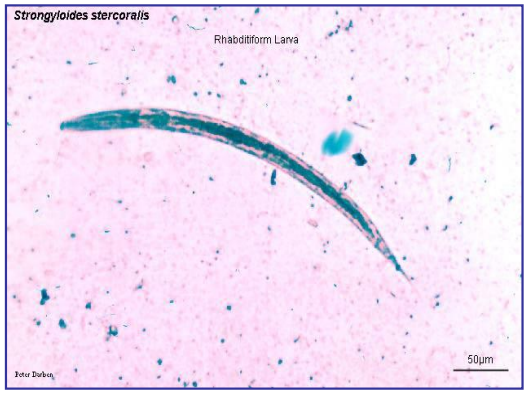
Hooked worms



3

Strongyloides stercolaris

larvae

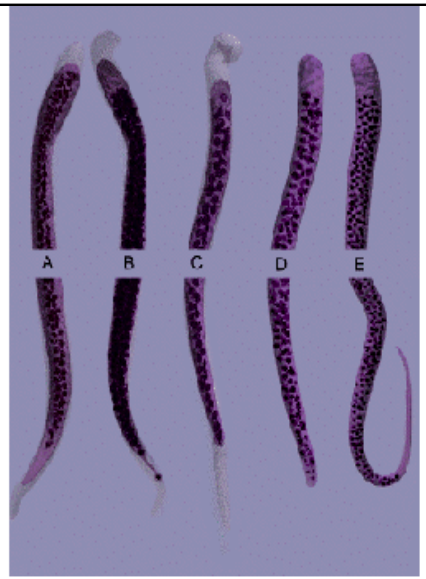


Filiariform

Rhabditiform

5

Microfilaria



A: *Wuchereria bancrofti*

B: *Brugia malayi*

C: *Loa loa*

D: *Onchocerca volvulus*

E: *Mansonella* species

Molecular Methods

Nucleic acid extraction

Nucleic acid extraction is the initial step required in genetic analysis of viruses, bacteria and other organisms. In a clinical setting it must be uniform amongst different specimens, efficient to ensure an accurate result and easy to perform. For this reason, commercially available quality-controlled kits are often used. For this practical, we will use such a kit, available from Qiagen.

You will be provided with a serum sample containing heat inactivated Hepatitis C Virus to extract and purify its RNA. A standard HCV^+ serum specimen would contain viable virus particles and thus require more appropriate handling, such as in a Class I microbiological safety cabinet, until lysed.

Follow the protocol provided (QIAamp viral RNA Mini spin protocol) in the additional handout from step 1, including the optional step 9a.

Reagent aliquots / materials are as follows:

Cap label	*Contents*
AVL	600 µl of AVL buffer (560 µl required)
P	150 µl Plasma (140 µl required)
E	600 µl Ethanol (560 µl required)
AW1	550 µl of prepared AW1 Buffer (500 µl required)
AW2	550 µl of prepared AW2 Buffer (500 µl required)
AVE	100 µl of AVE buffer (60 µl required)

1x 1.5ml eppendorf with cap attached – for mixing AVL, Plasma and ethanol

2x 1.5ml eppendorf without cap attached – for elution of RNA and step 9a

1x QIAamp spin column

3x round bottomed 2ml collection tubes

cDNA synthesis

RNA viruses such as HCV and HIV must be converted into complementary DNA (cDNA) to act as a template for PCR amplification. This process is performed by the Reverse Transcriptase enzyme, an integral component of RNA (and some DNA) virus genomes, which was been cloned for *in vitro* use.

Reverse transcription requires that the RNA template is primed by a DNA primer and extended with dNTPS by the RT enzyme to create a cDNA molecule comprised of one nascent DNA strand hybridised to the original RNA molecule. The following protocol provides you with a typical method to generate cDNA from your extracted virus, starting at step 2.

1. Take your RNA (usually stored at -70^{o}C post extraction) and place it on ice to defrost. In addition, thaw your cDNA synthesis reagents (hexamer primers, dNTPs, RT buffer) at room temperature then place on ice and place your RT enzyme directly on ice.

2. Assemble the following cDNA synthesis reagents in a new tube:

Labelled:		
5x	5x RT Buffer	2.5 μl
dN	dNTPS (10mM)	3 μl
RT	MMLV RT enzyme (200u / μl)	1 μl
H_2O	Water (certified RNase Free)	5 μl 11.5 μl

Notes:

RNase inhibitors can also be included as an extra precaution against RNase contamination.

After all reagents are added, set a Gilson pipette to 10 μl and pipette the mixture up and down gently to ensure adequate mixing of reagents without introduction of bubbles.

3. Pipette 10 μl of your RNA into a clean 0.2 ml tube and add 2.5 μl of 5x RT buffer and 1μl of Hexamer Primer (200ng / μl, labelled **HEX**). Place the tube in a PCR machine with a heated lid set to 70°C for 5 minutes.

4. Take your RNA sample off the hot block ensuring the temperature has not dropped from 70 °C and place immediately and firmly into the ice. Allow 15 to 20 seconds to elapse to ensure the full 12.5 μl has become ice cold, and then add the 12.5 μl of mixed cDNA synthesis reagents, again mixing by gentle pipetting.

5. Label the tube with your initials and place the reaction back onto the PCR machine for the following cycle:

   22 °C for 10 minutes

   42 °C for 60 minutes

   4 °C soak

   Store the cDNA at 4 °C for short term or at -20 °C for longer periods.

Polymerase chain reaction (PCR)

Various methodologies have been developed to enable amplification of a variety of templates. In its simplest form, PCR involves three separate steps - template denaturation, primer annealing and primer extension. This three step process can be repeated for up to 55 cycles (perhaps even more, but usually around 35 cycles), resulting in near logarithmic amplification of the target DNA fragment. Increased sensitivity can be obtained by carrying out nested primer PCR - in this method, products from the first round PCR are used as DNA template for a second PCR using primers positioned internally to the first round primers.

Today you will set-up a standard PCR reaction to test your sample for HCV cDNA. The cDNA will also be diluted out to estimate the quantity present within your sample and in addition, you will include positive and negative controls.

Method:

a) Serial dilution of your patients' cDNA

1. Label four 0.2 ml tubes with 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} (or just -1,-2 etc to simplify).

2. Pipette 18 μl of PCR grade H_20 into each tube

3. Pipette 2 μl from your cDNA reaction into the tube labelled 10^{-1}. Close the tube, vortex and pulse centrifuge it.

4. Pipette 2 μl from the 10^{-1} cDNA dilution into the tube labelled 10^{-2}. Close the tube, vortex and pulse centrifuge it. Repeat this process for all the remaining tubes.

b) Master Mix preparation

1. Assemble an 8x reaction volume master mix in a 0.5 ml eppendorf sufficient for the 7 tubes you will be using (add volumes in bold below):

Labelled		Vol. Added to x 8 master mix	[Vol. per PCR tube]
10x	10 x Reaction buffer	20 μl	[2.5 μl]
H_20	Sterile Water	139 μl	[17.375 μl]
P1	Primer 1 (5 pmols/μl)	8.0 μl	[1.0 μl]
P2	Primer 2 (5 pmols/μl)	8.0 μl	[1.0 μl]
N	dNTP mix (2.5 mM each dNTP)	8.0 μl	[1.0 μl]
Enz	Hot star Taq polymerase	1.0 μl	[0.125 μl]
	(+ cDNA template*	-	[2.0 μl])
		25μl total	

Notes:

Due to pipetting calibration and errors, the master mix is typically assembled with approximately 10 % more than is required to ensure no shortfalls arise. Here 7 tubes are required, therefore a master mix for 8 tubes is assembled.

After adding the Hot star Taq polymerase, ensure that the PCR mix is adequately mixed by pipetting the mixture up and down several times. This is important for any mixture, particularly those containing enzymes, which have a high glycerol concentration. Ensure that the reaction mix does not foam / bubble, so be careful during the mixing stage!

Most enzymes stocks are sensitive to temperature variations and must be incubated on ice during use (e.g. the reverse transcriptase used in the first session). However, the Taq enzyme used here is a specially engineered recombinant version that is unusually stable at room temperature and thus the exception to the rule.

2. Dispense 23 μl into each of 7 x 0.2 ml microtubes. Label these C1-C7 as follows (and with a further label unique to your work e.g. your initials):

C1) neat cDNA

C2) 10^{-1} cDNA

C3) 10^{-2} cDNA

C4) 10^{-3} cDNA

C5) 10^{-4} cDNA

C6) cDNA negative control

C7) cDNA positive control

3. Add 2 μl of each cDNA to the appropriate PCR amplification, as indicated above.

4. Add 2 μl of water to PCR amplification tube C6.

5. Add 2 μl of the +ve control (labelled **+ve**) to PCR amplification tube C7.

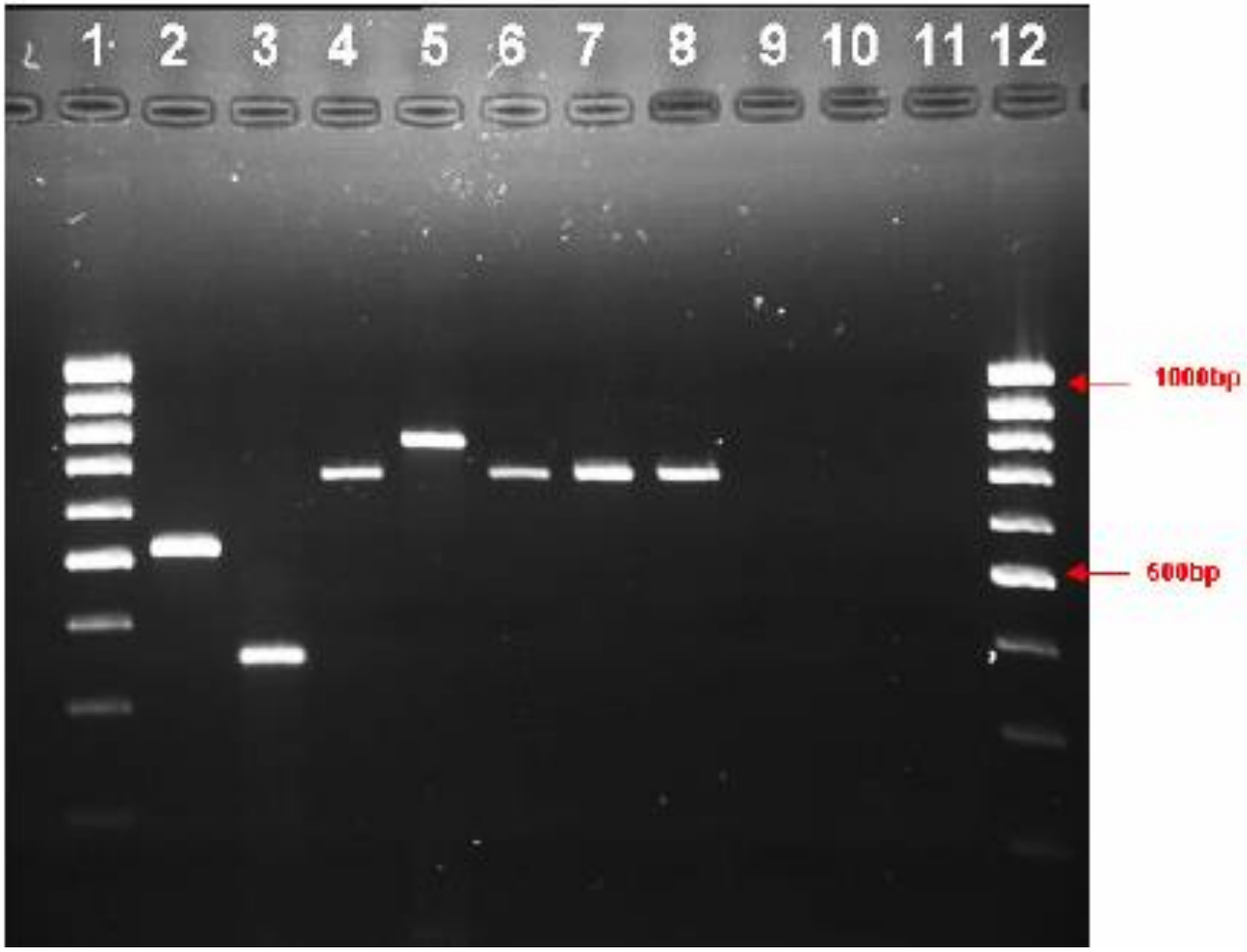
6. Your tubes will then be collected by a demonstrator and cycled as follows:

95 ^{o}C for 10 minutes (Hot start step required for this particular Taq type)

94 ^{o}C for 30 seconds
55 ^{o}C for 30 seconds — for 40 cycles
72 ^{o}C for 30 seconds

The samples will then be electrophoresised on a 2% gel following the procedure outlined in part 4a 'Agarose gel Electrophoresis' below, and you'll

get a photo of this in the next session. However today you can see the equipment involved and practice loading some samples.



Real-time polymerase chain reaction (Rt-PCR)

Recent advances in PCR machinery allow detection to be performed during amplification, a process called Real-time PCR, as opposed to amplification then separate examination of the product on an agarose gel. Real-time PCR is of particular importance in a clinical setting as the immediate detection improves turnaround time for assay results, and potential contamination problems are drastically reduced as PCR tubes need not be opened after amplification.

Central to the quantitative Real-time PCR approach is the use of standards of known copy number. Co-amplification of a control plasmid containing the cloned target sequence over a range of starting quantities allows for the generation of a standard curve from which unknown samples, e.g. your extracted HCV cDNA, can be calculated.

Today we will simply generate a standard curve for the assay by logarithmically diluting a plasmid control, then setting up a Real-time PCR to quantify your sample more accurately

Method:

a) Serial dilution of control plasmid

1. Label six 0.2 ml eppendorf tubes with 10^7, 10^6, 10^5, 10^4, 10^3, 10^2.

2. Pipette 18 μl of PCR grade H_20 into each tube

3. Pipette 2 μl from the 10^8 copies per μl standard provided (labelled **10^8**) into the tube labelled 10^7. Close the tube, vortex and pulse centrifuge it.

4. Pipette 2 μl from the 10^7 copies per μl standard into the tube labelled 10^6. Close the tube, vortex and pulse centrifuge it. Repeat this process for all the remaining tubes.

b) Master Mix preparation

1. Assemble a 10x reaction volume master mix in a 0.5 ml eppendorf sufficient for the row of 8 strip tubes you will be using (add volumes in bold below):

Tube labelled	*Reagent*	*Volume Per tube*	*10x master mix*
2x	2x Real-time common reagent mix	12.5μl	**125 μl**
p3	Forward primer	1 μl	**10 μl**
p4	Reverse primer	1 μl	**10 μl**
H_20	Molecular grade water	9.5 μl	**95 μl**

2. Dispense 24 μl into each of the 8 tubes

3. Add 1μl of the 10^7 copies per μl standard to the left hand tube of the strip (mark the side of your tube clearly to orientate it, **not the lid** as this will interfere with the fluorescence detection)

4. Add 1μl of the 10^6 standard to the next tube and so on until the 10^2 standard is added to the sixth in the row of 8

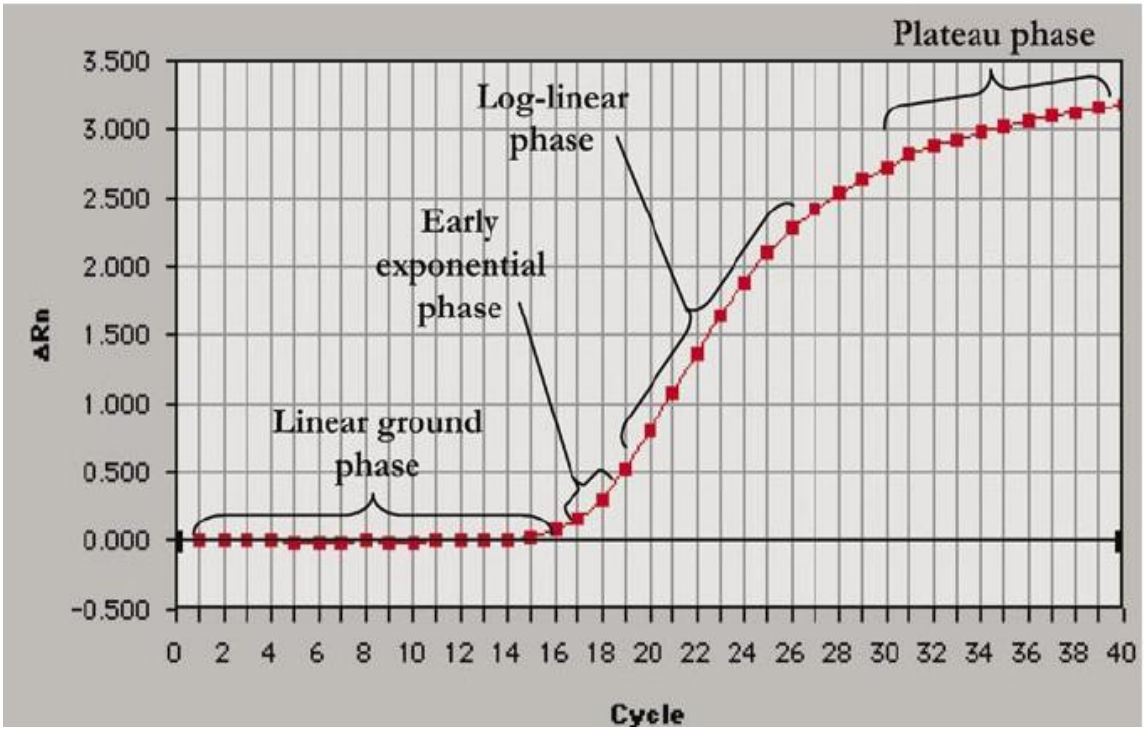
5. Add 1μl of your cDNA to your seventh tube.

6. Add 1μl of H_20 to your eighth and final tube – this will serve as your negative control

7. Your tubes will then be collected by a demonstrator and cycled as follow on a Stratagene MX4000 Real-time machine:

95 °C for 10 minutes (Hot start step required for this particular Taq type)

94 °C for 30 seconds
55 °C for 30 seconds — for 40 cycles
72 °C for 30 seconds



References

1. https://microbiologysociety.org/publication/education-outreach-resources/basic-practical-microbiology-a-manual.html
2. https://www.futurelearn.com/courses/basic-concepts-in-microbiology-and-clinical-pharmacology-of-antimicrobials
3. https://www.ase.org.uk/resources/basic-practical-microbiology-manual
4. https://www.sciencedirect.com/science/article/pii/S1876034115002452
5. https://www.wiley.com/enus/Practical+Immunology%2C+4th+Edition-p-9781405146739

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2022