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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – 1 – COLLECTION ANF TRANSPORT OF SMPLE – SMB 3201

Unit:1 **Specimen collection**

1.Selection, Collection and Transport of blood

Blood Specimen Collection and Processing

The first step in acquiring a quality lab test result for any patient is the specimen collection procedure. The venipuncture procedure is complex, requiring both knowledge and skill to perform. Several essential steps are required for every successful collection procedure.

Venipuncture Procedure:

1.A phlebotomist must have a professional, courteous, and understanding manner in all contact with all patients.

2 The first step to the collection is to positively identify the patient by two forms of identification; ask the patient to state and spell his/her name and give you his/her birth date. Check these against the requisition (paper or electronic).

3.Check the requisition form for requested tests, other patient information and any special draw requirements. Gather the tubes and supplies that you will need for the draw.

4.Position the patient in a chair, or sitting or lying on a bed.

5.Wash your hands.

6.Select a suitable site for venipuncture, by placing the tourniquet 3 to 4 inches above the selected puncture site on the patient. See below for venipuncture site selection “notes.”

7.Do not put the tourniquet on too tightly or leave it on the patient longer than 1 minute.

8.Next, put on non-latex gloves, and palpate for a vein.

9.When a vein is selected, cleanse the area in a circular motion, beginning at the site and working outward. Allow the area to air dry. After the area is cleansed, it should not be touched or palpated again. If you find it necessary to reevaluate the site by palpation, the area needs to be re-cleansed before the venipuncture is performed.

10. the patient to make a fist; avoid “pumping the fist.” Grasp the patient’s arm firmly using your thumb to draw the skin taut and anchor the vein. Swiftly insert the needle through the skin into the lumen of the vein. The needle should form a 15-30 degree angle with the arm surface. Avoid excess probing.

1.0 Angle of venipuncture

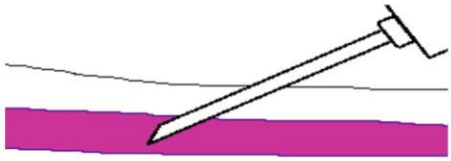


Figure 1: Angle of Venipunture

11.When the last tube is filling, remove the tourniquet.

12. the needle from the patient's arm using a swift backward motion.

13. gauze immediately on the puncture site. Apply and hold adequate pressure to avoid formation of a hematoma. After holding pressure for 1-2 minutes, tape a fresh piece of gauze or Band-Aid to the puncture site.

14.Dispose of contaminated materials/supplies in designated containers.

Note: The larger median cubital and cephalic veins are the usual choice, but the basilic vein on the dorsum of the arm or dorsal hand veins are also acceptable. Foot veins are a last resort because of the higher probability of complications.

Fingerstick Procedure:

1.Follow steps #1 through #5 of the procedure for venipuncture as outlined above.

2 The best locations for fingersticks are the 3rd (middle) and 4th (ring) fingers of the non-dominant hand. Do not use the tip of the finger or the center of the finger. Avoid the side of the finger where there is less soft tissue, where vessels and nerves are located, and where the bone is closer to the surface. The 2nd (index) finger tends to have thicker, callused skin. The fifth finger tends to have less soft tissue overlying the bone. Avoid puncturing a finger that is cold or cyanotic, swollen, scarred, or covered with a rash.

3.When a site is selected, put on gloves, and cleanse the selected puncture area.

4. the finger toward the selected site prior to the puncture.

5.Using a sterile safety lancet, make a skin puncture just off the center of the finger pad. The puncture should be made perpendicular to the ridges of the fingerprint so that the drop of blood does not run down the ridges.

6.Wipe away the first drop of blood, which tends to contain excess tissue fluid.

Fingerstick

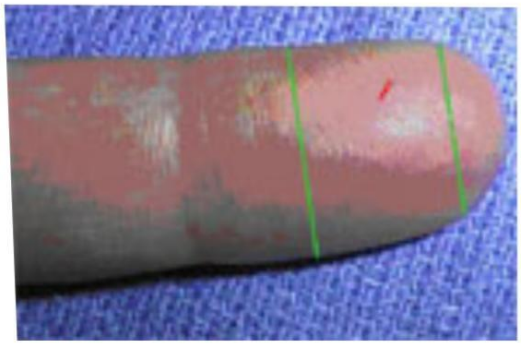


Figure 1.1: Fingerstick

7. drops of blood into the collection tube/device by gentle pressure on the finger. Avoid excessive pressure or “milking” that may squeeze tissue fluid into the drop of blood.

8.Cap, rotate and invert the collection device to mix the blood collected.

9 Have the patient hold a small gauze pad over the puncture site for a few minutes to stop the bleeding.

10.Dispose of contaminated materials/supplies in designated containers.

11 Label all appropriate tubes at the patient bedside.

Heelstick Procedure (infants):

The recommended location for blood collection on a newborn baby or infant is the heel. The diagram below indicates the proper area to use for heel punctures for blood collection.

1.1 Heelstick

Figure 1.2: Heelstick

1.Prewarming the infant’s heel (42° C for 3 to 5 minutes) is important to increase the flow of blood for collection.

2.Wash your hands, and put gloves on. Clean the site to be punctured with an alcohol sponge. Dry the cleaned area with a dry gauze pad.

3.Hold the baby’s foot firmly to avoid sudden movement.

4.Using a sterile blood safety lancet, puncture the side of the heel in the appropriate regions shown above. Make the cut across the heel print lines so that a drop of blood can well up and not run down along the lines.

5.Wipe away the first drop of blood with a piece of clean, dry cotton gauze. Since newborns do not often bleed immediately, use gentle pressure to produce a rounded drop of blood. Do not use excessive pressure because the blood may become diluted with tissue fluid.

6.Fill the required microtainer(s) as needed.

7.When finished, elevate the heel, place a piece of clean, dry cotton on the puncture site, and hold it in place until the bleeding has stopped. Apply tape or Band-Aid to area if needed.

8.Be sure to dispose of the lancet in the appropriate sharps container. Dispose of contaminated materials in appropriate waste receptacles.

9. your gloves and wash your hands.

Order of Draw:

Blood collection tubes must be drawn in a specific order to avoid cross-contamination of additives between tubes. The recommended order of draw is:

1.First – blood culture bottle or tube (yellow or yellow-black top)

2 Second – coagulation tube (light blue top).

3.Third – non-additive tube (red top)

4.Last draw – additive tubes in this order:

- SST (red-gray or gold top). Contains a gel separator and clot activator.
- Sodium heparin (dark green top)
- PST (dark green green top with gold rim). Contains lithium heparin anticoagulant and a gel separator.
- EDTA (lavender top)
- Oxalate/fluoride (light gray top) or other additives

NOTE: Tubes with additives must be thoroughly mixed. Clotting or erroneous test results may be obtained when the blood is not thoroughly mixed with the additive.

Labeling The Sample

All specimens must be received by the laboratory with a legible label containing at least two (2) unique identifiers.

The specimen must be labeled with the patient's full name (preferably last name first, then first name last) and one of the following:

- Geisinger medical record number (MRN) – for Geisinger locations, this is the required second identifier
- Patient's full date of birth (must include the month, day, and year)

- ❖ Unique requisition identifier/label

Areas to Avoid When Choosing a Site for Blood Draw:

Certain areas are to be avoided when choosing a site for blood draw:

- Extensive scars from burns and surgery – it is difficult to puncture the scar tissue and obtain a specimen.
- The upper extremity on the side of a previous mastectomy – test results may be affected because of lymphedema.
- Hematoma – may cause erroneous test results. If another site is not available, collect the specimen distal to the hematoma.
- Intravenous therapy (IV) / blood transfusions – fluid may dilute the specimen, so collect from the opposite arm if possible.
- Cannula/fistula/heparin lock – hospitals have special policies regarding these devices. In general, blood should not be drawn from an arm with a fistula or cannula without consulting the attending physician.
- Edematous extremities – tissue fluid accumulation alters test results.

Techniques to Prevent Hemolysis (which can interfere with many tests):

- Mix all tubes with anticoagulant additives gently (vigorous shaking can cause hemolysis) 5-10 times.
- Avoid drawing blood from a hematoma; select another draw site.
- If using a needle and syringe, avoid drawing the plunger back too forcefully.
- Make sure the venipuncture site is dry before proceeding with draw.
- Avoid a probing, traumatic venipuncture.
- Avoid prolonged tourniquet application (no more than 2 minutes; less than 1 minute is optimal).
- Avoid massaging, squeezing, or probing a site.
- Avoid excessive fist clenching.
- If blood flow into tube slows, adjust needle position to remain in the center of the lumen.

Blood Sample Handling and Processing:

Pre-centrifugation Handling – The first critical step in the lab testing process, after obtaining the sample, is the preparation of the blood samples. Specimen integrity can be maintained by following some basic handling processes:

- Fill tubes to the stated draw volume to ensure the proper blood-to-additive ratio. Allow the tubes to fill until the vacuum is exhausted and blood flow ceases.
- Tubes should be stored at 4-25°C (39-77°F).
- Tubes should not be used beyond the designated expiration date.
- Mix all gel barrier and additive tubes by gentle inversion 5 to10 times immediately after the draw. This assists in the clotting process. This also assures homogenous mixing of the additives with the blood in all types of additive tubes.
- Serum separator tubes should clot for a full 30 minutes in a vertical position prior to centrifugation. Short clotting times can result in fibrin formation, which may interfere with complete gel barrier formation.

Blood Sample Centrifugation – It is recommended that serum be physically separated from contact with cells as soon as possible, with a maximum time limit of 2 hours from the time of collection.

- Complete gel barrier formation (gel barrier tubes) is time, temperature and G-force dependent. The uniformity of the barrier is time dependent; an incomplete barrier could result from shortened centrifugation times.

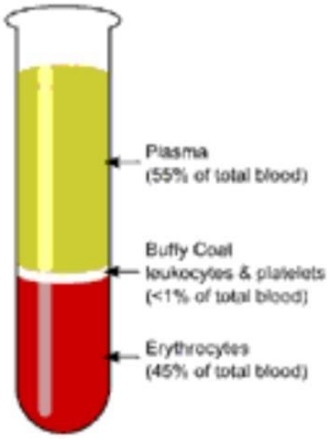


Figure 1.3: Blood components after centrifugation

1.3 Blood after separation

- In general, for a horizontal, swing-bucket centrifuge, the recommended spin time is 10 minutes. For a fixed-angle centrifuge, the recommended spin time is 15 minutes.
- NOTE: Gel flow may be impeded if chilled before or after centrifugation.
- Tubes should remain closed at all times during the centrifugation process.
- Place the closed tubes in the centrifuge as a "balanced load" noting the following:
- ➢ Opposing tube holders must be identical and contain the same cushion or none at all.
- ➢ Opposing tube holders must be empty or loaded with equally weighted samples (tubes of the same size and equal in fill).
- ➢ If an odd number of samples is to be spun, fill a tube with water to match the weight of the unpaired sample and place it across from this sample.

Centrifuge Safety

- Interference with an activated centrifuge by an impatient employee can result in bodily injury in the form of direct trauma or aerosolization of hazardous droplets.
- Centrifuges must never be operated without a cover in place.
- Uncovered specimen tubes must not be centrifuged.
- Centrifuges must never be slowed down or stopped by grasping part(s) of the device with your hand or by applying another object against the rotating equipment.
- Be sure the centrifuge is appropriately balanced before activating. If an abnormal noise, vibration, or sound is noted while the centrifuge is in operation, immediately stop the unit (turn off the switch) and check for a possible load imbalance.
- Clean the centrifuge daily with a disinfectant and paper towel. Broken tubes or liquid spills must be cleaned immediately.

2.selection, collection and transport of Urine;

URINE SPECIMENS

TYPES AND COLLECTION PROCEDURES;

PRINCIPLE

Collection and transportation of urine specimens to the clinical laboratory are important because Variables such as collection method, container, transportation, and storage affect the analysis Outcome and consequently diagnostic and therapeutic decisions based on the results. Clinical staff Are responsible for patient instruction, collection and labeling of urine specimens and timely Transportation of specimens to the Laboratory.

SPECIMEN RREQUIREMEN

A. Specimen types

1. Random specimen

For chemical and microscopic examination, a voided specimen is usually more Suitable. A randomly collected specimen may be collected at unspecified times And is often more convenient for the patient. A random specimen is suitable for Most screening purposes.

2. First morning specimen or 8-hour specimen

The patient should be instructed to collect the specimen immediately upon rising From a night's sleep. Other 8-hour periods may be used to accommodate Insomniacs, night-shift workers, and in certain pediatric situations. The bladder Is emptied before lying down and the specimen is collected on arising so that the Urine collected only reflects the recumbent position. Any urine voided during The night should be collected and pooled with the first morning voided specimen.

3. Fasting specimen

This differs from a first morning specimen by being the second voided specimen After a period of fasting.

4. 2-Hour postprandial specimen

The patient should be instructed to void shortly before consuming a routine meal And to collect a specimen 2 hours after eating.

5. 24-hour (or timed) specimenTo obtain an accurately timed specimen, it is necessary to begin and end the Collection period with an empty bladder. The following instructions forCollecting a 24-hour specimen can be applied to any timed collection (consult Test requirements to determine if a special preservative is required):

Day 1 – 7 AM: Patient voids and discards specimen. Patient collects all urine

For the next 24 hours.

Day 2 – 7 AM: Patient voids and adds this urine to the previously collected

Urine.

6. Catheterized specimen

This specimen is collected under sterile conditions by passing a hollow tube

Through the urethra into the bladder.

7. Midstream “clean catch” specimen

This specimen provides a safer, less traumatic method for obtaining urine for

Bacterial culture. It also offers a more representative and less contaminated

Specimen for microscopic analysis than the random specimen. Adequate

Cleansing materials and a sterile container must be provided for the patient. The

Procedure for the collection of a “clean catch” urine is described below in

Section VI of this policy.

8. Suprapubic aspiration

Urine may be collected by external introduction of a needle into the bladder. It

Is free of extraneous contamination and may be used for cytologic examination.

9. Pediatric specimens

This may be a sterile specimen obtained by catheterization or by suprapubic

Aspiration. The random specimen may be collected by attaching a soft, clear

Plastic bag with adhesive to the general area of both boys and girls.

B. Transportation of specimens

Urine specimens should be delivered to the within 2 hours of collection or refrigeratedAnd transported to the lab as soon as possible.

REAGENTS AND SUPPLIES (for collection of “clean catch” specimens)

- Disposable, clean, dry, leak-proof container (sterile container with lid required for

Microbiological cultures)

- Screw top specimen tube
- Disposable gloves
- Betadine swabs (Hibiclens if allergic t.o betadine)
- Dry, clean gauze
- Patient's bedpan or urinal, if patient is unable to go the bathroom**.**

CALIBRATION

No calibration is required for this procedure.

QUALITY CONTROL

Identification of the patient must be performed by asking a conscious patient his or her full name

And birthdate. Verify by checking the identification band if available.

PROCEDURE

Patient preparation:For FEMALE patients:

1. hands thoroughly before beginning the procedure and put on disposable

Gloves.

2. betadine swabs or Hibiclens to cleanse the perineal area.

- Separate the folds of the labia and wipe the betadine swab or Hibiclens from

Front to back (anterior to posterior) on one side, then discard swab or

Towelette.

- Using a second betadine swab or Hibiclens, wipe the other side from front to

Back, then discard.

- Using a third betadine swab or Hibiclens, wipe down the middle from front to

Back, then discard.

- Pat dry periurethral area with clean dry gauze to remove excessive betadineWhile keeping the labia separated.

For MALE patients:

1. Wash hands thoroughly before beginning the procedure and put on disposable

Gloves.

2. If the patient is not circumcised, pull the foreskin back (retract the foreskin) on

The penis to clean and hold it back during urination.

3. Using a circular motion, clean the head of the penis with betadine swabs or

Hibiclens. Discard the swab or towelette.

- Urination should begin, passing the first portion into the bedpan, urinal, or toilet.

After the flow of urine has started, the urine specimen container should be placed under

The patient collecting the midportion (midstream "clean catch") without contaminating the

Container.

- Any excess urine can pass into the bedpan, urinal, or toilet.

Cover the urine container immediately with the lid being careful not to touch the inside of

The container or the inside of the lid.

- Transfer urine to specimen tube if tubes are used for transport instead of urine containers.
- Attach label to tube or container and place specimen in the transport bag.
- Remove gloves and wash hands.
- Record date and time of collection and initials of the person collecting (or submitting) the

Specimen on the specimen container. Transport specimen to the Laboratory within 2

Hours of collection or refrigerate and transport to the lab as soon as possible.

CALCULATIONS

Not applicable**.**

REPORTING RESULTS

Not applicable.

PROCEDURAL NOTES

- Specimens submitted for routine urinalysis should be collected in clean, dry containers. The

Specimen may be random, first morning, fasting, 2-hour postprandial, 24-hour (or timed),

Catheterized, midstream, clean-catch, or suprapubic aspiration. The specimen should be submitted

To the lab in a plastic screw-top transfer tube or specimen cup. Specimens submitted in syringes

Will not be accepted by the laboratory.

- The specimen containers must be properly labeled with appropriate patient identification

Including: name, medical record number, date of birth/age, the date and time of collection, and

Initials of the person collecting (or submitting) the sample

Specimens should be submitted to the laboratory immediately. A specimen for urinalysis should

Be examined while fresh. Specimens left at room temperature will begin to decompose resulting in

Chemical and microscopic changes.

- A minimum of 12 ml of urine should be submitted for analysis. Smaller sample quantities will be

Analyzed but the statement "QNS FOR ACCURACY: < 5 ML SUBMITTED FOR ANALYSIS"

Will accompany results of those specimens with volumes < 5 ml., e.g., babies or newborns.

SPECIMENS FOR PREGNANCY TESTING

First morning specimens are the best for pregnancy testing because the urine is more concentrated.

SPECIMENS FOR OSMOLALITY

No special sample preparation is required. Whole blood, serum, plasma, or urine may be used.

LIMITATIONS OF PROCEDURE

A. Specimens submitted in syringes will not be accepted.

B. Specimens improperly labeled must be discarded and recollected.

C. Urine osmolality cannot be collected with preservatives.

D. Urine samples leaking in the collection bag are unacceptable.

3.**selection, collection and transport of Sputum;**

Sputum collection techniques:

Sputum obtained spontaneouslyTwo specimens are to be collected. When possible, specimens should be collected outside in the open air and far away from other people.The first sample is collected on the spot, at the consultation, when the patient is identified as suspected TB case. If the patient has recently eaten, ask him/her to rinse his/her mouth with water in order to avoid the presence of food in the sample.The second sample is collected the day after, in the early morning, right after the patient wakes up and before eating. The second sample may be collected at home then the patient brings it to the health facility.Alternatively, two sputum specimens can be collected one hour apart (frontloaded microscopy).

Collection technique:

- The patient must be given a labelled sputum container (or a Falcon® tube, if the sample is to be shipped by air).
- Have the patient take a deep breath, hold for a few seconds, exhale, repeat two or three times, then cough: sputum is material brought up from the lungs after a productive cough. One or two minutes of chest clapping are of benefit.
- Collect at least 3 ml and close the container hermetically.

The quality of sample determines the reliability of the result. Always check that the sample contains solid or purulent material and not only saliva. Take a new sample if unsatisfactory.

If the sample is collected at home, make sure that the patient has understood the technique, including closing the container hermetically after collecting the sputum.

Sputum induction

Sputum induction is sometimes used in children when sputa cannot be spontaneously expectorated, and only in order to perform cultures or Xpert MTB/RIF.

Sputum induction must be performed under close medical supervision. The child should be observed for respiratory distress during, and for 15 minutes after, the procedure. Bronchospasm may occur. Salbutamol spray and oxygen must be ready at hand.

Equipment

- Gloves and respirator
- Suction catheter (6, 7, 8F)
- Sputum container
- 50 ml syringe, needle
- Mask and tubing for nebulizer
- Holding chamber with child's mask (to be sterilized between each patient)
- Sterile hypertonic solution of 5% sodium chloride (to be kept refrigerated)
- Sterile solution of 0.9% sodium chloride (for the specimen)
- Salbutamol spray
- Oxygen

Procedure

The child should fast for at least 2 hours before the procedure.

Prior to nebulization:

• Explain the procedure to the child and/or the person accompanying him/her (this person must wear a respirator).

• Place the child in a sitting position in the adult's arms.

• Administer 2 puffs of salbutamol via a holding chamber, 10 minutes before nebulization.

• Prepare a sputum container.

Nebulization

• Fill the nebulizer with 5 ml of 5% hypertonic saline solution (sputum inducer).

• Place the nebulizer mask over the child's mouth.

• Leave the child to inhale until the reservoir is empty.

Nasopharyngeal suction

• Do 1 to 2 minutes of clapping.

• Clean out the nasal cavity.

• During suction, the child is laid on his /her side, back to the operator, who is behind him/her.

• Fit a suction catheter to a 50 ml syringe. Lubricate the end of the catheter.

• Measure the distance from the tip of the nose to the angle of the jaw. Insert the suction catheter to that depth.

• When inserting and withdrawing the tube, pull on the plunger of the syringe to create suction.

• Once the syringe is filled with air and mucus, disconnect it from the suction catheter and purge the air (tip facing upward), so that only mucus is left in the syringe.

• To collect the mucus: draw 2 ml of 0.9% sodium chloride into the syringe to rinse, then empty contents into the sample container.

Gastric aspiration

Gastric aspiration is sometimes used in children when sputa cannot be spontaneously expectorated nor induced using hypertonic saline, and only in order to perform cultures or Xpert MTB/RIF.

Equipment

- Gloves and respirator
- Suction catheter (6, 7, 8F)
- Sputum container
- 50 ml syringe
- Sterile water

Procedure

Prior to inserting the suction catheter

• Explain the procedure to the child and/or the person accompanying him/her (this person must wear a respirator);

• Place the child in a half-sitting or sitting position in the adult's arms.

- Insert a nasogastric tube and check that it is correctly placed.
- First suction to collect the gastric fluid and place it in the sputum container, then rinse the stomach with 30 ml of sterile water and suction again. Add the suctioned fluid to the first sample.
- Start culture within 4 hours of collecting the sample. If there will be more than four hours' delay, neutralize with 100 mg of sodium bicarbonate.

Sputum specimen storage

When examinations are not performed on the site of collection:

Specimen for smear microscopy;

Smears should be performed within three-four days of collection and in the meanwhile stored refrigerated (2 to 8°C) and protected from light.

Contamination does not affect microscopy but heat make specimen liquefy, with selection of mucopurulent part of the sample more difficult.

Specimen for culture in liquid medium;

Keep the specimen refrigerated (2 to 8°C), protected from light. Do not use cethylpyrodinium chloride (CPC) as it is not compatible with MGIT.

The specimen should be processed as soon as possible.

Specimen for culture on Lowenstein-Jensen medium (LJ)

- Specimens that can be cultured in less than 3 days after collection:

Keep refrigerated (2 to 8°C) and protected from light until transport OR immediately transport to the laboratory for processing.

- Specimens that will be cultured more than 3 days after collection:

Use Falcon tubes and add 1% CPC to preserve the specimen for up to 2 weeks. Specimens with CPC should not be refrigerated, as the CPC will crystallize and be ineffective.

Samples with CPC can be inoculated on LJ. For inoculation on agar, they require prior neutralization by neutralizing buffer (Difco).

CPC can be used for specimens tested by Xpert MTB/RIF.

Sputum specimen shipment

To a local laboratory;

- Without CPC transport medium: between 2 and 8°C and protected from light;
- With CPC transport medium: should not be refrigerated because at low temperatures the CPC will crystallize and ruin the sample. Specimens should be kept at room temperature, protected from heat and light.

By air to a reference laboratory for culture;

Samples are collected and shipped in 50 ml Falcon® conical tubes with screw caps. The tubes are labelled UN 3373, corresponding to Category B infectious substances. If transport times are less than 12 hours, even specimens without CPC can be transported at room temperature.

Samples are triple-packaged, in accordance with IATA packing instruction 650:

1. Primary container holding the sputum sample: tube tightly closed and placed into a latex glove;

2. Secondary container intended to protect the primary container: leak-proof box with enough absorbent material to absorb the entire sample, should the primary container break;

3. Outer packaging intended to protect the secondary container, with UN 3373 labelling.

Information to be provided:

- Primary container: label with the patient's name or identification number and the sample collection date and location;
- Outer package: indicate the name of the receiving laboratory, the complete address (name, street, postal code, locality, country), and telephone number.

All samples must be accompanied by the corresponding laboratory test request form (including clinical information).

4.**Selection, collection and transport of CSF**

CSF Specimen Collection for Analysis

CSF Laboratory Orders

Obtain CSF for gram stain, cell count, protein, glucose and aerobic culture where able. Obtain kit 922257 (20G needle) or 922258 (22G needle) from Hospital Stores (356-1784). The kit should contain 4 pre-numbered tubes to be filled in chronological order. Avoid covering tube numbers with stickers to ensure appropriate routing of samples.

- With low volume, one-tube specimens not all testing may be possible and the clinician must determine which tests should be prioritized. If cultures are desired, Microbiology must receive the specimen first to ensure the culturing of a sterile specimen.
- Transport time <15 minutes. Do not refrigerate CSF for bacterial culture.

If adequate volume is obtained, orders are placed per tube as follows:

1 – Chemistry/Immunology Protein (LAB118) and Glucose (LAB611)

2– Microbiology Aerobic culture with gram stain (LAB4801). Select “CSF lumbar puncture”, “CSF shunt”, or “CSF ventricular trap” as source to ensure appropriate culturing. If cryptococcal meningitis is suspected, fungal cultures (LAB240) and cryptococcal antigen (LAB2233) should be ordered as well. The Meningitis/Encephalitis PCR Panel (LAB8514) should be ordered on all lumbar punctures where infections is being considered and can not be ordered on non-lumbar puncture samples (order individual tests instead).

3– Hematology If a pathologist review is needed, it can be obtained by ordering Cytospin, CSF (LAB1024) in addition to the CSF Cell Count and Differential (LAB1022).

4 – Specimen Control Specimen storage (LAB4890). Select “Spinal fluid” as source and request to hold for 30 days at -80°C unless 4°C or -20°C storage is needed for a specific test.

5.Selection, collection and transport of PUS

Possible pathogens in Pus

Gram positive	Gram negative
Staphylococcus aureus	*Pseudomonas aeruginosa*
Streptococcus pyogenes	*Escherichia coli*
Enterococcus species	*Proteus* species
Anaerobic streptococci	*Klebsiella* species
Other streptococci	Bacteriodes species
Clostridium perfringens and other clostridia	*Acinetobacter* species
Actinomycetes	Other enteric bacilli
Mycobacterium tuberculosis	
Others	
Fungi: *Histoplsama, Candida,* and fungi that cause mycetoma	
Parasites: *Entamoeba histolytica* (in pus aspirated from amoebic liver abscess)	
Viruses: Pox viruses and herpes viruses	

Table 1: Pus Sample: Collection, Processing, Staining and Culture;

Specimen Collection

As far as possible, collect specimens before antimicrobial therapy and/or before application of antiseptic dressing. The ideal specimen is an aspirate from a previously undrained abscess, or a tissue biopsy. Ideally, a minimum volume of 1mL (up to 5 mL) of pus should be collected. Large volumes of purulent material maintain the viability of anaerobes for longer.

The aspirate should be collected in a sterile syringe – any air bubbles should be expelled. Needle safely and tightly capped (needles should NOT be sent).

A tissue specimen should be placed in a sterile universal bottle (or any sterile leak proof container) and sent to the lab for immediate processing if anaerobes are suspected. If there will be a delay in transporting, the tissue should be placed in an anaerobic transport system.

Swabs are less desirable because of the smaller amount of specimen that is sampled and the fact that they are often contaminated with normal skin flora, making interpretation of results difficult. When using swabs, the deepest part of the wound should be sampled, avoiding the superficial microflora. Swabs should be well soaked in pus.

Laboratory examination of Pus sample

Describe the appearance of the specimen: Describe presence/absence of sulphur granules (needed only for the suspected cases of mycetoma or actinomycosis, when requested).

Preparation of the Smear:

If Pus swab is sent:

Only one aerobic pus swab: Inoculate the culture media first before using the swab to make smears for Gram staining

If swabs (one anaerobic and two aerobic) are submitted for culture, use the second swab for making gram stain

If tissue sample is submitted: make Gram stain from ground tissue.

Is pus aspirate is sent: using a sterile pipette place one drop of pus on to a clean microscope slide. Spread this using a sterile loop to make a thin smear for Gram staining.

Gram Staining:

Make an evenly spread smear of the specimen on a clean, grease-free slide. Allow the smear to air-dry in a safe place. Heat fix the specimen and stain by Gram staining technique. Examine the smear for the presence of bacteria and pus cells (PMNs) using 100x objective lens and look especially for:

Gram negative rods (Possible pathogens are E.coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus or Bacteroides species)

Gram positive cocci in pairs, chains or clusters (possible pathogens are Staphylococcus aureus, Streptococcus pyogenes, anaerobic streptococci or enterococci).

Gram positive large rods with square ends (possible pathogens are Clostridium perfringens or Bacillus anthracis)

In the case of anaerobic infections large number of pleomorphic bacteria (streptococci, Gram positive and Gram negative rods of various size and fusiform bacteria) may be seen. Sometimes, Gram positive yeast cells with psuedohyphae may be seen, which can be Candida albicans.

Pus Culture:

Culture Media: Wound specimens collected on aerobic swabs or pus aspirate should be plated on to the following media:

- Sheep blood agar (to isolate S. Aureus and Streptococcus pyogenes or other streptococci)
- MacConkey agar (to isolate Gram negative rods)

Incubation Condition:

Temperature: 35ºC -37ºC

Atmosphere: Blood Agar plate in carbon dioxide enriched atmosphere (e.g. 5% CO_2 incubator or in a candle jar) and MacConkey agar plate in ambient air (normal incubator)

Time: Up to 48 hours (observe the plate after 24 hours of incubation, if growth is seen do further processing, if not re-incubate for additional 24 hours.)

Examination and Reporting the Culture results;

If the growth is seen after 24/48 hours of culture, examination of the colony morphology and identification of the isolates should be done.

In the Blood Agar plate look for the hemolysis. Both Staphylococus aureus and Streptococcus pyogenes gives beta-hemolysis in Blood Agar (Some S. AuFor Streptococcus pyogenes and Enterococci antimicrobial sensitivity testing should be done in MHA supplemented with sheep blood. For the S.aureus and other gram-negative bacilli, Mueller-Hinton Agar (MHA) is used. The selection of the antibiotics panel depends on the isolated organism. Unless indicated routinely used (or first line) antibiotics should be used. If the patient is in Intensive Care Unit (SICU, PICU, NICU) or is receiving particular antibiotics, or the isolate is resistant to first-line antibiotics sensitivity testing should include requested antibiotics and/or second line antibiotics.reus isolates may not show hemolysis).

- S. aureus gives yellow to cream or white colonies. Colonies are slightly raised and easily emulsified.
- S.pyogenes produces beta-hemolytic colonies. Colonies are usually small, colourless, dry, shiny or mucoid.
- Enterococci gives non-hemolytic colonies in blood agar.

We can differentiate between streptococci and staphylococci by a very simple and rapid test-Catalase test (Staphylococcus-positive, Streptococcus-negative). For identification of suspected S. Aureus colonies perform coagulase test (to differentiate coagulase negative Staphylococci from S. Aureus) and for suspected Group A Streptococci (S.pyogenes) perform bacitracin sensitivity test (can be added in the blood agar plate with other antibiotics). If enterococci is suspected perform bile esculin test.

Pseudomonas aeruginosa gives large, flat, spreading pale colored colonies in MacConkey Agar. It is oxidase positive and can be identified by its pigments and/or distinctive smell (characteristics fruity smell).

Depending on the facilities available in the diagnostic laboratories, organisms can be identified using enterotube test or API-20E test or other newer diagnostics test available for the identification of isolates.

Antimicrobial Sensitivity Testing:

For *Streptococcus pyogen*es and Enterococci antimicrobial sensitivity testing should be done in MHA supplemented with sheep blood. For the *S.aureus* and other gram-negative bacilli, **Mueller-Hinton Agar (MHA)** is used. The selection of the antibiotics panel depends on the isolated organism. Unless indicated routinely used (or first line) antibiotics should be used. If the patient is in Intensive Care Unit (SICU, PICU, NICU) or is receiving particular antibiotics, or the isolate is resistant to first-line antibiotics sensitivity testing should include requested antibiotics and/or second line antibiotics.

6.Selection, collection and transport of Faeces;

Collection and transport of stool specimens:

Faecal specimens should be collected in the early stages of the diarrhoeal disease, when pathogens are present in the highest number, and preferably before antimicrobial treatment is started, if appropriate. The specimen should be collected in the morning to reach the

laboratory before noon, so that it can be processed the same day. Formed stools should be rejected. Ideally, a fresh stool specimen is preferred to a rectal swab, but a rectal swab is acceptable if the collection cannot be made immediately or when transportation of the stool to the laboratory is delayed.

1.4 Stool sample collection and transport

Figure 1.4: Stool Sample Collection and Transport

Procedure for collecting stool samples:

1. the patient with two small wooden sticks and a suitable container with a leakproof lid (e.g. a clean glass cup, a plastic or waxed-cardboard box, or a special container with a spoon attached to the lid).

2.The use of penicillin bottles, matchboxes and banana leaves should be discouraged as they expose the laboratory staff to the risk of infection.

3.Instruct the patient to collect the stool specimen on a piece of toilet tissue or old newspaper and to transfer it to the container, using the two sticks.

4.The specimen should contain at least 5 g of faeces and, if present, those parts that contain blood, mucus or pus. It should not be contaminated with urine.

5. the specimen has been placed in the specimen container, the lid should be sealed.

Transport of specimen:

1.The patient should be asked to deliver the specimen to the clinic immediately after collection.

2. it is not possible for the specimen to be delivered to the laboratory within 2 hours of its collection, a small amount of the faecal specimen (together with mucus, blood and epithelial threads, if present) should be collected on two or three swabs and placed in a container with transport medium (Cary–Blair, Stuart or Amies) or 33 mmol/l of glycerol–phosphate buffer.

3.For cholera and other Vibrio spp., alkaline peptone water is an excellent transport (and enrichment) medium.

4.Pathogens may survive in such media for up to 1 week, even at room temperature, although refrigeration is preferable.

Procedure for collecting rectal swabs:

1.Moisten a cotton-tipped swab with sterile water. Insert the swab through the rectal sphincter, rotate, and withdraw.

2.Examine the swab for faecal staining and repeat the procedure until sufficient staining is evident.

3.The number of swabs to be collected will depend on the number and types of investigation required.

4.Place the swab in an empty sterile tube with a cotton plug or screw-cap, if it is to be processed within 1–2 hours.

5.If the swab must be kept for longer than 2 hours, place it in transport medium

Microscopic Examination of specimen for Bacterial pathogens-Simple, Differential staining and motility

Staining Microscopic Specimens

In their natural state, most of the cells and microorganisms that we observe under the microscope lack color and contrast. This makes it difficult, if not impossible, to detect important cellular structures and their distinguishing characteristics without artificially treating specimens. We have already alluded to certain techniques involving stains and fluorescent dyes, and in this section we will discuss specific techniques for sample preparation in greater detail. Indeed, numerous methods have been developed to identify specific microbes, cellular structures, DNA sequences, or indicators of infection in tissue samples, under the microscope. Here, we will focus on the most clinically relevant techniques.

Preparing Specimens for Light Microscopy

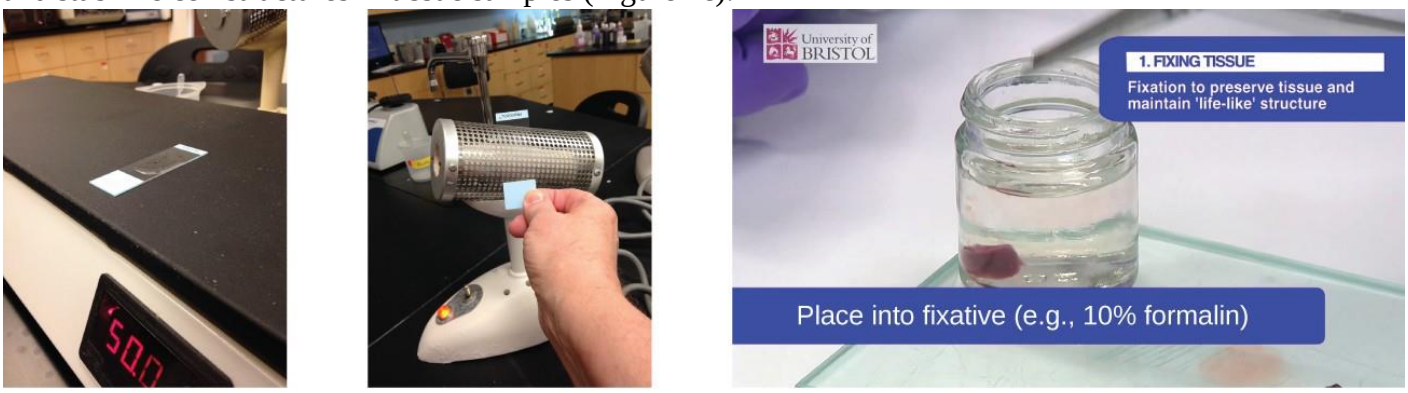
In clinical settings, light microscopes are the most commonly used microscopes. There are two basic types of preparation used to view specimens with a light microscope: wet mounts and fixed specimens.

The simplest type of preparation is the **wet mount**, in which the specimen is placed on the slide in a drop of liquid. Some specimens, such as a drop of urine, are already in a liquid form and can be deposited on the slide using a dropper. Solid specimens, such as a skin scraping, can be placed

on the slide before adding a drop of liquid to prepare the wet mount. Sometimes the liquid used is simply water, but often stains are added to enhance contrast. Once the liquid has been added to the slide, a coverslip is placed on top and the specimen is ready for examination under the microscope.

The second method of preparing specimens for light microscopy is **fixation**. The "fixing" of a sample refers to the process of attaching cells to a slide. Fixation is often achieved either by heating (**heat fixing**) or chemically treating the specimen. In addition to attaching the specimen to the slide, fixation also kills microorganisms in the specimen, stopping their movement and metabolism while preserving the integrity of their cellular components for observation.

To heat-fix a sample, a thin layer of the specimen is spread on the slide (called a **smear**), and the slide is then briefly heated over a heat source (Figure 1b). **Chemical fixatives** are often preferable to heat for tissue specimens. Chemical agents such as acetic acid, ethanol, methanol, formaldehyde (formalin), and glutaraldehyde can denature proteins, stop biochemical reactions, and stabilize cell structures in tissue samples (Figure 1c).



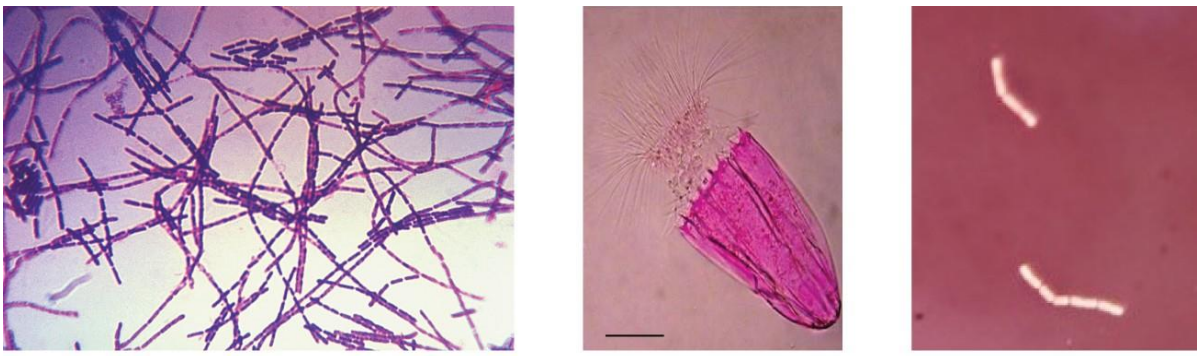
(a) (b) (c)

Figure 1.5. (a) A specimen can be heat-fixed by using a slide warmer like this one. (b) Another method for heat-fixing a specimen is to hold a slide with a smear over a microincinerator. (c) This tissue sample is being fixed in a solution of formalin (also known as formaldehyde). Chemical fixation kills microorganisms in the specimen, stopping degradation of the tissues and preserving their structure so that they can be examined later under the microscope. (credit a: modification of work by Nina Parker; credit b: modification of work by Nina Parker; credit c: modification of work by "University of Bristol"/YouTube)

In addition to fixation, **staining** is almost always applied to color certain features of a specimen before examining it under a light microscope. Stains, or dyes, contain salts made up of a positive ion and a negative ion. Depending on the type of dye, the positive or the negative ion may be the chromophore (the colored ion); the other, uncolored ion is called the counterion. If the chromophore is the positively charged ion, the stain is classified as a **basic dye**; if the negative ion is the chromophore, the stain is considered an **acidic dye**.

Dyes are selected for staining based on the chemical properties of the dye and the specimen being observed, which determine how the dye will interact with the specimen. In most cases, it is preferable to use a **positive stain**, a dye that will be absorbed by the cells or organisms being

observed, adding color to objects of interest to make them stand out against the background. However, there are scenarios in which it is advantageous to use a **negative stain**, which is absorbed by the background but not by the cells or organisms in the specimen. Negative staining produces an outline or silhouette of the organisms against a colorful background (Figure 2).



(a) (b) (c)

Figure 1.6. (a) These *Bacillus anthracis* cells have absorbed crystal violet, a basic positive stain. (b) This specimen of *Spinoloricus*, a microscopic marine organism, has been stained with rose bengal, a positive acidic stain. (c) These *B. megaterium* appear to be white because they have not absorbed the negative red stain applied to the slide. (credit a: modification of work by Centers for Disease Control and Prevention; credit b: modification of work by Roberto Danovaro, Antonio Pusceddu, Cristina Gambi, Iben Heiner, Reinhardt Mobjerg Kristensen; credit c: modification of work by Anh-Hue Tu)

Because cells typically have negatively charged cell walls, the positive chromophores in basic dyes tend to stick to the cell walls, making them positive stains. Thus, commonly used basic dyes such as **basic fuchsin**, **crystal violet**, **malachite green**, **methylene blue**, and **safranin** typically serve as positive stains. On the other hand, the negatively charged chromophores in acidic dyes are repelled by negatively charged cell walls, making them negative stains. Commonly used acidic dyes include **acid fuchsin**, **eosin**, and **rose bengal**. Table 2 provides more detail.

Some staining techniques involve the application of only one dye to the sample; others require more than one dye. In **simple staining**, a single dye is used to emphasize particular structures in the specimen. A simple stain will generally make all of the organisms in a sample appear to be the same color, even if the sample contains more than one type of organism. In contrast, **differential staining** distinguishes organisms based on their interactions with multiple stains. In other words, two organisms in a differentially stained sample may appear to be different colors. Differential staining techniques commonly used in clinical settings include Gram staining, acid-fast staining, endospore staining, flagella staining, and capsule staining. Table 3 provides more detail on these differential staining techniques.

Gram Staining

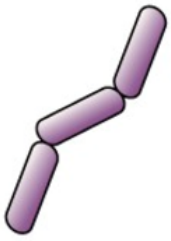
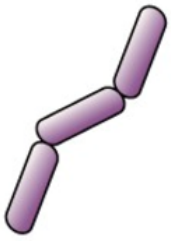
The **Gram stain procedure** is a differential staining procedure that involves multiple steps. It was developed by Danish microbiologist Hans Christian **Gram** in 1884 as an effective method to distinguish between bacteria with different types of cell walls, and even today it remains one of

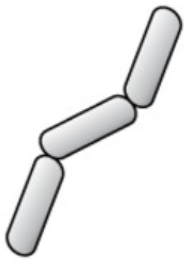
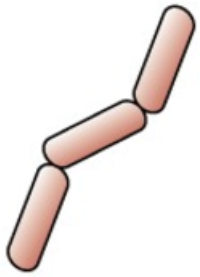
the most frequently used staining techniques. The steps of the Gram stain procedure are listed below and illustrated in Table 1.

1. First, **crystal violet**, a **primary stain**, is applied to a heat-fixed smear, giving all of the cells a purple color.
2. Next, **Gram's iodine**, a **mordant**, is added. A mordant is a substance used to set or stabilize stains or dyes; in this case, Gram's iodine acts like a trapping agent that complexes with the crystal violet, making the crystal violet–iodine complex clump and stay contained in thick layers of peptidoglycan in the cell walls.
3. Next, a **decolorizing agent** is added, usually ethanol or an acetone/ethanol solution. Cells that have thick peptidoglycan layers in their cell walls are much less affected by the decolorizing agent; they generally retain the crystal violet dye and remain purple. However, the decolorizing agent more easily washes the dye out of cells with thinner peptidoglycan layers, making them again colorless.
4. Finally, a secondary **counterstain**, usually **safranin**, is added. This stains the decolorized cells pink and is less noticeable in the cells that still contain the crystal violet dye.

Gram-staining is a differential staining technique that uses a primary stain and a secondary counterstain to distinguish between gram-positive and gram-negative bacteria.

Table 1.2. Gram stain process

Gram staining stems	Cell effects	Gram-positive	Gram-negative
Step 1: Crystal Violet *primary stain added to the specimen smear*	Stains cells purple or blue.		
Step 2: Iodine *mordant, makes the dye less soluble so it adheres to cell walls.*	Cells remain purple or blue.		

Step 3: Alcohol *the decolorizer, washes away stain from gram-negative cell walls*	Gram-positive cells remain purple or blue, Gram-negative cells are colorless.		
Step 4: Safranin *counterstain allows dye adherence to gram-negative cells*	Gram-positive cells remain a purple or blue. Gram-negative cells appear pink or red		

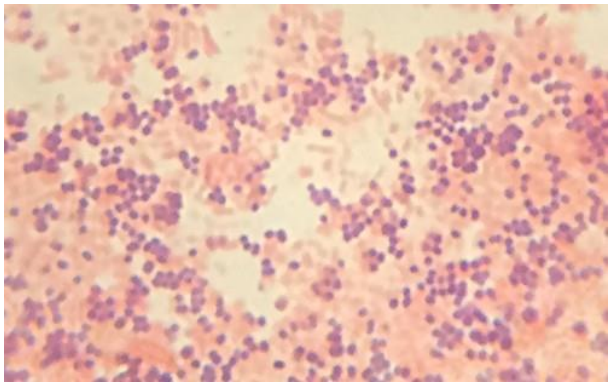


Figure 1.7: In this specimen, the gram-positive bacterium Staphylococcus aureus retains crystal violet dye even after the decolorizing agent is added. Gram-negative Escherichia coli, the most common Gram stain quality-control bacterium, is decolorized, and is only visible after the addition of the pink counterstain safranin. (credit: modification of work by Nina Parker)

The purple, crystal-violet stained cells are referred to as gram-positive cells, while the red, safranin-dyed cells are gram-negative (Figure 3). However, there are several important considerations in interpreting the results of a Gram stain. First, older bacterial cells may have damage to their cell walls that causes them to appear gram-negative even if the species is gram-positive. Thus, it is best to use fresh bacterial cultures for Gram staining. Second, errors such as leaving on decolorizer too long can affect the results. In some cases, most cells will appear gram-positive while a few appear gram-negative (as in Figure 3). This suggests damage to the individual cells or that decolorizer was left on for too long; the cells should still be classified as gram-positive if they are all the same species rather than a mixed culture.

Besides their differing interactions with dyes and decolorizing agents, the chemical differences between gram-positive and gram-negative cells have other implications with clinical relevance. For example, Gram staining can help clinicians classify bacterial pathogens in a sample into

categories associated with specific properties. Gram-negative bacteria tend to be more resistant to certain antibiotics than gram-positive bacteria. We will discuss this and other applications of Gram staining in more detail in later chapters.

Clinical Focus: Nathan, Part 3

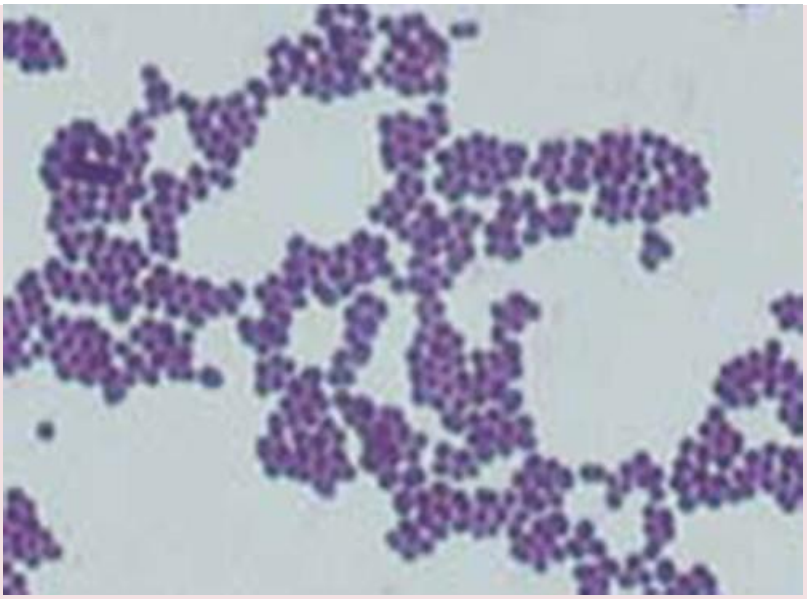


Figure 1.8: (credit: modification of work by American Society for Microbiology)

This example continues Nathan's story that started in The Properties of Light and Instruments of Microscopy.

Viewing Nathan's specimen under the darkfield microscope has provided the technician with some important clues about the identity of the microbe causing his infection. However, more information is needed to make a conclusive diagnosis. The technician decides to make a Gram stain of the specimen. This technique is commonly used as an early step in identifying pathogenic bacteria. After completing the **Gram stain procedure**, the technician views the slide under the brightfield microscope and sees purple, grape-like clusters of spherical cells

- Are these bacteria gram-positive or gram-negative?
- What does this reveal about their cell walls?

We'll conclude Nathan's example later on this page.

Acid-Fast Stains

Acid-fast staining is another commonly used, differential staining technique that can be an important diagnostic tool. An **acid-fast stain** is able to differentiate two types of gram-positive cells: those that have waxy mycolic acids in their cell walls, and those that do not. Two different methods for acid-fast staining are the **Ziehl-Neelsen technique** and the **Kinyoun technique**. Both use **carbolfuchsin** as the primary stain. The waxy, acid-fast cells retain the carbolfuchsin

even after a decolorizing agent (an acid-alcohol solution) is applied. A secondary counterstain, methylene blue, is then applied, which renders non–acid-fast cells blue.

The fundamental difference between the two carbolfuchsin-based methods is whether heat is used during the primary staining process. The Ziehl-Neelsen method uses heat to infuse the carbolfuchsin into the acid-fast cells, whereas the Kinyoun method does not use heat. Both techniques are important diagnostic tools because a number of specific diseases are caused by **acid-fast bacteria** (AFB). If AFB are present in a tissue sample, their red or pink color can be seen clearly against the blue background of the surrounding tissue cells (Figure 5).

Using Microscopy to Diagnose Tuberculosis

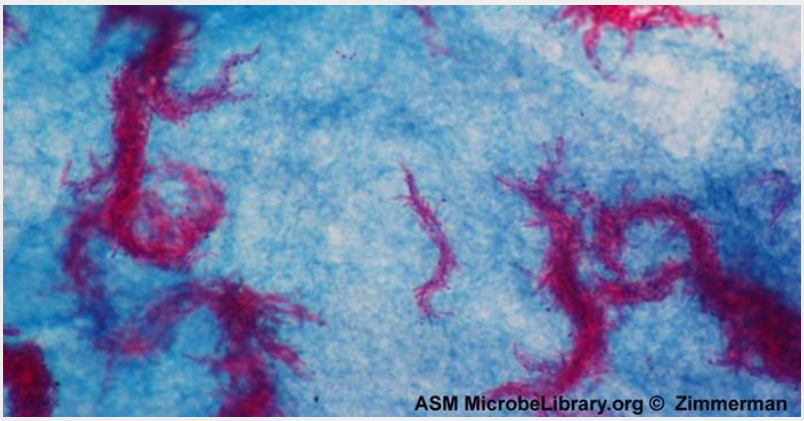


Figure 1.9: Ziehl-Neelsen staining has rendered these Mycobacterium tuberculosis cells red and the surrounding growth indicator medium blue. (credit: modification of work by American Society for Microbiology)

Mycobacterium tuberculosis, the bacterium that causes **tuberculosis**, can be detected in specimens based on the presence of acid-fast bacilli. Often, a smear is prepared from a sample of the patient's sputum and then stained using the Ziehl-Neelsen technique (Figure 5). If acid-fast bacteria are confirmed, they are generally cultured to make a positive identification. Variations of this approach can be used as a first step in determining whether *M. tuberculosis* or other acid-fast bacteria are present, though samples from elsewhere in the body (such as urine) may contain other *Mycobacterium* species.

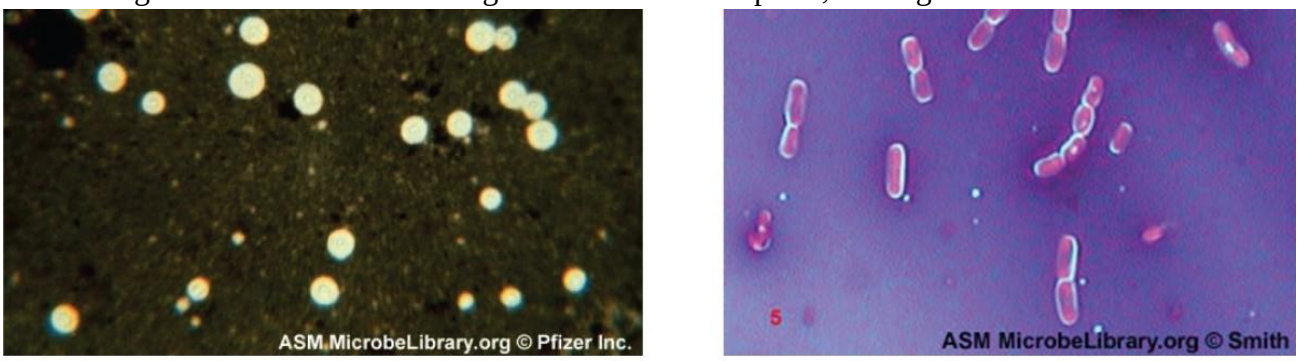
An alternative approach for determining the presence of *M. tuberculosis* is immunofluorescence. In this technique, fluorochrome-labeled antibodies bind to *M. tuberculosis*, if present. Antibody-specific fluorescent dyes can be used to view the mycobacteria with a fluorescence microscope.

Capsule Staining

Certain bacteria and yeasts have a protective outer structure called a capsule. Since the presence of a capsule is directly related to a microbe's virulence (its ability to cause disease), the ability to determine whether cells in a sample have **capsules** is an important diagnostic tool. Capsules do not absorb most basic dyes; therefore, a negative staining technique (staining around the cells) is typically used for **capsule staining**. The dye stains the background but does not penetrate the

capsules, which appear like halos around the borders of the cell. The specimen does not need to be heat-fixed prior to negative staining.

One common negative staining technique for identifying encapsulated yeast and bacteria is to add a few drops of **India ink** or **nigrosin** to a specimen. Other capsular stains can also be used to negatively stain encapsulated cells (Figure 6). Alternatively, positive and negative staining techniques can be combined to visualize capsules: The positive stain colors the body of the cell, and the negative stain colors the background but not the capsule, leaving halo around each cell.



(a) (b)

Figure 1.10: (a) India-ink was used to stain the background around these cells of the yeast *Cryptococcus neoformans*. The halos surrounding the cells are the polysaccharide capsules. (b) Crystal violet and copper sulfate dyes cannot penetrate the encapsulated *Bacillus* cells in this negatively stained sample. Encapsulated cells appear to have a light-blue halo. (credit a: modification of work by American Society for Microbiology; credit b: modification of work by American Society for Microbiology)

Endospore Staining

Endospores are structures produced within certain bacterial cells that allow them to survive harsh conditions. Gram staining alone cannot be used to visualize endospores, which appear clear when Gram-stained cells are viewed. **Endospore staining** uses two stains to differentiate **endospores** from the rest of the cell. The **Schaeffer-Fulton method** (the most commonly used endospore-staining technique) uses heat to push the primary stain (**malachite green**) into the endospore. Washing with water decolorizes the cell, but the endospore retains the green stain. The cell is then counterstained pink with **safranin**. The resulting image reveals the shape and location of endospores, if they are present. The green endospores will appear either within the pink vegetative cells or as separate from the pink cells altogether. If no endospores are present, then only the pink vegetative cells will be visible (Figure 7).

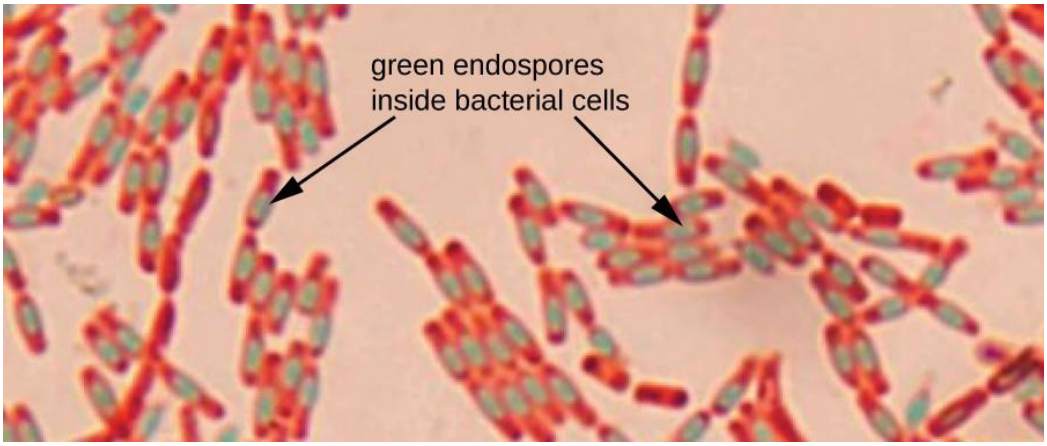


Figure 1.11: A stained preparation of *Bacillus subtilis* showing endospores as green and the vegetative cells as pink. (credit: modification of work by American Society for Microbiology)

Endospore-staining techniques are important for identifying *Bacillus* and *Clostridium*, two genera of endospore-producing bacteria that contain clinically significant species. Among others, ***B. anthracis*** (which causes **anthrax**) has been of particular interest because of concern that its spores could be used as a bioterrorism agent. ***C. difficile*** is a particularly important species responsible for the typically hospital-acquired infection known as "C. diff."

Flagella Staining

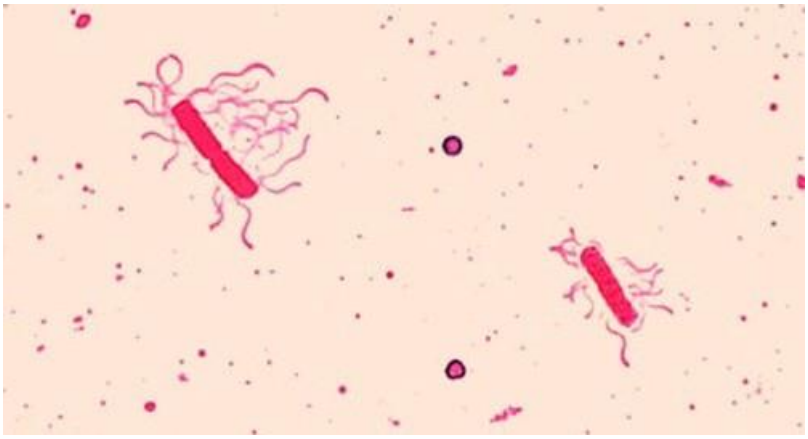


Figure 1.12: A flagella stain of Bacillus cereus, a common cause of foodborne illness, reveals that the cells have numerous flagella, used for locomotion. (credit: modification of work by Centers for Disease Control and Prevention)

Flagella (singular: flagellum) are tail-like cellular structures used for locomotion by some bacteria, archaea, and eukaryotes. Because they are so thin, flagella typically cannot be seen under a light microscope without a specialized **flagella staining** technique. Flagella staining thickens the flagella by first applying mordant (generally tannic acid, but sometimes potassium alum), which coats the flagella; then the specimen is stained with **pararosaniline** (most commonly) or **basic fuchsin** (Figure 8).

Though flagella staining is uncommon in clinical settings, the technique is commonly used by microbiologists, since the location and number of flagella can be useful in classifying and identifying bacteria in a sample. When using this technique, it is important to handle the specimen with great care; flagella are delicate structures that can easily be damaged or pulled off, compromising attempts to accurately locate and count the number of flagella.

Table 1.3. Simple Stains[1]

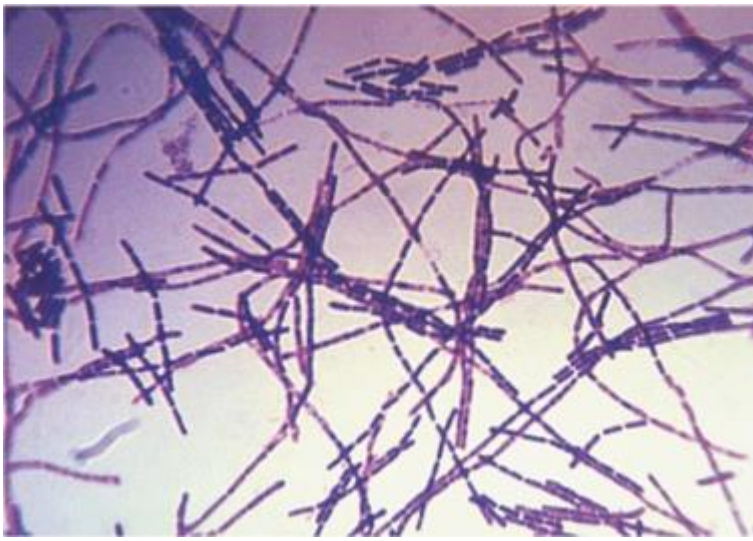
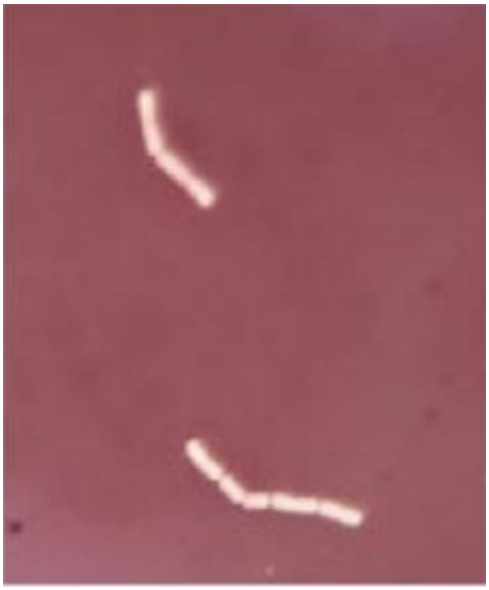
Stain Type	Specific Dyes	Purpose	Outcome	Sample Images 1.14
Basic stains	Methylene blue, crystal violet, malachite green, basic fuschsin, carbolfuschsin, safranin	Stain negatively charged molecules and structures, such as nucleic acids and proteins	Positive stain	

Table 1.3. Simple Stains[1]

Stain Type	Specific Dyes	Purpose	Outcome	Sample Images
Acidic stains	Eosine, acid fuchsin, rose bengal, Congo red	Stain positively charged molecules and structures, such as proteins	Can either be positive or negative stain, depending on the cell's chemistry	**1.14**  **1.15** 2.4

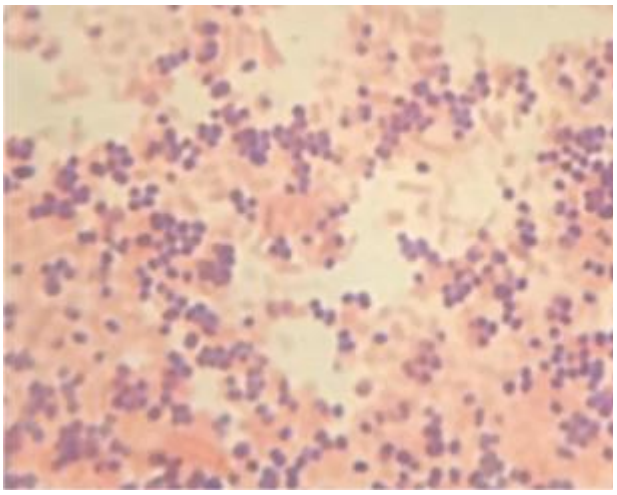
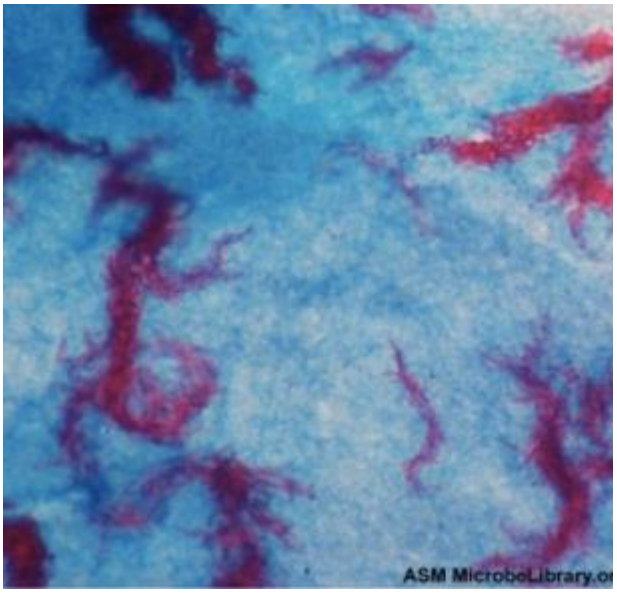
Table 1.3. Simple Stains[1]

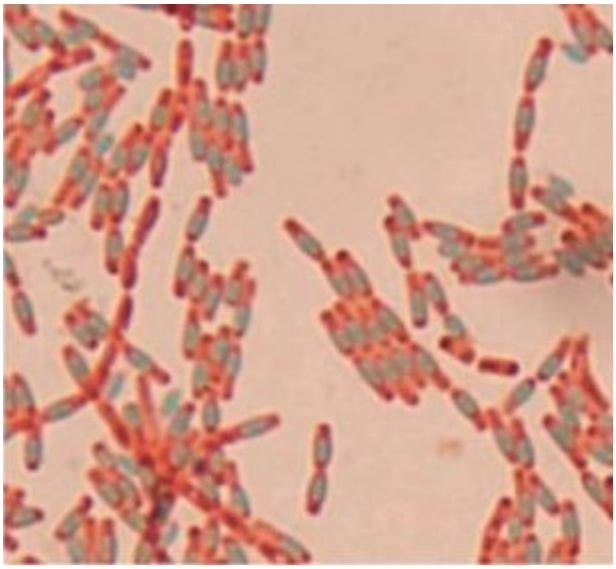
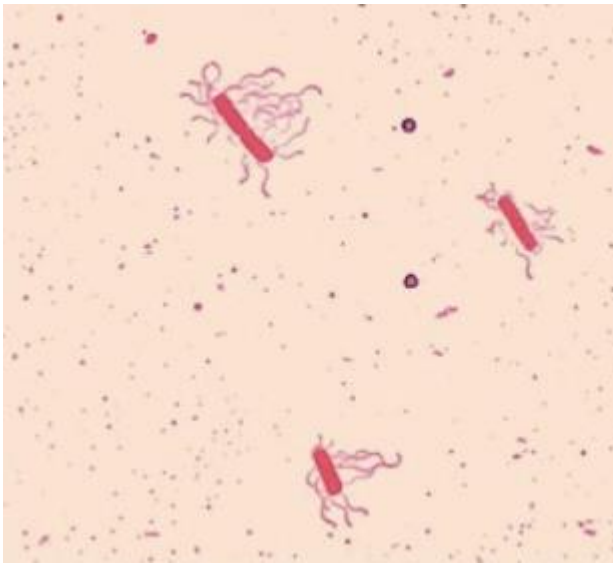
Stain Type	Specific Dyes	Purpose	Outcome	Sample Images 1.14
Negative stains	India ink, nigrosine	Stains background, not specimen	Dark background with a light specimen	

1.16

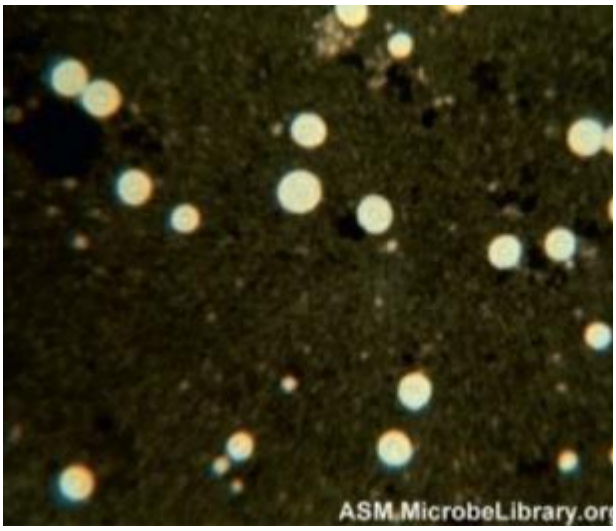
Table 3. Differential Stains

Stain Type	Specific Dyes	Purpose	Outcome	Sample Images
Gram stain	Uses crystal violet, Gram's iodine,	Used to distinguish cells by cell-wall	Gram-positive cells stain purple/violet. Gram-negative	1.12

	ethanol (decolorizer), and safranin	type (gram-positive, gram-negative)	cells stain pink	
Acid-fast stain	After staining with basic fuchsin, acid-fast bacteria resist decolonization by acid-alcohol. Non-acid-fast bacteria are counterstained with methylene blue.	Used to distinguish acid-fast bacteria such as *M. tuberculosis*, from non-acid-fast cells.	Acid-fast bacteria are red; non-acid-fast cells are blue.	

Endospore stain	Uses heat to stain endospores with malachite green (Schaeffer-Fulton procedure), then cell is washed and counterstained with safranin.	Used to distinguish organisms with endospores from those without; used to study the endospore	Endospores appear bluish-green; other structures appear pink to red.	
Flagella stain	Flagella are coated with a tannic acid or potassium alum mordant, then stained using either pararosaline or basic fuchsin	Used to view and study flagella in bacteria that have them.	Flagella are visible if present	
Capsule stain	Negative staining with India ink or nigrosine is used to stain the background, leaving a	Used to distinguish cells with capsules from those without.	Capsules appear clear or as halos if present.	1.15

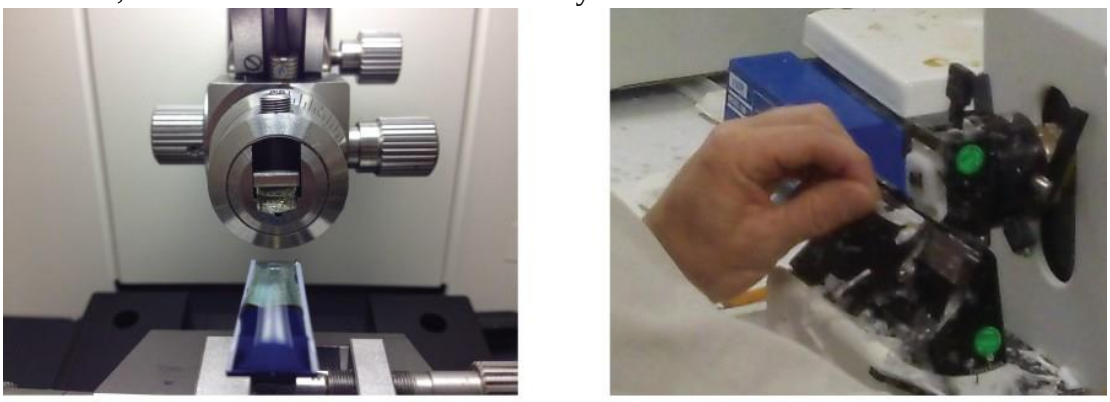
clear area of the cell and the capsule
Counterstaining can be used to stain the cell while leaving the capsule clear



1.16

Preparing Specimens for Electron Microscopy

Samples to be analyzed using a TEM must have very thin sections. But cells are too soft to cut thinly, even with diamond knives. To cut cells without damage, the cells must be embedded in plastic resin and then dehydrated through a series of soaks in ethanol solutions (50%, 60%, 70%, and so on). The ethanol replaces the water in the cells, and the resin dissolves in ethanol and enters the cell, where it solidifies. Next, **thin sections** are cut using a specialized device called an **ultramicrotome** (Figure 9). Finally, samples are fixed to fine copper wire or carbon-fiber grids and stained—not with colored dyes, but with substances like uranyl acetate or osmium tetroxide, which contain electron-dense heavy metal atoms.



(a) (b)

Figure 1.17: (a) An ultramicrotome used to prepare specimens for a TEM. (b) A technician uses an ultramicrotome to slice a specimen into thin sections. (credit a: modification of work by "Frost Museum"/Flickr; credit b: modification of work by U.S. Fish and Wildlife Service Northeast Region)

When samples are prepared for viewing using an SEM, they must also be dehydrated using an ethanol series. However, they must be even drier than is necessary for a TEM. Critical point drying with inert liquid carbon dioxide under pressure is used to displace the water from the specimen. After drying, the specimens are sputter-coated with metal by knocking atoms off of a palladium target, with energetic particles. Sputter-coating prevents specimens from becoming charged by the SEM's electron beam.

Using Microscopy to Diagnose Syphilis

The causative agent of **syphilis** is ***Treponema pallidum***, a flexible, spiral cell (spirochete) that can be very thin (<0.15 μm) and match the refractive index of the medium, making it difficult to view using brightfield microscopy. Additionally, this species has not been successfully cultured in the laboratory on an artificial medium; therefore, diagnosis depends upon successful identification using microscopic techniques and serology (analysis of body fluids, often looking for antibodies to a pathogen). Since fixation and staining would kill the cells, darkfield microscopy is typically used for observing live specimens and viewing their movements. However, other approaches can also be used. For example, the cells can be thickened with silver particles (in tissue sections) and observed using a light microscope. It is also possible to use fluorescence or electron microscopy to view *Treponema* (Figure 10).

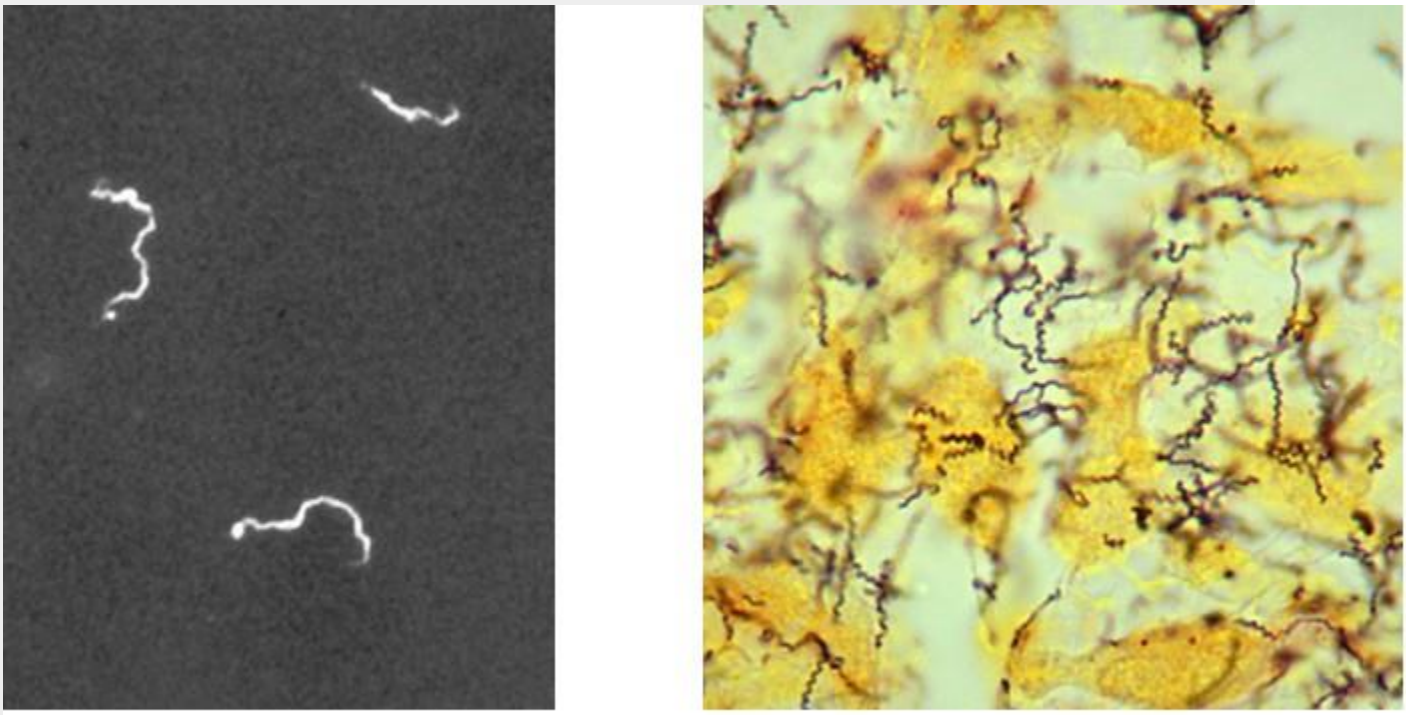


Figure 1.18: (a) Living, unstained Treponema pallidum spirochetes can be viewed under a darkfield microscope. (b) In this brightfield image, a modified Steiner silver stain is used to visualized T. pallidum spirochetes. Though the stain kills the cells, it increases the contrast to make them more visible. (c) While not used for standard diagnostic testing, T. pallidum can also be examined using scanning electron microscopy. (credit a: modification of work by Centers for Disease Control and Prevention; credit b:

modification of work by Centers for Disease Control and Prevention; credit c: modification of work by Centers for Disease Control and Prevention)

In clinical settings, indirect **immunofluorescence** is often used to identify *Treponema*. A primary, unstained antibody attaches directly to the pathogen surface, and secondary antibodies "tagged" with a fluorescent stain attach to the primary antibody. Multiple secondary antibodies can attach to each primary antibody, amplifying the amount of stain attached to each *Treponema* cell, making them easier to spot (Figure 1.19).

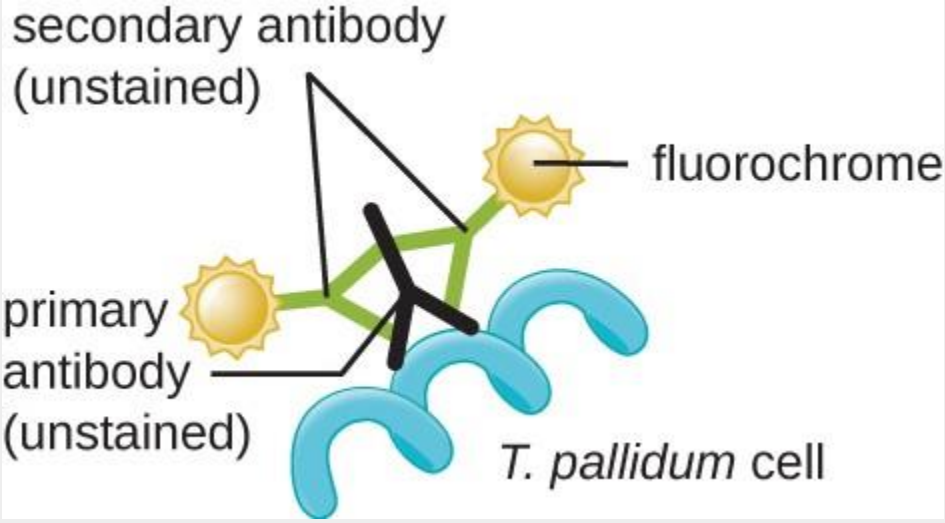


Figure 1.19: Indirect immunofluorescence can be used to identify *T. pallidum*, the causative agent of syphilis, in a specimen.

Preparation and Staining for Other Microscopes

Samples for fluorescence and confocal microscopy are prepared similarly to samples for light microscopy, except that the dyes are fluorochromes. Stains are often diluted in liquid before applying to the slide. Some dyes attach to an antibody to stain specific proteins on specific types of cells (**immunofluorescence**); others may attach to DNA molecules in a process called **fluorescence in situ hybridization (FISH)**, causing cells to be stained based on whether they have a specific DNA sequence.

Sample preparation for two-photon microscopy is similar to fluorescence microscopy, except for the use of infrared dyes. Specimens for STM need to be on a very clean and atomically smooth surface. They are often mica coated with Au(111). Toluene vapor is a common fixative.

Focus: Nathan, Resolution

This example concludes Nathan's story that started in The Properties of Light, Instruments of Microscopy, and above.

From the results of the Gram stain, the technician now knows that Nathan's infection is caused by spherical, gram-positive bacteria that form grape-like clusters, which is typical of staphylococcal bacteria. After some additional testing, the technician determines that these bacteria are the medically important species known as ***Staphylococcus aureus***, a common culprit in wound infections. Because some strains

of *S. aureus* are resistant to many antibiotics, skin infections may spread to other areas of the body and become serious, sometimes even resulting in amputations or death if the correct antibiotics are not used.

After testing several antibiotics, the lab is able to identify one that is effective against this particular strain of *S. aureus*. Nathan's doctor quickly prescribes the medication and emphasizes the importance of taking the entire course of antibiotics, even if the infection appears to clear up before the last scheduled dose. This reduces the risk that any especially resistant bacteria could survive, causing a second infection or spreading to another person.

Microscopy and Antibiotic Resistance

As the use of antibiotics has proliferated in medicine, as well as agriculture, microbes have evolved to become more resistant. Strains of bacteria such as methicillin-resistant *S. aureus* (**MRSA**), which has developed a high level of resistance to many antibiotics, are an increasingly worrying problem, so much so that research is underway to develop new and more diversified antibiotics.

Fluorescence microscopy can be useful in testing the effectiveness of new antibiotics against resistant strains like MRSA. In a test of one new antibiotic derived from a marine bacterium, MC21-A (bromophene), researchers used the fluorescent dye **SYTOX Green** to stain samples of MRSA. SYTOX Green is often used to distinguish dead cells from living cells, with fluorescence microscopy. Live cells will not absorb the dye, but cells killed by an antibiotic will absorb the dye, since the antibiotic has damaged the bacterial cell membrane. In this particular case, MRSA bacteria that had been exposed to MC21-A did, indeed, appear green under the fluorescence microscope, leading researchers to conclude that it is an effective antibiotic against MRSA.

Of course, some argue that developing new antibiotics will only lead to even more antibiotic-resistant microbes, so-called **superbugs** that could spawn epidemics before new treatments can be developed. For this reason, many health professionals are beginning to exercise more discretion in prescribing antibiotics. Whereas antibiotics were once routinely prescribed for common illnesses without a definite diagnosis, doctors and hospitals are much more likely to conduct additional testing to determine whether an antibiotic is necessary and appropriate before prescribing.

A sick patient might reasonably object to this stingy approach to prescribing antibiotics. To the patient who simply wants to feel better as quickly as possible, the potential benefits of taking an antibiotic may seem to outweigh any immediate health risks that might occur if the antibiotic is ineffective. But at what point do the risks of widespread antibiotic use supersede the desire to use them in individual cases?

Differential Staining Techniques

Viewing Bacterial Cells

The microscope is a very important tool in microbiology, but there are limitations when it comes to using one to observe cells in general and bacterial cells in particular. Two of the most important concerns are resolution and contrast. Resolution is a limitation that we can't do much about, since most bacterial cells are already near the resolution limit of most light microscopes. Contrast, however, can be improved by

either using a different type of optical system, such as phase contrast or a differential interference contrast microscope, or by staining the cells (or the background) with a chromogenic dye that not only adds contrast, but gives them a color as well.

There are many different stains and staining procedures used in microbiology. Some involve a single stain and just a few steps, while others use multiple stains and a more complicated procedure. Before you can begin the staining procedure, the cells have to be mounted (smeared) and fixed onto a glass slide.

A bacterial smear is simply that—a small amount of culture spread in a very thin film on the surface of the slide. To prevent the bacteria from washing away during the staining steps, the smear may be chemically or physically "fixed" to the surface of the slide. Heat fixing is an easy and efficient method, and is accomplished by passing the slide briefly through the flame of a Bunsen burner, which causes the biological material to become more or less permanently affixed to the glass surface.

Heat fixed smears are ready for staining. In a simple stain, dyes that are either attracted by charge (a cationic dye such as methylene blue or crystal violet) or repelled by charge (an anionic dye such as eosin or India ink) are added to the smear. Cationic dyes bind the bacterial cells which can be easily observed against the bright background. Anionic dyes are repelled by the cells, and therefore the cells are bright against the stained background. See Figures 1 and 2 for examples of both.

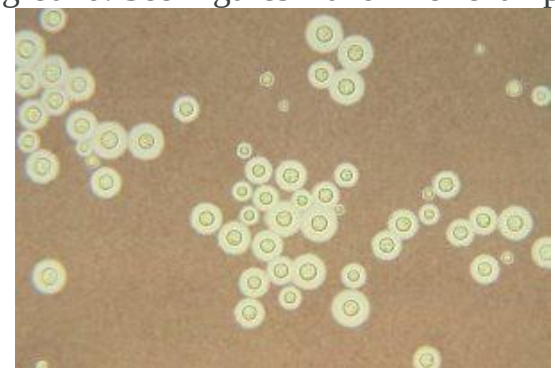


Figure 1.20: Negative stain of *Cyptococcus neoformans*, an encapsulated yeast

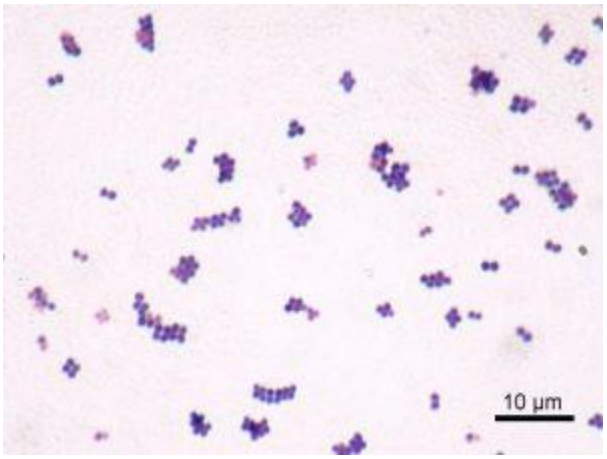


Figure 1.21: Positive stain of *Staphylococcus aureus.*

Probably the most important feature made obvious when you stain bacterial cells is their **cellular morphology** (not to be confused with colonial morphology, which is the appearance of bacterial colonies on an agar plate). Most heterotrophic and culturable bacteria come in a few basic shapes: spherical cells (coccus/cocci), rod-shaped cells (bacillus/bacilli), or rod-shaped cells with bends or twists (vibrios and spirilla, respectively). There is greater diversity of shapes among Archaea and other bacteria found in ecosystems other than the human body.

Often bacteria create specific **arrangements** of cells, which form as a result of binary fission by the bacteria as they reproduce. Arrangements are particularly obvious with non-motile bacteria, because the cells tend to stay together after the fission process is complete. Both the shape and arrangement of cells are characteristics that can be used to distinguish among bacteria. The most commonly encountered bacterial shapes (cocci and bacilli) and their possible arrangements are shown in Figures 3 and 4.

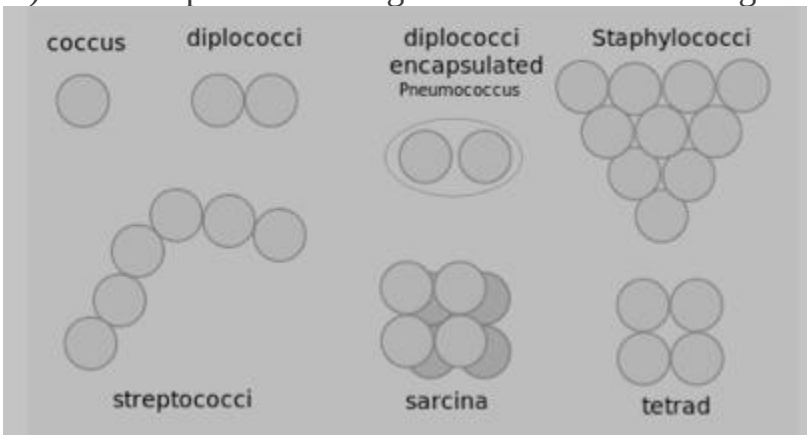


Figure 1.22: Possible bacterial cell arrangements for cocci

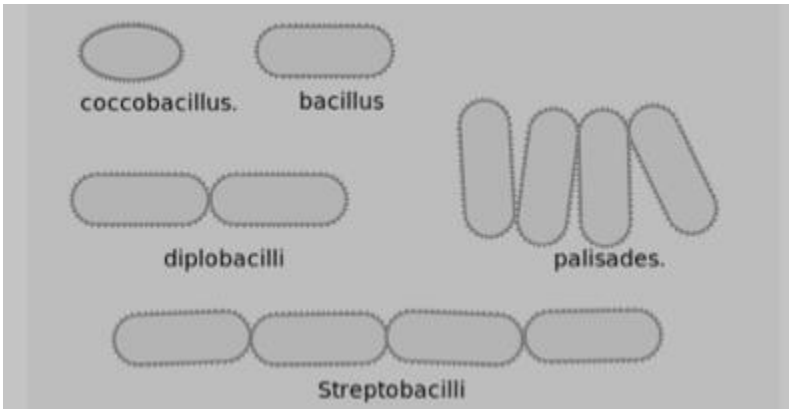


Figure 1.23: Possible bacteria cell arrangements for bacilli

Differential Staining Techniques

In microbiology, differential staining techniques are used more often than simple stains as a means of gathering information about bacteria. Differential staining methods, which typically require more than one stain and several steps, are referred to as such because they permit the differentiation of cell types or cell structures. The most important of these is the Gram stain. Other differential staining methods include the endospore stain (to identify endospore-forming bacteria), the acid-fast stain (to discriminate *Mycobacterium* species from other bacteria), a metachromatic stain to identify phosphate storage granules, and the capsule stain (to identify encapsulated bacteria). We will be performing the Gram stain and endospore staining procedures in lab, and view prepared slides that highlight some of the other cellular structures present in some bacteria.

Gram Stain

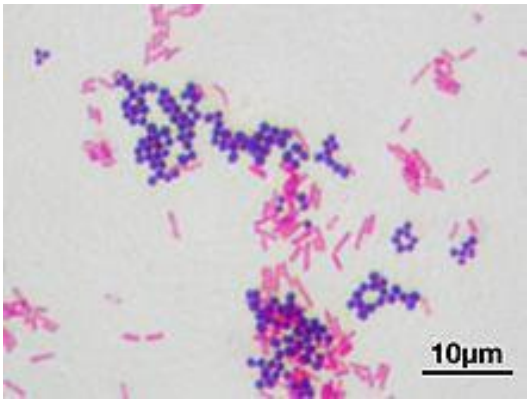


Figure1.24: Bacteria stained with Gram stain.

In 1884, physician Hans Christian Gram was studying the etiology (cause) of respiratory diseases such as pneumonia. He developed a staining procedure that allowed him to identify a bacterium in lung tissue taken from deceased patients as the etiologic agent of a fatal type of pneumonia. Although it did little in the way of treatment for the disease, the Gram stain method made it much easier to diagnose the cause of a person's death at autopsy. Today we use Gram's staining techniques to aid in the identification of bacteria, beginning with a preliminary classification into one of two groups: **Gram positive** or **Gram negative**.

The differential nature of the Gram stain is based on the ability of some bacterial cells to retain a primary stain (crystal violet) by resisting a decolorization process. Gram staining involves four steps. First cells are stained with crystal violet, followed by the addition of a setting agent for the stain (iodine). Then alcohol is applied, which selectively removes the stain from only the Gram negative cells. Finally, a secondary stain, safranin, is added, which counterstains the decolorized cells pink.

Although Gram didn't know it at the time, the main difference between these two types of bacterial cells is their cell walls. Gram negative cell walls have an outer membrane (also called the envelope) that dissolves during the alcohol wash. This permits the crystal violet dye to escape. Only the decolorized cells take up the pink dye safranin, which explains the difference in color between the two types of cells. At the conclusion of the Gram stain procedure, Gram positive cells appear purple, and Gram negative cells appear pink.

When you interpret a Gram stained smear, you should also describe the morphology (shape) of the cells, and their arrangement. In Figure 5, there are two distinct types of bacteria, distinguishable by Gram stain reaction, and also by their shape and arrangement. Below, describe these characteristics for both bacteria:

	Gram positive bacterium:	**Gram negative bacterium:**
Morphology		
Arrangement		

Acid Fast Stain

Some bacteria produce the waxy substance **mycolic acid** when they construct their cell walls. Mycolic acid acts as a barrier, protecting the cells from dehydrating, as well as from phagocytosis by immune system cells in a host. This waxy barrier also prevents stains from penetrating the cell, which is why the Gram stain does not work with mycobacteria such as *Mycobacterium*, which are pathogens of humans and animals. For these bacteria, the **acid–fast staining** technique is used.

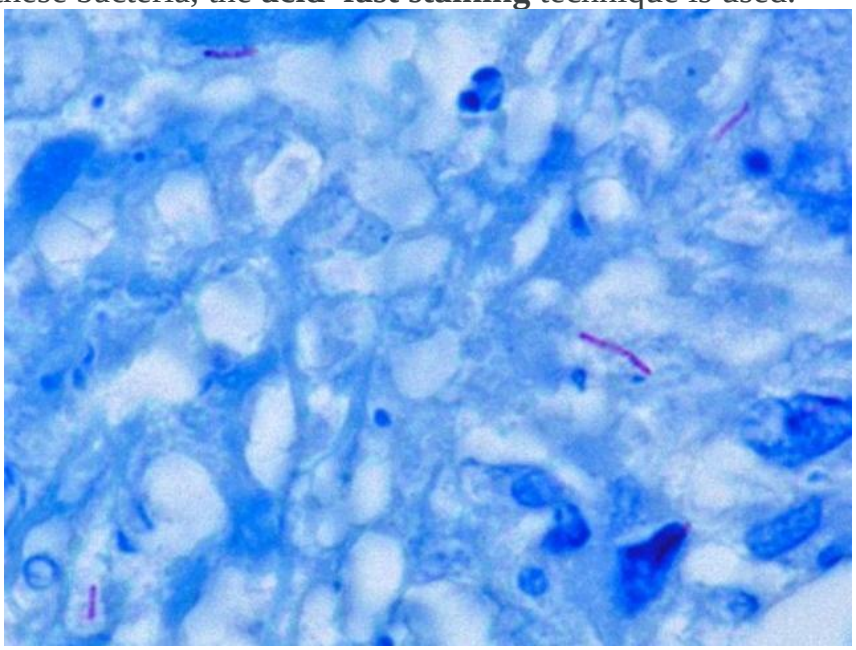


Figure 1.25: Acid-fast bacilli in sputum

To perform the acid-fast stain, a heat-fixed smear is flooded with the primary stain carbol fuchsin, while the slide is heated over a steaming water bath. The heat "melts" the waxy cell wall and permits the absorption of the dye by the cells. Then the slide is allowed to cool and a solution of acid and alcohol is added as a decolorizer. Cells that are "acid-fast" because of the mycolic acid in their cell wall resist decolorization and retain the primary stain. All other cell types will be decolorized. Methylene blue is then used as a counterstain. In the end, acid-fast bacteria (AFB) will be stained a bright pink color, and all other cell types will appear blue.

Staining Methods to Highlight Specific Cell Structures

Capsule: The polysaccharide goo that surrounds some species of bacteria and a few types of eukaryotic microbes is best visualized when the cells are negative stained. In this method, the bacteria are first mixed with the stain, and then a drop of the mixture is spread across the surface of a slide in the thin film. With this method, capsules appear as a clear layer around the bacterial cells, with the background stained dark.

Metachromatic granules or other intracytoplasmic bodies: Some bacteria may contain storage bodies that can be stained. One example is the Gram positive bacilli *Corynebacterium*, which stores phosphate in structures called "volutin" or metachromatic granules that are housed within the cell membrane. Various staining methods are used to visualize intracytoplasmic bodies in bacteria, which often provide an identification clue when observed in cells.

Endospore Stain

Endospores are dormant forms of living bacteria and should not be confused with reproductive spores produced by fungi. These structures are produced by a few genera of Gram-positive bacteria, almost all bacilli, in response to adverse environmental conditions. Two common bacteria that produce endospores are *Bacillus* or *Clostridum*. Both live primarily in soil and as symbionts of plants and animals, and produce endospores to survive in an environment that change rapidly and often.

The process of **endosporulation** (the formation of endospores) involves several stages. After the bacterial cell replicates its DNA, layers of peptidoglycan and protein are produced to surround the genetic material. Once fully formed, the endospore is released from the cell and may sit dormant for days, weeks, or years. When more favorable environmental conditions prevail, endospores **germinate** and return to active duty as vegetative cells.

Mature endospores are highly resistant to environmental conditions such as heat and chemicals and this permits survival of the bacterial species for very long periods. Endospores formed millions of years ago have been successfully brought back to life, simply by providing them with water and food.

Because the endospore coat is highly resistant to staining, a special method was developed to make them easier to see with a brightfield microscope. This method, called the **endospore stain**, uses either heat or long exposure time to entice the endospores to take up the primary stain, usually a water soluble dye such as malachite green since endospores are permeable to water. Following a decolorization step which removes the dye from the vegetative cells in the smear, the counterstain safranin is applied to provide color and contrast. When stained by this method, the endospores are green, and the vegetative cells stain pink, as shown in Figure 1.24.

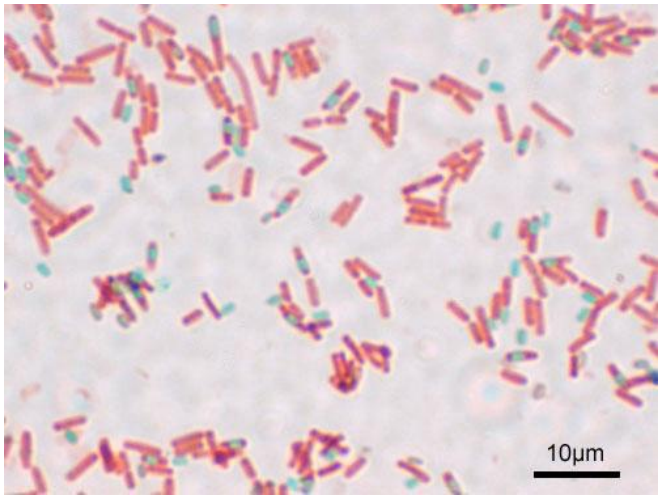


Figure 1.26: Bacterial cells with endospores, stained with the endospore stain.

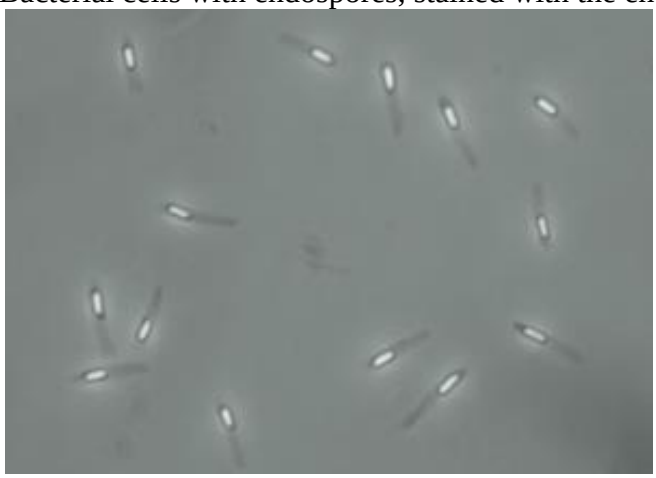


Figure 1.27: Bacilli with endospores viewed by phase-contrast microscopy.

Although endospores themselves are resistant to the Gram stain technique, bacterial cells captured in the process of creating these structures can be stained. In this case, the endospores are seen as clear oval or spherical areas within the stained cell. Endospores can also be directly observed in cells by using phase contrast microscopy, as shown in Figure 8.

Method

Because many differential staining methods require several steps and take a long time to complete, we will not be performing all of the differential staining methods discussed above.

Pre-stained slides will be used to visualize bacterial capsules, metachromatic granules, and acid-fast bacilli. Obtain one slide of each of the three bacteria listed in the table below. As you view these slides, make note of the “highlighted” structures. Your

environmental isolate may have one or more of these cellular features, and learning to recognize them will aid in identification. These should all be viewed using the oil immersion objective lens.

Bacterium	Stain	Description or sketch of cells with the specified feature
Flavobacterium capsulatum	Capsule stain	
Corynebacterium diphtheria	Methylene blue(metachromatic granules)	
Mycobacterium tuberculosis	Acid fast stain	

Gram Stain

All staining procedures should be done over a sink. The Gram stain procedure will be demonstrated, and an overview is provided in Table 1.

Table 1. Gram stain procedural steps.

Step	**Procedure**	**Outcome**
Primary stain(crystal violet)	Add several drops of crystal violet to the smear and allow it to sit for 1 minute. Rinse the slide with water.	Both Gram-positive and Gram-negative cells will be stained purple by the crystal violet dye.

Mordant (iodine)	Add several drops of iodine to the smear and allow it to sit for 1 minute. Rinse the slide with water.	Iodine "sets" the crystal violet, so both types of bacteria will remain purple.
Decolorization (ethanol)	Add drops of ethanol **one at a time** until the runoff is clear. Rinse the slide with water.	Gram-positive cells resist decolorization and remain purple. The dye is released from Gram-negative cells.
Counterstain(safranin)	Add several drops of safranin to the smear and allow it to sit for one minute. Rinse the slide with water and blot dry.	Gram-negative cells will be stained pink by the safranin. This dye has no effect on Gram-positive cells, which remain purple.

A volunteer from your lab bench should obtain cultures of the bacteria you will be using in this lab, as directed by your instructor. One of the cultures will be a Gram positive bacterium, and the other will be Gram negative. Below, write the names of the bacteria you will be using, along with the BSL for each culture:

_____

_____

Obtain two glass slides, and prepare a smear of each of the two bacterial cultures, one per slide, as demonstrated. Allow to COMPLETELY air dry and heat fix. Stain both smears using the Gram stain method. Observe the slides with a light microscope at 1,000X and record your observations in the table below.

Name of culture	Gram stain reaction	Cellular morphology	Arrangement

Gram Stain "Final Exam": prepare a smear that contains a mixture of the Gram-positive AND Gram-negative bacteria by adding a small amount of each bacterium to a single drop of water on a slide. Heat fix the smear and Gram stain it. You should be able to determine the Gram stain reaction, cellular morphology and arrangement of BOTH bacteria in this mixed smear. Your instructor may ask to see this slide and offer constructive commentary.

Endospore Stain

Only a few genera of bacteria produce endospores and nearly all of them are Gram-positive bacilli. Most notable are *Bacillus* and *Clostridium* species, which naturally live in soil and are common contaminants on surfaces. The growth of *Clostridium* spp. is typically limited to anaerobic environments; *Bacillus* spp. may grow aerobically and anaerobically. Endospore-forming bacteria are distinct from other groups of Gram positive bacilli and distinguishable by their endospores.

An overview of the endospore stain procedure is provided in Table 2.

Table 2. Endospore stain procedural steps.

Step	Procedure	Outcome
Primary stain(malachite green)	Add several drops of malachite green to the smear and allow it to sit for 10 minutes. If the stain starts to dry out, add additional drops.	Vegetative cells will immediately take up the primary stain. Endospores are resistant to staining but eventually take up the dye.
Decolorization(water)	Rinse the slide under a gentle stream of water for 10-15 seconds.	Once the endospores are stained, they remain green. A thorough rinse with water will decolorize the vegetative cells.
Counterstain(safranin)	Add several drops of safranin to the smear and allow it to sit for 1 minute. Rinse the	Decolorized vegetative cells take up the counterstain and appear pink; endospores

	slide and blot dry.	are light green.

After staining, endospores typically appear as light green oval or spherical structures, which may be seen either within or outside of the vegetative cells, which appear pink.

The shape and location of the endospores inside the bacterial cells, along with whether the sporangium is either distending (D) or not distending (ND) the sides of the cell, are important characteristics that aid in differentiating among species (see Figure 9).

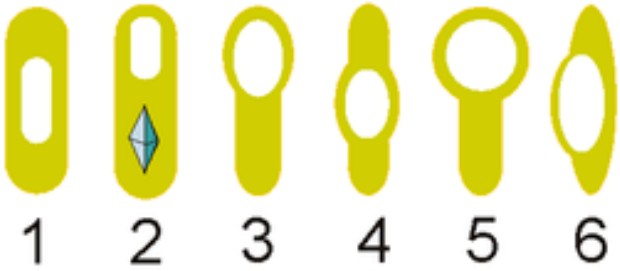


Figure 1.28:

Oval, central, not distended (ND)

1. Oval, terminal, ND (and parasporal crystal)
2. Oval, terminal, distended (D)
3. Oval, central, D
4. Spherical, terminal, D
5. Oval, lateral, D

Endospores are quite resistant to most staining procedures; however, in a routinely stained smear, they may be visible as "outlines" with clear space within. If you observe "outlines" or what appear to be "ghosts" of cells in a Gram stained smear of a Gram-positive bacilli, then the endospore stain should also be performed to confirm the presence or absence of endospores.

A volunteer from your lab bench should obtain bacterial cultures for endospore staining, as directed by your instructor. Note that these will all be species of *Bacillus*. Prepare smears and stain each using the endospore staining technique. Observe the slides and note the shape and location of the endospore and the appearance of the sporangium (swollen or not swollen) in the table below:

Name of culture	Endospore Shape	Location	Sporangium

In addition, choose ONE of the cultures from above and Gram stain it. Record your results below in the spaces provided:

Name of Gram stained culture: _____

Gram stain reaction and cellular morphology: _____

Are endospores visible in the Gram stained smear? _____ If you see endospores, describe how they appear in the Gram stained preparation, and how this is similar to and different from what you see in the endospore stained preparation.

Cultivation of bacteria

- A microbial culture ,is a method of multiplying microorganism by letting them reproduce in predetermined culture media under controlled laboratory condition
- Microbial cultures are used to determine the type of organism ,its abundance in the sample being tested ,or both

purpose of culturing

- Isolation of bacteria
- properties of bacteria i.e. culturing bacteria is the initial step in studying its morphology and its identification
- maintenance of stock cultures
- Estimate viable counts
- To test for antibiotic sensitivity
- To create antigen for laboratory use
- Certain genetic studies and manipulation of the cell also need that bacteria to be cultured in vitro

Culture media

- An artificial culture media must provide environmental and nutrition conditions that exist in the natural habitat of a bacterium
- A culture medium contains water ,a source of carbon and energy source of nitrogen ,trace elements and some growth factors .
- The PH of the medium must be set accordingly

Pure culture

- In the laboratory bacteria are isolated and grown in pure culture in order to study the function of a particular specie
- A pure culture is a population of cell or growing in the absence of the absence of other species or types .A pure culture may originate from a single cell or single organism.

Classification of culture media

- Bacteria culture media can be classified in at least three ways
    - ✓ Consistency
    - ✓ Nutritional component
    - ✓ Function use

Classification based on consistency

A . liquid media : these are available for use in test -tubes ,bottle or flasks .liquid media are sometimes referred as "broths "(eg. Nutrient broth).In liquid medium ,bacteria grow uniformly producing general turbidity .no agar is added ,mostly used for inoculums preparation

B. solid media :An agar plate is a petri dish that contain a growth medium used to culture microorganism .2%of agar is added .agar is the most company used solidifying agent .

Classification based on nutrition components

1.simple media

2.complex media

3.synthetic or chemically defined media

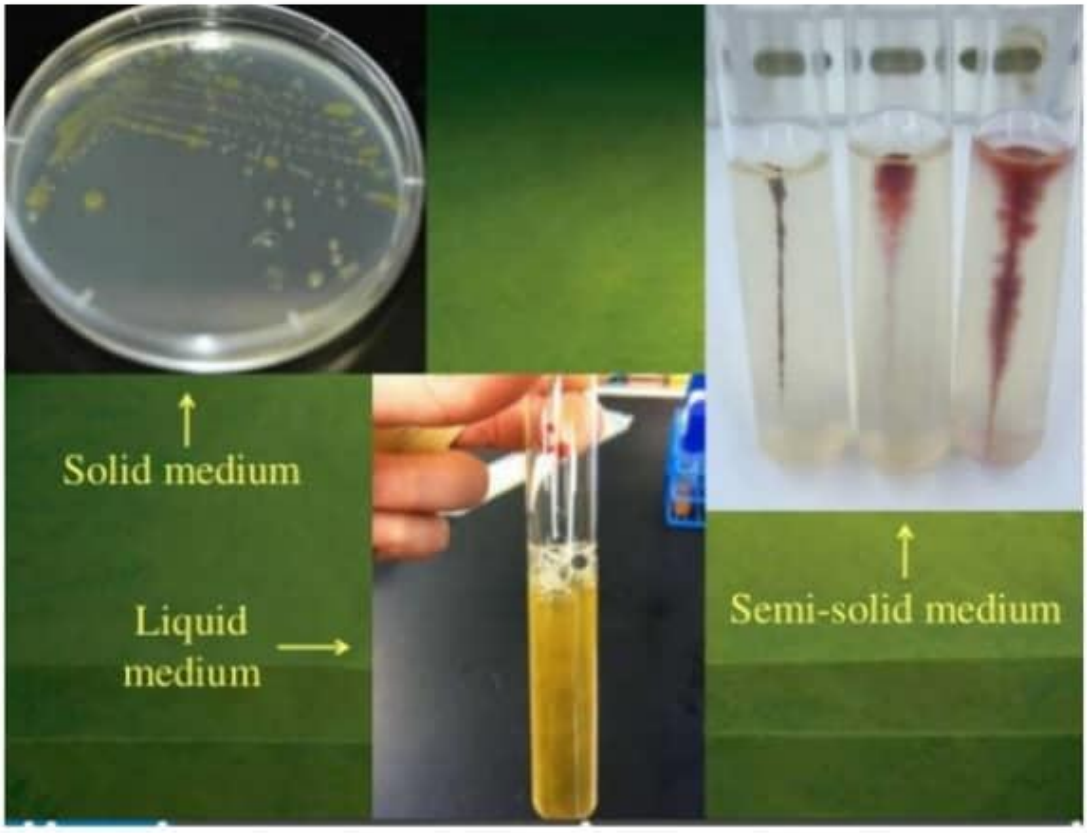


Figure1.29: Classification based on functional use or application

1.Enriched media

2.selective media

3.differential media

4.transport media

5.indicator media

Enriched media:

- Addition of extra nutrients in the form blood ,serum ,egg yolk etc to basal medium makes makes them enriched media

Figure 1.30: Media used to isolate pathogens from a mixed culture

Media used to isolate pathogens from a mixed culture .

- Stimulate growth of desired bacterium and inhibit growth of unwanted bacterium
- Media is incorporated with inhibitory substance to suppress the unwanted organism ,thus increase in number of desired bacteria

Chocolate Agar

- Chocolate agar is a non -selective enriched growth medium used for growing fastidious bacteria ,such as Haemophilus influenzae

Blood agar

- Blood agar contains mammalian blood (usually sheep or horse)typically at a concentration of 5-10%BAP are enriched differential media used to isolate fastidious organisms and detect hemolytic activity
  1.29 Media

Figure 1.31: Enriched Media

2.selective media

- The inhibitory substance is added to a solid media thus causing an increases in number of colonies of desired bacterium
- Selective media and enrichment media are designed to inhibit unwanted commensal or contamination bacteria and help to recover pathogen from a mixture of bacteria

- Any agar media can be made selective 1.3011.

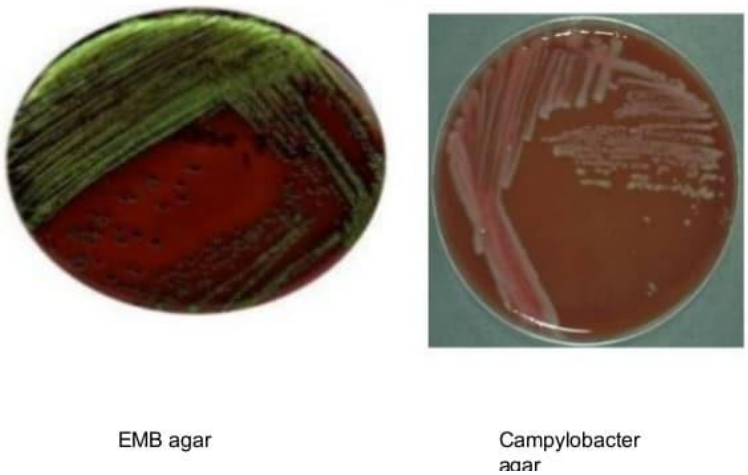


Figure 1.32: EMB agar and Campylobacter agar

Examples

- Thayer Martin medium selective for Neisseria gonorrhoeae

- EMB agar is selective for gram negative bacteria .the dye methylene blue in the medium inhibits the growth of gram positive bacteria ,small amounts of this dye effectively inhibit the growth of most gram positive bacteria
- Campylobacter agar (CAMPY) is used for the selective isolation of campylobacter Jejuni

Differential Media

- Certain media are designed in such a way that different bacteria can be recognized on the basis of the colony colour various approaches, include incorporation of dyes metabolic substrates etc ,so that those bacteria that utilize them appear as differently Coloured colonies. substances incorporated in it enable it to distinguish between bacteria.

Examples of differential media ;MacConkey's agar ,CLED agar ,XLD agar etc.

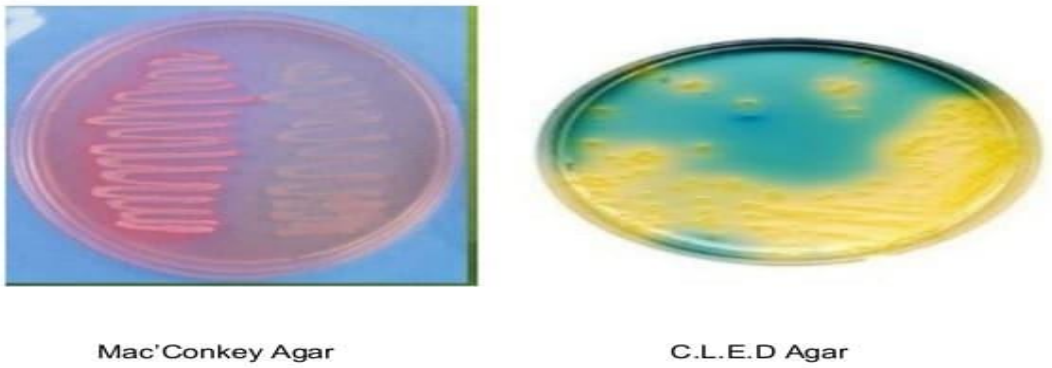


Figure 1.33: MacConkey's agar ,CLED agar

- CYSTEINE LACTOSE ELECTROLYTE DIFFECIENT AGAR C.L.E.D Agar is a non selective solid medium for cultivation of pathogens from urine specimens. Lack of salts (electrolytes)inhibits swarming of proteus spp.

- MacConkey Agar culture medium designed to grow gram negative bacteria and differentiate them for lactose fermentation it contains bile salts (to inhibit most gram-positive bacteria),crystal violet dye(which also inhibits certain gram-positive bacteria). Lactose fermenter-pink

Culture method

- Streak culture

- Lawn culture
- Stroke culture
- Stab culture
- Pour culture

Streak culture

Used for the isolation of bacteria in pure culture from clinical specimen

- Platinum wire is used
- One loop full of the specimen is transferred onto the surface of a well dried plate
- Spread over a small area at the periphery
- The inoculum is then distributed thinly over the plate by streaking it with a loop in a series of parallel lines in different segments of the

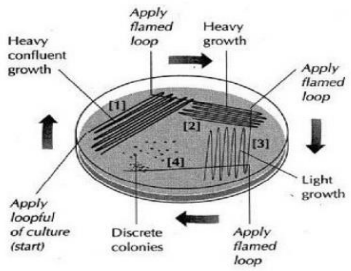


Figure 1.34: Lawn culture plate

- Provides a uniform surface growth of the bacterium
- Lawn culture are prepared by flooding the surface of the plate with a liquid suspension of the bacterium

- Uses For bacteriophage typing

Figure 1.35: Stroke culture

- Stroke culture is made in tubes containing agar slope /slant
  Uses
  -demonstrate of gelatin liquefaction
  -oxygen requirement of the bacterium

  Pour plate culture
- 1ml of the inoculum is added to the molten agar
- Mix well and pour to a sterile petri dish
- Allow it to set

- Depth of the medium

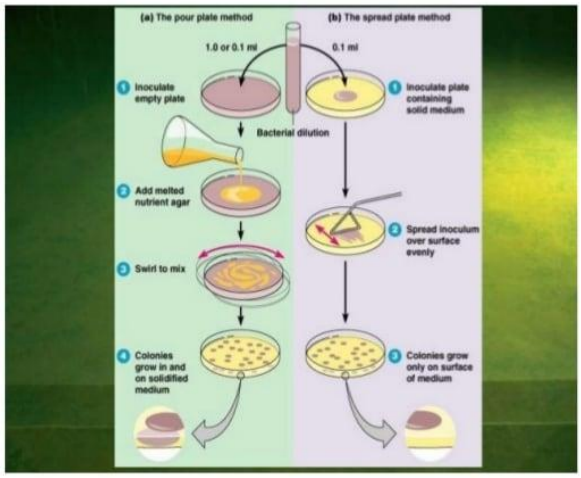


Figure 1.36: Pour Plate and Spread Plate method

BIOCHEMICAL TEST OF BACTERIA

1) Carbohydrate Fermentation Test (Sugar Fermentation Test)

Carbohydrate fermentation test is used to determine the ability of microbes to ferment carbohydrates with production of an acid/gas. Sugars are metabolized through different metabolic pathways. If fermenting bacteria are grown in liquid culture medium containing carbohydrate, they may produce organic acids as by-products of fermentation. These acids released into the medium and medium pH is lowered. If pH indicator such as phenol red is included, acid production will change the medium from its original colour to yellow. Gases produced during fermentation can be detected by using Durham tube within the liquid culture medium.

Results: If the medium changes from colorless to yellow and gas bubble is found in Durham's tube, then it indicates acid and gas production. If no changes are observed, then no sugar is utilizied by the organism.

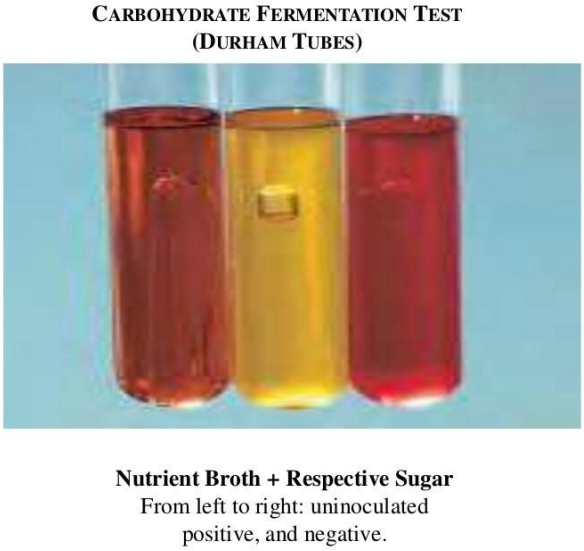


Figure 1.37: Carbohydrate Fermentation Test

2) INDOLE PRODUCTION TEST

Indole production test is used to determine the ability of microbe to degrade the amino acid tryptophan.

Tryptophanase

Tryptophan ⟶ Indole+ Pyruvic acid+Ammonia

HCl, alcohol

P-Dimethylaminobenzaldehyde + Indole ⟶ Quinoidal red-violet compound

Results: Development of cherry red colour at the interface of the reagent and the broth, within seconds after adding Kovac's reagent indicates presence of indole and test is positive. If no color change is observed, then test is negative and so organisms are not capable of producing tryptophanase.

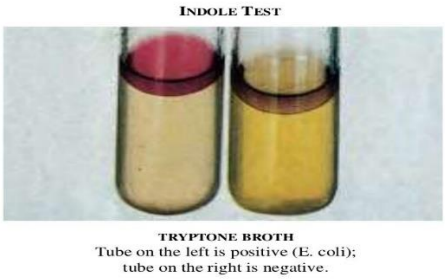


Figure 1.38: Indole Test

3) **Methyl Red Test (MR test)**
Methyl red test is used to differentiate E.coli and E.aerogen and to determine the ability of microorganisms to oxidize glucose with production and stabilization of high content of acid end product.

Principle:

E.coli:

Glucose + H_2O ⟶ Lactic acid, Acetic acid, Formic acid + Methyl Red ⟶ CO_2 + H_2 + Red colour (positive test) (pH 4)

E. aerogen:

Glucose + H_2O ⟶ Acetic acid ⟶ 2,3butanediol + CO_2 + H_2O + Methyl Red ⟶ Yellow colour (negative test) (pH 6)

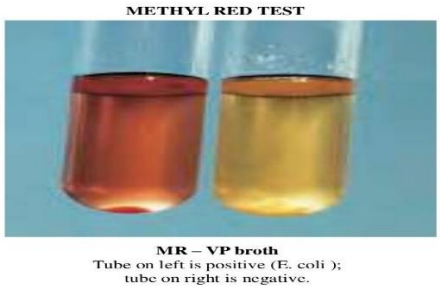
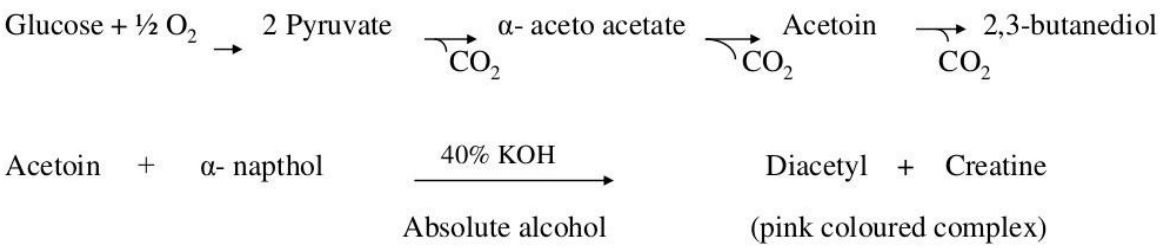


Figure 1.39: Methylred Test

4) **Voges-Proskauer Test (VP test)**

Voges-Proskauer test is used to differentiate E.coli and E.aerogen by the production of 2,3-butanediol and acetoin via glucose fermentation. This test determines the capability of some organisms to produce non-acidic or neutral end products such as acetoin, from organic acid that results from glucose metabolism. This test characterizes E.aerogen. Test identifies bacteria that ferment glucose and leading to 2,3-butanediol accumulation in the medium.

Results: Development of crimson red color indicates positive test for E.aerogen. No color change indicates negative test.



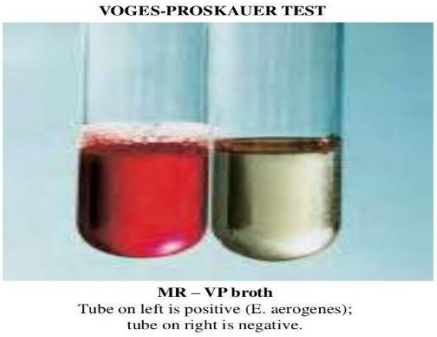


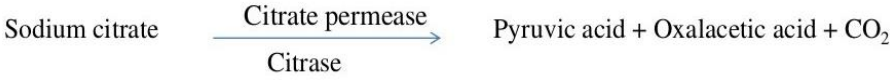
Figure 1.40: VOGES-PROSKAUER TEST

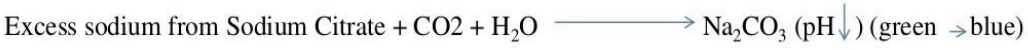
5) **Citrate Utilization Test**

Citrate utilization test is used to determine ability of microbes to ferment citrate as sole carbon source.

- ❖ Citrate as sole carbon source for their energy needs
- ❖ Presence of citrate permease that facilitates transport of citrate into bacterium

- Sodium citrate as carbon source, NH4+ as nitrogen source
- pH indicator- Bromothymol blue
- This test is done on slants since O2 is necessary for citrate utilization
- When bacteria oxidize citrate, they remove it from medium and liberate CO2
- CO2 combines with sodium and water to form sodium carbonate-an alkaline product
- This raises pH and turns indicator blue- represents positive citrate test, absence of color change is negative citrate test

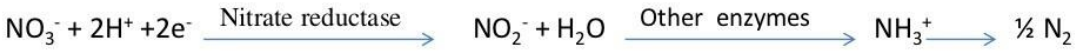




6) **Nitrate Reduction Test**

Nitrate reduction test is used to determine ability of microbes to reduce nitrate to nitrite or beyond the nitrite stage.

- Certain organisms like Chemolithoautotrophic bacteria and many Chemoorganoheterotrophs can use nitrate as terminal electron acceptor during anaerobic respiration.
- In this process, nitrate is reduced to nitrite by nitrate reductase
- Further reduce nitrite to either ammonium ion or molecular nitrogen
- Nitrate broth medium contains 0.5% potassium nitrate (KNO3)
- Examined for presence of gas and nitrite ions in the medium



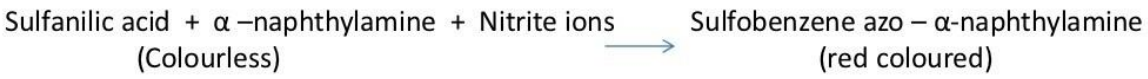


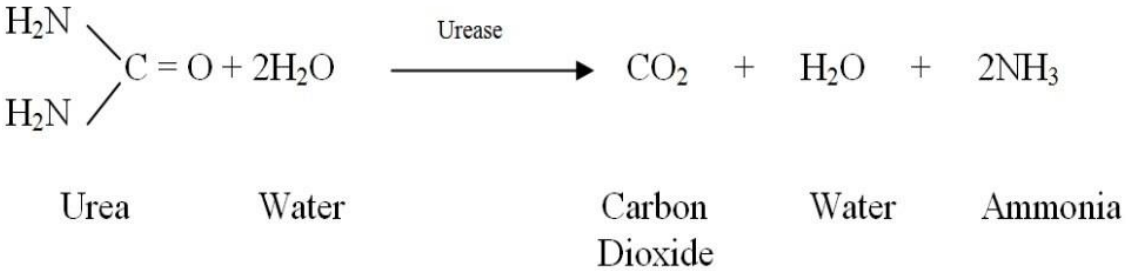
Figure 1.41: Nitrate Reduction Test

7) **Urease Test**

Urease test used to determine ability of microbes to degrade urea by urease.

- Urea is diamide carbonic acid often referred as carbamide
- The hydrolysis of urea is catalyzed by specific enzyme urease to yield 2 moles of ammonia
- Urease attacks the nitrogen and carbon bond in urea and forms ammonia
- Presence of urease is detected when organisms are grown in urea broth.
- Medium contains pH indicator- Phenol Red
- Splitting of urea creates alkaline condition which turns phenol red into deep pink color
- Mainly used for identification of Proteus sp from other genus of lactose non fermenting enteric organisms

Results: If urea is present in the medium, then it will be degraded which creates alkaline condition in the medium that results in color change from reddish pink to deep pink.



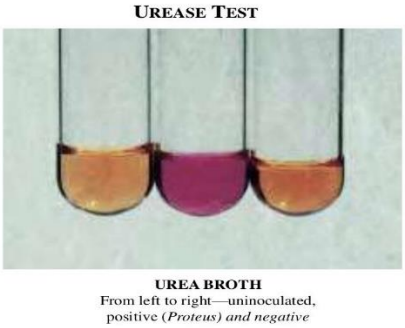


Figure 1.42: Urease Test

8) **Triple Sugar Iron (TSI) Agar Test**

TSI test is done to differentiate between the members of family Enterobacteriaceae family. It is also used to distinguish Enterobacteriaceae from other gram- negative enteric bacteria. TSI agar slants contain 1% of lactose and sucrose and 0.1% glucose. The pH indicator phenol red is incorporated in the medium to detect acid production from carbohydrate fermentation. The uninoculated medium is red in color due to presence of phenol red dye. Yeast extract and peptone provide nitrogen, sulfur, trace elements, vitamin B complex etc.

NaCl maintains osmotic equilibrium. Sodium thiosulfate and ferrous sulfate make H2S indicator system. Thiosulfate is reduced toH2S by several species of bacteria, H2S combine with FeSO4 present in medium and forms black precipitate. Blackening occurs in butt of the tube. Incubation done for 18-24hrs to detect sugar fermentation, gas and H2S production.

The indicator is pink at alkaline pH, yellow at acidic pH and red at neutral pH.

Results:

- Alkaline slant/Acidic butt- Only glucose is fermented
- Acidic slant Acidic butt -Glucose, sucrose and lactose, all 3 sugars are fermented
- Bubbles or cracks present – Gas production
- Black precipitate present- H2S production

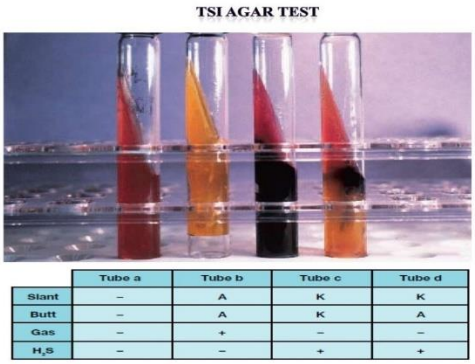


Figure 1.43: TSI agar test

9) **Oxidase Test**

Oxidase test is done to determine ability of microbes to produce oxidase enzyme.

Oxidase enzyme plays a key role in electron transport chain during aerobic respiration. Cytochrome Oxidase catalyzes the oxidation of reduced cytochrome by molecular oxygen, resulting in formation of H_2o and H_2O_2. Aerobic as well as some facultative anaerobes and microaerophilic bacteria shows oxidase activity.

IN CELL:

2 reduced Cytochrome C + 2 H^+ + ½ O_2 —Cytochrome Oxidase→ 2 oxidized Cytochrome C

Purple

IN TUBE:

2 oxidized Cytochrome C + *p* – aminodimethylalanine oxalate → 2 reduced Cytochrome C + Wurster's Blue (dark purple)

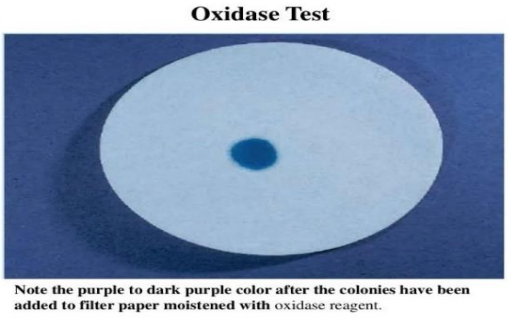


Figure 1.44: Oxidase Test

10) <u>Catalase test</u>

Catalase test is used to determine the ability of microbes to produce catalase.

Certain organisms produce Hydrogen Peroxide during aerobic respiration and sometimes extremely toxic superoxide radicals. These are toxic because they are powerful oxidizing agents and destroy cellular components very rapidly. Many bacteria possess enzymes that afford protection against toxic O_2 products. Obligate aerobes and facultative anaerobes contain superoxidase dismutase enzymes. Catalase production and activity can be detected by adding substrate H_2O_2 to an appropriately incubated tryptic soy agar slant culture. If catalase was produced by bacteria, they will liberate free O_2 gas on reaction.

<u>Results:</u> Bubbles of O_2 represent positive catalase test. Absence of bubble formation is negative catalase test.

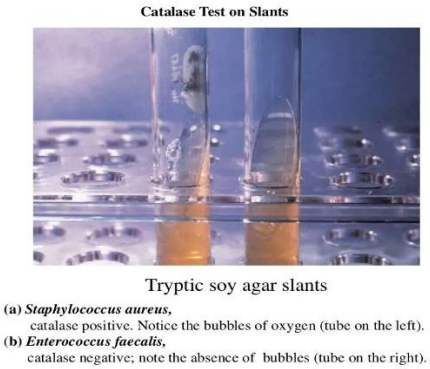


Figure 1.45: Catalase Test on Slants

11) <u>Starch Hydrolysis</u>

Many bacteria produce enzymes called hydrolases. Hydrolase catalyze splitting of organic molecules into smaller molecules in presence of water. The starch molecule consists of two constituents:

- Amylose, an unbranched glucose polymer (200-300 units)
- Amylopectin, a large branched polymer

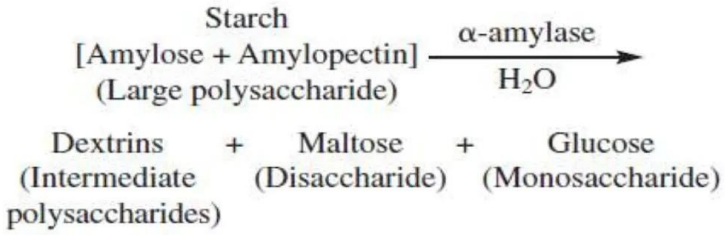
Both Amylose and Amylopectin are rapidly hydrolyzed by some bacteria, using their α-amylases to yield dextrin, glucose and maltose

<u>Interpretation:</u>

Gram's iodine can be used to indicate presence of starch. When in contacts starch it forms blue to brown complex. Hydrolyzed starch doesn't produce a color change.

Positive result: If clear area appears after adding gram's iodine to medium, Amylase has been produced by bacteria.

Negative result: If there is no clearing, starch has not been hydrolyzed.

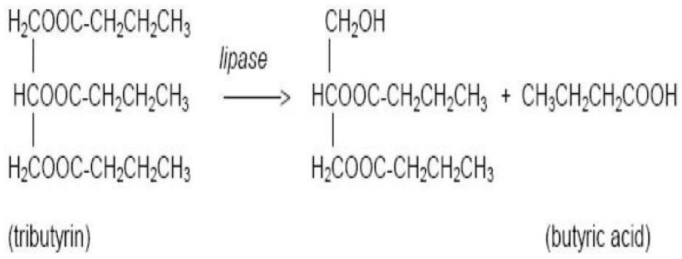


12) **<u>Lipid Hydrolysis</u>**

The two common bacteria catabolized by bacteria are the triglycerides and phospholipids. Triglycerides are hydrolyzed by enzymes lipases into glycerol and free fatty acid molecules. Glycerol and free fatty acids can be taken up by bacterial cell and further metabolized by reactions of-

- Glycolysis, β-oxidation pathway and citric acid cycle.
- These lipids can also enter other metabolic pathways where they are used for synthesis of cell membrane phospholipids.

Since phospholipids are functional components of all cells, the ability of bacteria to hydrolyze host cell phospholipids is an important factor in the spread of pathogenic bacteria. The lipolytic bacteria hydrolyze the lipids, causing spoilage called rancidity. The culture medium contains tributyrin as a reactant, degradation of this compound gives rise to clear zones surrounding the lipolytic colonies in the otherwise turbid culture medium.



ANTIMICROBIAL SUSCEPTIBILITY TEST

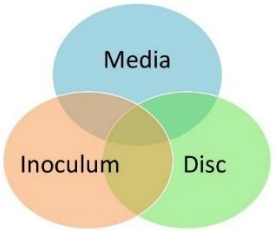
Introduction:

Once an organism is isolated, characterization includes test to detect antimicrobial resistance. The procedures used to produce antimicrobial susceptibility profiles and detect resistance to therapeutic agents are referred to as antimicrobial susceptibility testing (AST).

Testing methods:

- Methods that directly measure the activity of one or more antimicrobial agents against a bacterial isolate
- Methods that directly detect the presence of a specific resistance mechanism in a bacterial isolate
- Special methods that measure complex antimicrobial organism interactions

CLSI 2014 and EUCAST guidelines are followed



Media in AST:

Best medium is Mueller Hinton Agar (MHA) because-

- Shows acceptable batch to batch reproducibility for susceptibility testing
- Low in sulfonamide, trimethoprim and tetracycline inhibitors
- Gives satisfactory growth of non-fastidious pathogens

MHA preparation:

- Store the plates at 2-8°C
- Use within 7days of preparation
- Each batch of MHA plates should be checked for sterility control
- MHA added with 2% NaCl
- MHA added with defibrinated sheep blood 5%

Factors affecting AST are pH, moisture and effects of thymidine or thymine

Antibiotic stock solution:

- ✓ Buy commercial pure source of antibiotics
- ✓ Don't use injectable solutions
- ✓ Accurate weighing of powders is must
- ✓ Standard strains of stock culture should be used to evaluate stock solution
- ✓ After preparing stock solution, make 5ml aliquots and freeze it

Calculation of stock solution:

$$\frac{1000}{P} * V * C = W$$

Dried filter paper discs:

Whatmann filter paper is made to form disc size of 6mm. Keep in petridish and sterilize in hot air oven. With the help of antibiotic delivery loop which has a 20G wire with a diameter of 2mm, the antibiotic is delivered.

Storage of discs:

Refrigerate at 2-8°C. Beta lactam discs should be frozen. The drugs must be kept outside at RT1 to 2 hrs before work. The dispensing apparatus used to deliver drugs should also be refrigerated. Check the expiry date of drugs.

Inoculum-standard:

- 0.5 Macfarland standard.

- Prepared by 0.5ml of 0.048mol/L $BaCl_2$ and 99.5ml of 0.18mol/L of H_2SO_4
- Added with constant stirring
- Turbidity standard is checked by spectrophotometer -625nm-absorbance should be 0.008 to 0.10
- Seal the tubes containing Mcfarland standards and store in dark at RT
- The standard turbidity should be mixed thoroughly everytime before use
- Check the density monthly and replace it monthly

Methods of AST:

Disk diffusion method, MIC method, E test, Automated system

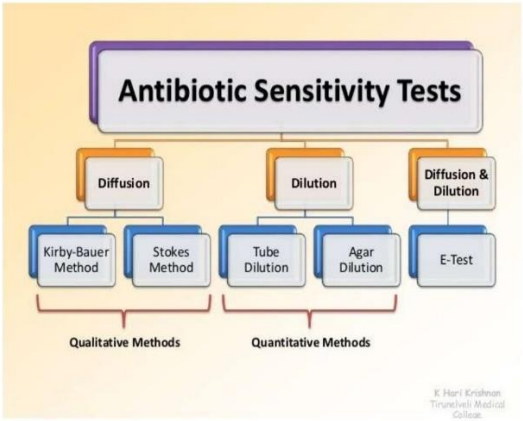


Figure 1.46: Antibiotic Sensitivity Tests

Conventional AST:

Disk diffusion method is the most convenient, widely used everywhere. It's developed by Kirby, Sherris, Baeur and Turk in 1966.

Types: 1. Kirby-Baeur method, 2. Stokes method

Kirby-Baeur method:

Types- Direct colony suspension method, Inoculum (log phase) method

Application of discs:

- 150 mm plate → 12 discs
- 100 mm plate → 6 discs
- Drug should not be relocated
- Distance from the lid edge → 15mm
- Distance between two drug from center to center → 24mm
- Inoculum → disc placement → incubation (only 15 minutes delay is acceptable each)

Kirby baeur disc method

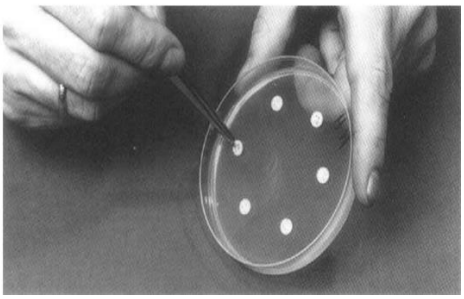


Figure 1.47: Kirby baeur disc method

Interpretation of results:

After 18hrs, confluent lawn of growth observed. Zones of inhibition are uniformly circular. Read results with reflected light and for MRSA, read in transmitted light. When proteus is tested, thin veil of swarming growth after zone of inhibition should be ignored.

Stokes method:

- Built in controls against many variables and provide dependable results
- A standard sensitive strain of the bacterium is inoculated in the middle third of the culture plate
- Standard strains are S. aureus ATCC 25923 and E. coli ATCC 25922

Stoke method

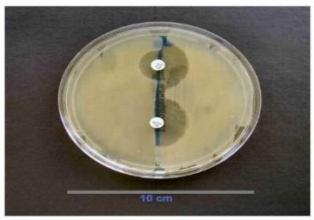


Figure 1.48: Stoke method

- The test bacterium is inoculated in the upper and lower third of the plate
- Antibiotic disks are placed between the standard and test inoculum so that zone of inhibition formed around each disc are composed of standard and test bacteria
- The results are reported as Susceptible, Intermediate susceptible and Resistant

Dilution method:

- ✓ The minimum concentration of antimicrobial to inhibit or to kill the microorganism is determined.
- ✓ MIC, Broth dilution method and Agar dilution method

Broth dilution method:

Media used is cation adjusted MH broth with a pH of 7.2 to 7.4.

- ✓ For Haem. Influenzae- HTM
- ✓ For TMP-SMX-Thymidine free medium
- ✓ For oxacillin resistance- MH broth with 2% NaCl

Making of working antibiotic solution:

Take original stock solution. Prepare stock dilutions of the antibiotic of concentrations 1000 and 100µg/ml. Arrange two rows of 12 sterile 7.5 * 1.3cm capped tubes in the rack. Take a 30ml universal screw capped tube. Add 8ml broth and required antibiotic concentration and mix the contents. Take 2ml + 2ml and put it in first tube in both rows. Add 4ml fresh broth. Transfer 2ml+2ml and put it in second tube. Continue this dilution up to 11 tubes. 12^{th} tube is the control tube.

Inoculation:

Inoculate 1^{st} row with one drop of overnight broth culture 1 in 1000 dilutions. 2^{nd} row with control with known sensitivity organisms.

Final inoculum= $5 * 10^5$ cfu/ml

Incubate at 37˚C for 16 to 18hrs. Inoculate another tube with 2ml broth and keep 4˚C in a fridge overnight to be used as standard for determination of complete inhibition.

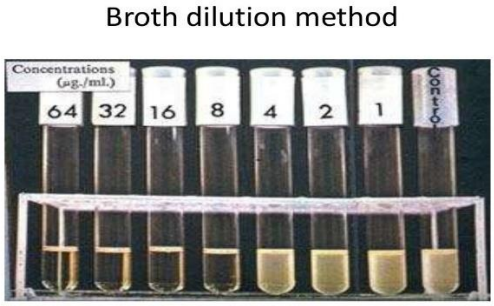


Figure 1.49: Broth dilution method

Interpretation:

- Positive control tube- Turbidity
- Negative control tube- Clear
- MIC end point is read

Microbroth dilution method:

Use double strength Mueller Hinton broth. 4x strength antibiotic solutions prepared as serial 2fold dilutions. Test organism is $2x10^6$/ml. Done in 96 well plate.

Agar dilution method:

Add 1ml concentration of the drug with 24ml of MHA. Make 1:10 dilution of 0.5 Macfarland standard inoculum which delivers 10^7 cfu/ml. Using pipette, deliver 0.001ml on the surface of the agar giving the final inoculum of 10^4 cfu/spot. Inoculate a control plate.

Figure 1.50: Agar dilution method

Interpretation:

Examine the drug free control growth of the test organism for viability and purity. Place the plate on a dark background and examine them for the lowest concentration that inhibits visible growth. A single colony of a faint haze is not recorded as growth.

E. Test (Epsilometer test):

It is an exponential gradient testing methodology. A predefined stable antimicrobial gradient is present on a thin inert carrier strip. Following incubation, the E strip releases drug and a symmetrical inhibition ellipse is produced.

- ✓ MIC= Intersection of inhibitory zone edge and calibrated carrier strip.

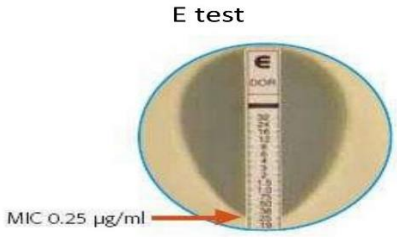


Figure 1.51: E test

Quality control in AST:

Quality control (QC) in a lab designed to monitor the analytical phase of testing procedures to ensure that tests are working properly. CLSI recommends use of ATCC strains for QC in AST. QC strains should be included daily with the test ideally. Not more than 1 in 20 results should be outside the accuracy limits. No zone should be more than 4SD away from midpoint between the stated limits. Due to expense, it can be switched as once weekly testing. Perform QC for 30days and with less than 10% inaccuracy, continue weekly. Test repeated for each new drug included. All documentation should be maintained indefinitely.

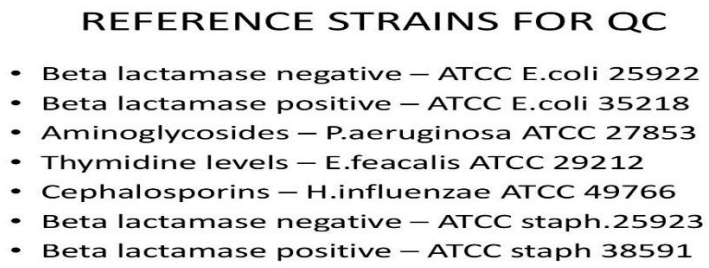


Figure 1.52: Reference strains for QC

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SCHOOL OF BIO AND CHEMICAL ENGINEERING

DEPARTMENT OF BIOTECHNOLOGY

UNIT – 2 – FUNGAL AND PARASITIC SAMPLE COLLECTION - SMB 3201

Laboratory Diagnosis of Fungal Infections

Introduction

- To confirm clinical suspicion to establish fungal cause of disease.
- To help in –
  - Choosing a therapeutic agent
  - Monitoring the course of diseases
  - Confirming mycological cure

Types and collection of Specimens

- Specimen collection depends on the corresponding disease
- Very important to proceed for a final diagnosis.

(a) Superficial Mycosis

- Clean the part with 70% alcohol
- Collect the material in a sterile paper or a sterile petridish to –
  - Allow drying of the specimen
  - Reduce bacterial contamination
  - Maintain viability

- **Dermatophytic lesion** – spreads outward in a concentric fashion with healing in the center – scrape outwards from the edge of the lesion with a scalpel blade at 90° angle or use Cellophane tape (when scaling is less).

- **Scalp lesion** – scraping with a blunt scalpel, including hair stubs, scales & contents of plugged follicles. Cut hair r seldom useful.

- **Scalp lesion –** Wood lamp's examination of infected hair produce fluorescence if infected with ringworm infection.

Hairbrush sampling technique esp for culture.

- **Onychomycosis** – stop antifungals one week prior to collection. Sample should be taken near the base of the nail as fungus in distal end is non viable; include full thickness of the nail

- **Mucosal infections** – mucosal scrapings preferred over swabs

(b) Subcutaneous Mycosis

- Scrapings or crusts from the superficial parts of lesions. Usually contaminants r there in these.

- Pus aspirates and Biopsy are valuable. Biopsy should be avoided in sporotrichosis as it leads to spread of infection and hinder healing.

(c) Systemic Mycosis

- Feces
- Urine
- Pus
- Sputum
- Biopsy
- CSF
- Blood
- Scraping or swabs from the edge of lesions

Collection & Transport of specimen

- ❖ Proper collection of specimen and in adequate quantity.
- ❖ Early transport to the lab to avoid overgrowth of contaminant

◆ **Respiratory specimens**

- Sputum – early morning sample, after mouthwash, flakes to be used for culturing
- Bronchoscopy – if non productive cough, BAL can be taken
- Bronchial brushings or lung biopsy – to rule out invasion or colonisation

◆ **Blood**

■ In biphasic Brain Heart Infusion agar

■ Inoculated in 2 bottles – for dimorphic fungi. Subculture is done after two days and seven days.

◆**Cerebrospinal fluid**

■ Should be immediately processed else stored at RT or at 30°C in an incubator
■ Centrifuge & use sediment for culture

◆ **Skin,Hair& Nail**

- ▪ Taken for dermatophytic infections
- ▪ Hair – plucked with forceps

◆ **Tissue, BM & Body fluids**

■ Tissues – grind or mince before culturing

■ Body fluids – centrifuge & use sediment for culture

◆ **Urine** – centrifuge & use sediment for culture

◆ **Stool**-Not suitable. Intestinal biposy or HPE r better.

◆ **Eye**- In keratomycosis, scrapings from base and margins of ulcer r taken using kimura's spatula. Aspirate can b taken from hypopyon or endophthalmitis

Diagnosis

◆ Direct examination

Fungal culture

- Serological tests
- Skin tests
- PCR & other molecular methods

Direct Examination

- Very decisive in the diagnosis of fungal infections
- Wet mount's -
  - Slide & tube KOH mounts – 10 to 20% KOH for 5-20 mins-digests protein debris, dissolves keratin. DMSO can be added to KOH to hasten clearing in skin scrapings & nail clippings
  - Calcofluor white – fluorescent stain – excellent morphology of pathogenic fungi. Stain binds to glucan and chitins which r abundant in fungal cell wall. If supplemented with KOH, useful for corneal scrapingswhich has scanty fungal elements.
  - India ink-capsulated fungi.
- Gram stain – fungi are gram positive
- Histopathology
  - Superficial infection – acute, subacute or chronic dermatitis with folliculitis
  - Subcutaneous & systemic infections – granulomatous reaction with fibrosis or pyogenic inflammation
  - Routine stain – Hematoxylin & Eosin (HE)
  - Special stains – PAS (Per lodic acid), GMS (Grocott Gomori Methanamine Silver), Mayer's mucícarmine, Gridley's stain
- Fluorescent – antibody Staining
  - To detect fungal Ag in clinical specimen such as pus, blood, CSF, tissue sections
  - Adv-can detect fungus even when few organisms are present

Fungal Culture

- Sabouraud Dextrose Agar (SDA)
  - Contains 2% dextrose, antibiotics (gentamicin, chloramphenicol) and cycloheximide. Cycloheximide is nt used when cryptococcus, aspergillus or penicillium r suspected.
- Selective media
  - Corn meal agar (CMA) – sporulation, chlamydospore formation
  - Bird seed agar-cryptococcus, forms brown colonies
  - Brain Heart Infusion (BHI) agar – dimorphic & other fastidious fungi
- Temperature requirement
  - Majority of fungi – 37°C
  - Superficial mycosis – 30°C

- Dimorphic fungi – 25°C & 37°C

◆ Incubation time

- At least 4 weeks
- Usually positive cultures are obtained in 7-10 days
- Candida & Aspergillus – 24 to 72 hrs

◆ Specimens should be cultured on agar slants:

- Safe
- Require less space
- More resistant to drying during prolonged incubation
- Blood cultures should be inoculated in to biphasic blood culture bottles

Interpretation of Fungal Culture

◆ Isolation of an established pathogen like H. Capsulatum or C. Neoformans – evidence of infection

◆ Isolation of commensal or opportunistic fungi like Candida or Aspergillus – consider following points:

- Isolation of same strain in all culture tubes
- Repeated isolation of same strain in multiple specimen
- Isolation of same strain from different sites
- Immune status
- Serological evidence

Identification of fungal cultures

◆ Colony morphology – colour, texture,pigment

◆ Fungal morphology under microscope – using Lactophenol Cotton Blue (LPCB) stain

◆ Composition of LPCB

- Lactic acid – preserves fungal structure
- Phenol-kills any live organism
- Glycerol – prevents drying
- Cotton blue – imparts blue color to structures

◆ Special culture techniques – Slide culture to see sporing structures & spore arrangement, CHROM agar for candida sps

◆ Biochemicals – ability to assimilate carbon & nitrogen, sugar fermentation

Serology

◆ Detection of Ag or Ab in serum or body fluids

■ Ab detection:

- Diagnosis of systemic & subcutaneous mycoses
- Assess prognosis of the disease
- Assess response to treatment

■ Ag detection:

- Early stages of infection
- In patients with impaired immunity.
- Latex particle agglutination (LPA) for cryptococcosis, candidiasis and aspergillosis.

Immunohistochemistry: Application of fluorochromes is effective for localisation of fungal elements

Serological tests used in Medical Mycology

◆ Agglutination

- Whole cell agglutination
- Latex particle agglutination
- Passive haemagglutination

◆ Immunodiffusion – most widely used

◆ Counter immunoelectrophoresis (CIEP)

◆ Indirect fluorescent Ab detection

◆ELISA, RIA

Skin tests

*Detects CMI and done in vivo and in vitro.

*Detects delayed hypersensitivity. Shown by occurrence of induration and erythema within 24 to 72 hours following intradermal inoculation of fungal antigen.

◆ Histoplasmosis Histoplasmin

◆ Blastomycosis Blastomycin

◆ Sporotrichosis Sporotrichin

◆ Dermatophytosis Trichophytin

◆ Candidiasis Candidin

In vitro

◆ Macrophage migration inhibition.

◆ Blast formation of sensitized lymphocytes in the presence of specific fungal antigen

Other Methods

- PCR – Polymerase Chain Reaction
- RFLP -Restriction fragment length polymorphism
- Protein electrophoresis
- Nucleic acid probes
- Serotyping
- Karyotyping

Human Parasitology

Basic Terminology and principles

- **Symbiosis**: Living together
- **Commensalism**: One symbiont benefits, other unaffected
- **Mutualism**: Both symbionts benefit
- **Parasitism**: One symbiont benefits, other is damaged.

Reality of Parasites

- 1.3 billion persons infected with Ascaris (1:4 persons on earth)
- 300 million with schistosomiasis
- 100 million new malaria cases/Year

Laboratory Methods for Parasites in faeces

- No technique is 100% successful in detecting parasites by a single stool examination, and at least three serial stools must be examined before a patient can be considered free from infections in which stages of parasites would be expected to be found in the faeces.
- Whilst clinical symptoms or a case history may provide clues as to which parasites may be present, each faecal specimen should be treated as an unknown, as parasite stages unrelated to the clinical picture may be present.

Faecal specimens may contain several stages of Parasites

- Faecal specimens are examined for the presence of protozoa and helminthes larvae or eggs.
- The stages of protozoa found in stools are trophozoites and cysts. The stages of helminthes usually found in stools are eggs and larvae, though whole adult's worms or segments of worms may also be seen. Adult worms and segments of tapeworms are usually visible to the naked eye, but eggs, larvae, trophozoites, and cysts can be seen only with the microscope.

Collection of faecal specimens

1. Because of the fragile nature of many intestinal parasites, and the need to maintain their morphology for accurate identification, reliable microscopic diagnosis can't be made unless the stool is collected properly.

2. Approximately 10 grams of fresh faeces uncontaminated by urine, oil, water, dyes or radio opaque into a clean plastic container.
3. The container should be free from antiseptics and disinfectants.
4. Label all samples clearly with the patient's name, reference number, date, and time of collection.
5. All samples should be accompanied by a requisition form from the physician giving relevant clinical details and recent travel history.
6. Samples and forms from patients with a confirmed or suspected diagnosis of certain infectious diseases such as AIDS or hepatitis should be clearly labeled with "Risk of Infection" or "Biohazard"
7. Most viable parasites are susceptible to desiccation or temperature variation. If time lapse between collection and observation is considerable, i.e. more than 4 days, it may be necessary to add some form of preservative to the faeces to retain the morphology as near to the original as possible.
8. Formed samples can be kept in a refrigerator at + 4c for a short while, but not in incubator.
9. Any whole worms or segments passed should be placed in a separate container.

Collect the information of patient

Provisional diagnosis

- History (Age, occupation, residency, previous infection)
- Complaint
- Clinical examination

Confirm the diagnosis

- Investigation

  -Laboratory investigation

  - Radiology

  - Surgical intervention (Exploratory).

The Microscopy in Parasitology

- The Microscope is the parasitologist's main tool. If possible the Microscope-should be binocular; most suitable objectives are the x10, x40, and x100.
- The Microscope must be covered and immersion oil removed from the lens -with xylene or ether when not in use.
- Calibration of the Microscope Eyepiece Micrometer:
  - On many occasions measuring the size of 16-01-2018 suspected parasites in faeces is helpful for identification.(eyepiece micrometer)

Microscopic Examination of Wet Mount

- Wet mount is the simplest and easiest technique for the examination of faeces, and this method should be performed in all laboratories at the peripheral level.
- A wet mount can be prepared directly from faecal material or from concentrated specimens. The basic types of wet mount that should be used for each faecal examination are saline, iodine, and buffered methylene blue.

The Saline Wet Mount

- Is used for the initial microscopic examination of stools. It is employed primarily to demonstrate worm's eggs, larvae, protozoan trophozoites, and cysts.
- This type of mount can also reveal the presence of red blood cells and white blood cells.

The Iodine Wet Mount

- Is used mainly to stain glycogen and the nuclei of cysts, if present. Cysts can usually be specifically identified in this mount.
- The buffered methylene blue (BMB) wet mount should be prepared each time amoebic trophozoites are seen in a saline wet mount, or when their presence is suspected.

Direct Saline and iodine mounts

- With a wax pencil writes the patient's number and name or the date at the left-hand end of the slide.

Preparing a faecal specimen

- Place a drop of saline in the centre of the left half of the slide and place a drop of iodine solution in the centre of the right half of the slide.
- Note: If the presence of amoebic trophozoites is suspected, warm saline (37c) should be used.

Preparation of Wet flim

- With an applicator stick (match or tooth pick), pick up a small portion of the specimen (size of a match head) and mix the drop of saline.

Stool Examination

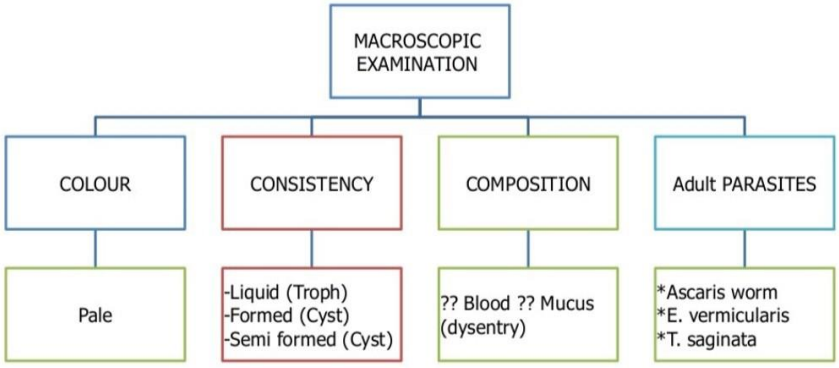


Figure 2: Macroscopic Examination

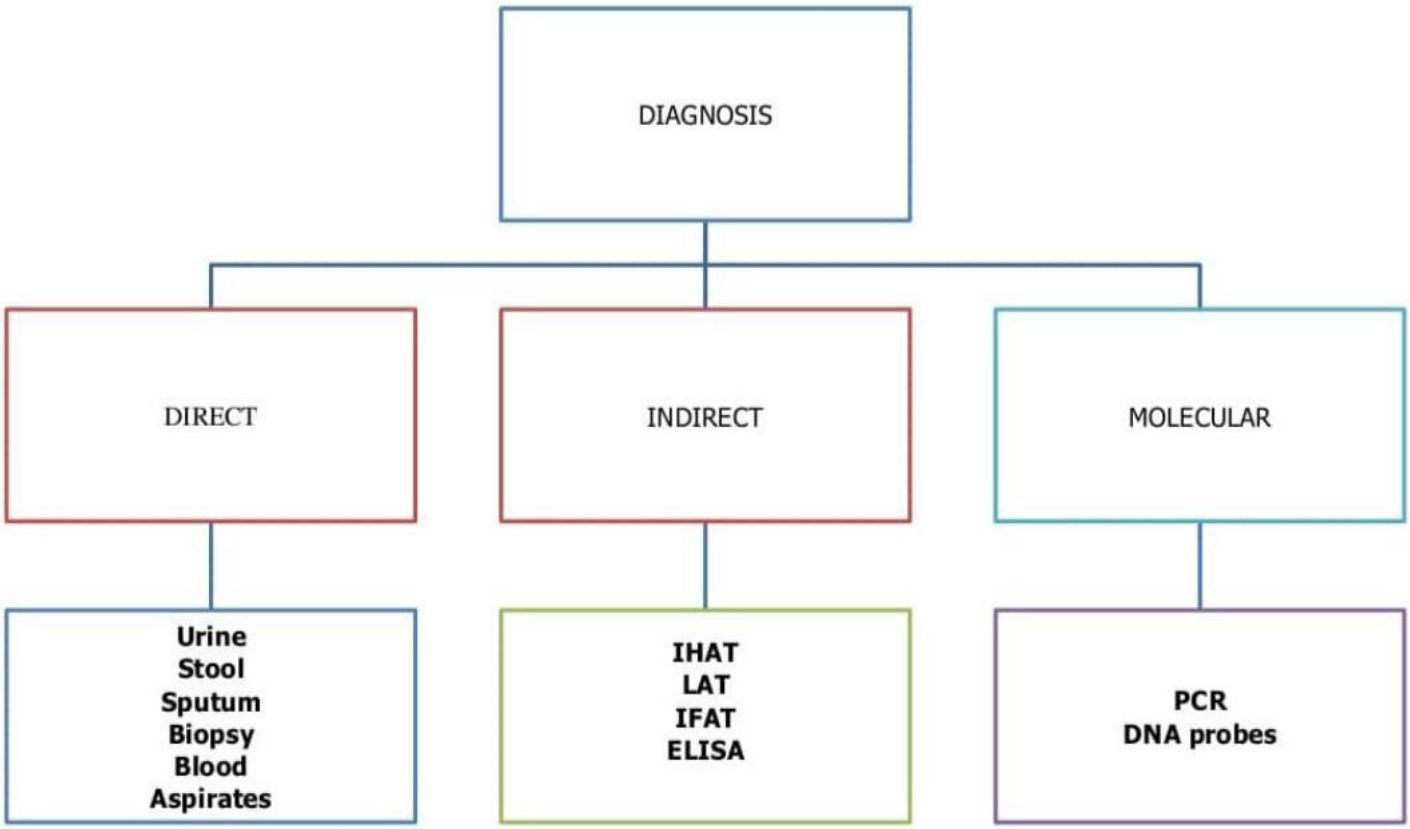


Figure 2.1: Types of Diagnosis

Examination

1. Put the slide with the mounts on the microscope stage and focus on the mount with the x10 or low-power objective.
2. Regulate the light in the microscope field with the sub stage diaphragm. You should be able to see objects in the field distinctly. Too much or too little light is not good.
3. Examine the entire coverslip area with the x10 objective; focus the objective on the top left-hand corner and move the slide systematically backwards and forwards, or up and down.
4. When organisms or suspicious material are seen, switch to the high-dry objective, and increase the light by opening the sub stage diaphragm to observe the detailed morphology.

-This is a systematic examination. If mounts are examined in this way, any parasites present will usually be found. If the mount is not examined systematically, parasites may be missed. Examine each microscope field carefully, focusing up and down, before moving to the next field.

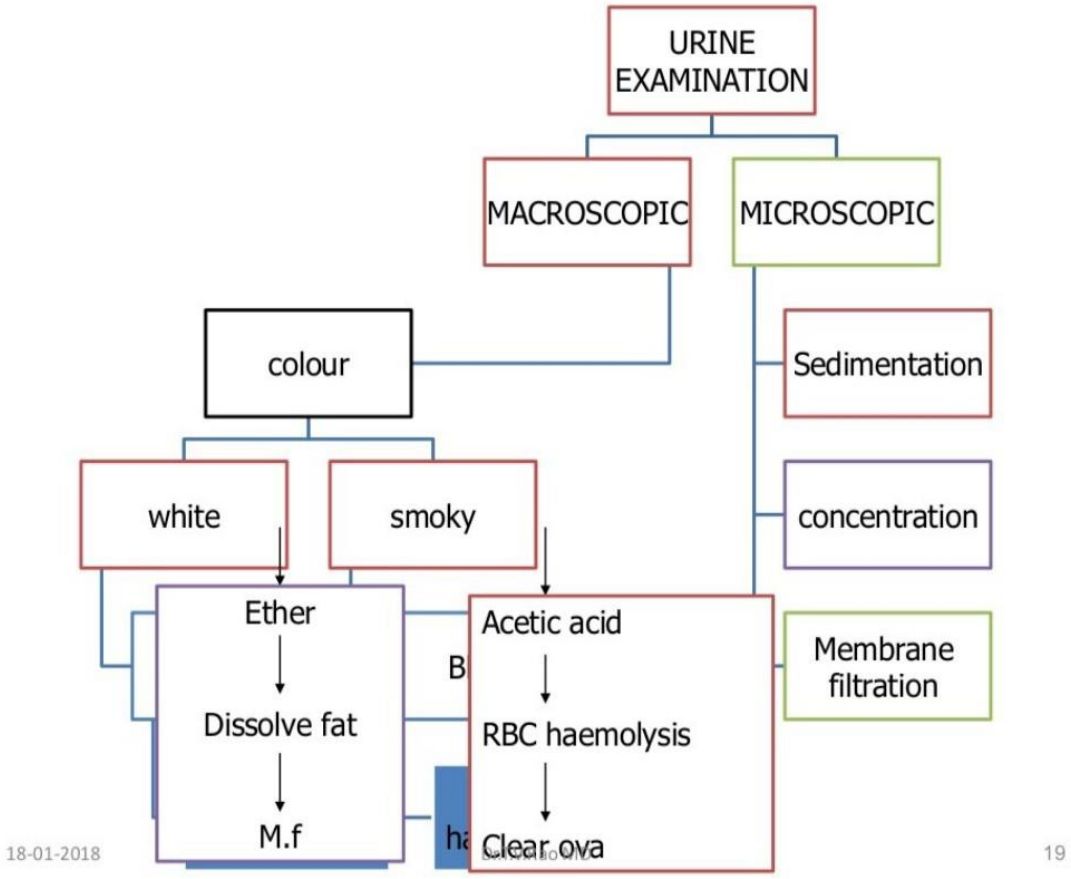
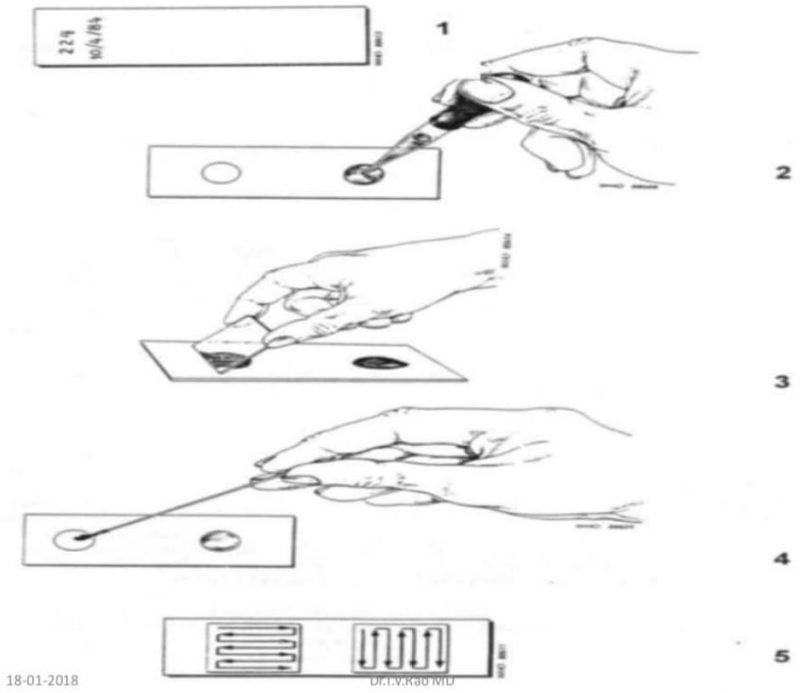


Figure 2.2: Urine Examination



Stool Examination A Rapid Methods

Saline smear

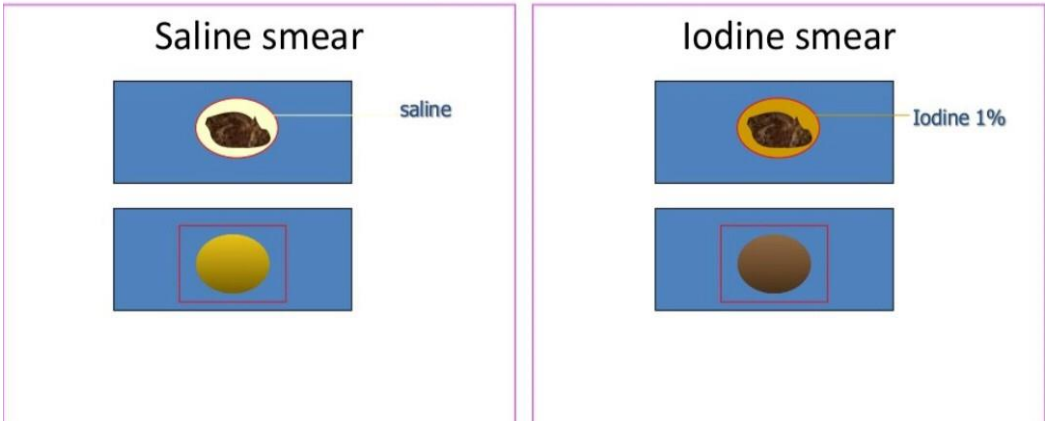


Figure 2.4: Saline smear and Iodine smear

Huge number of

- Eggs
- Protozoal trop, Motility (Amoeba, flagellates)

Huge number of

- Cyst morphological details

Need for concentration method for faecal examination

- A concentration procedure is performed mainly to separate the parasites from faecal debris. The concentration procedure not only increases the numbers of parasites in the sediment but it also unmasks them, making them more visible by removing organic and inorganic debris

Stool Examination scanty infection Concentration techniques

Sedimentation

- Heavy eggs (Ascaris egg)
- Operculated eggs (Trematodes)
- Larvae (Strong sterc.)
- Cysts

Flotation

- Non Operculated eggs
- Trematodes (S. M.)
- Cestode
- Nematode (Hookworms, Trichostong)
- Cysts

Stool Examination other techniques

- Stoll's technique for eggs of Ascaris, T. Trichiura., Hookworms, S. Mansoni
- Baermann's technique Detec. Of Nematode L. /stool, soil
- Cultures for Nematode larvae using Filter paper culture for Larvae of: St. Stercoralis (A,L) and Hookworms

Artifacts

- Artifacts other things, living or artificial, present in the stool that are not parasites and could mislead the laboratory worker.
- Note: "Artifacts not to be mistaken for cysts".

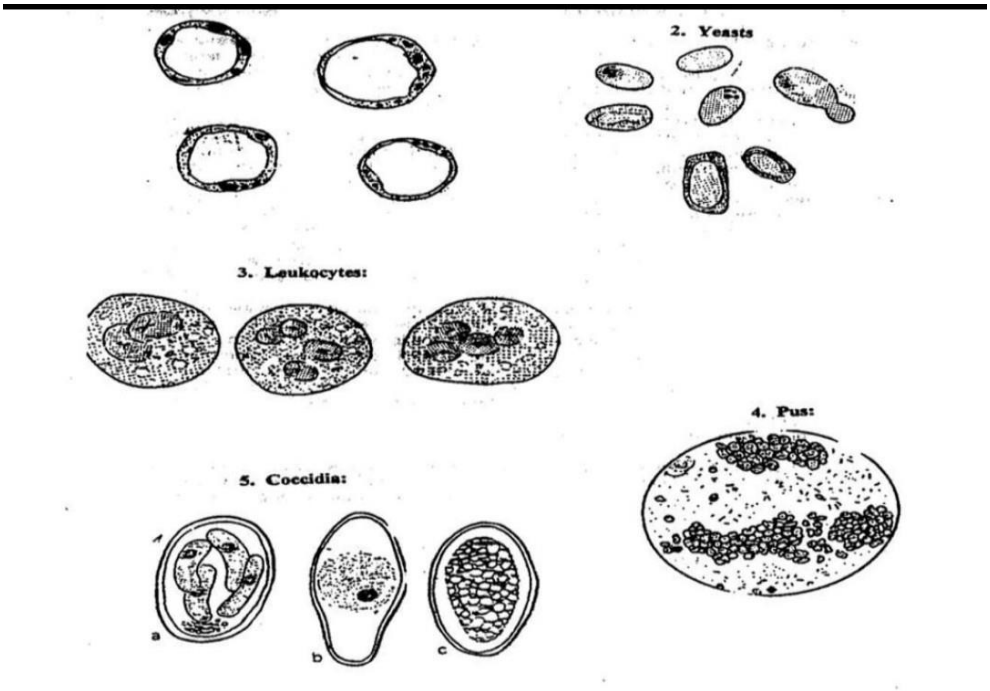


Figure 2.5: Air facts a) Blastocystis b) Yeasts c) Leukocytes d) Pus e) Coccidia

Parasite

- Round worm
- Hook worm
- Tape worm
- Pin worm
- Whipworm

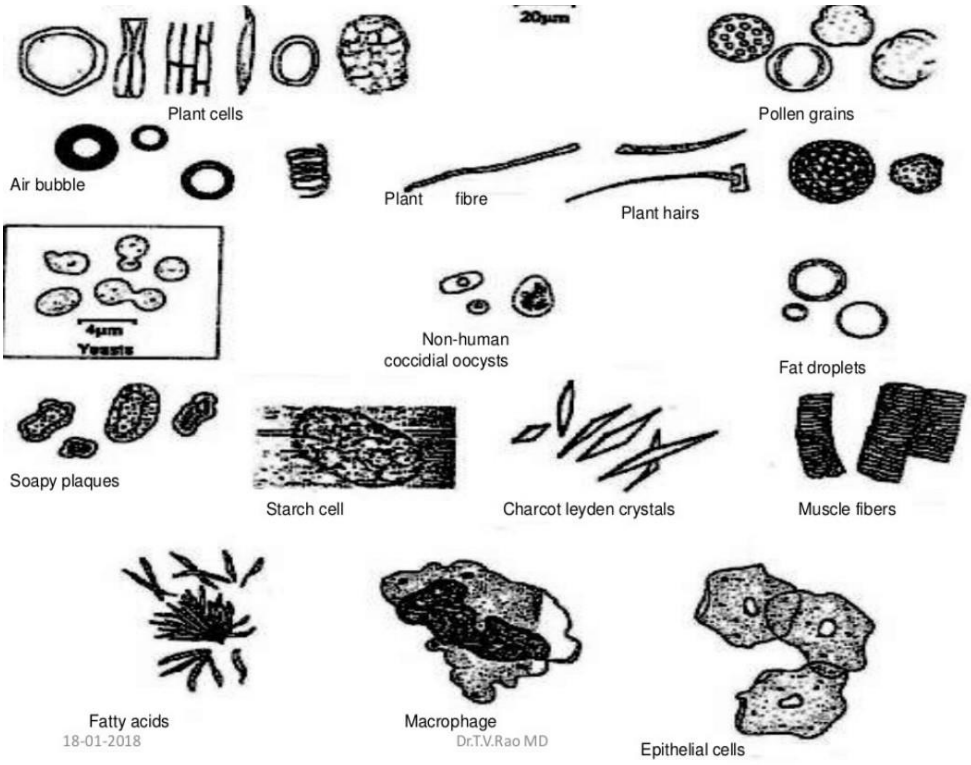


Figure 2.6: Parasites

Pin Worm Egg Collection

Eggs of Pin worm – Enterobius vermicularis rarely appear in stools. These are usually collected in the folds of skin in perianal region.

Collection

Cotton swab / Plaster patch – Anus especially in early morning – Dipped in Saline – Observed.

Examination of Parasites

- Warm stools are best for detecting Ova or parasites. Do not refrigerate the specimen.
- Because of cyclic life cycle of parasites, three separate random stool specimens are recommended for examination.

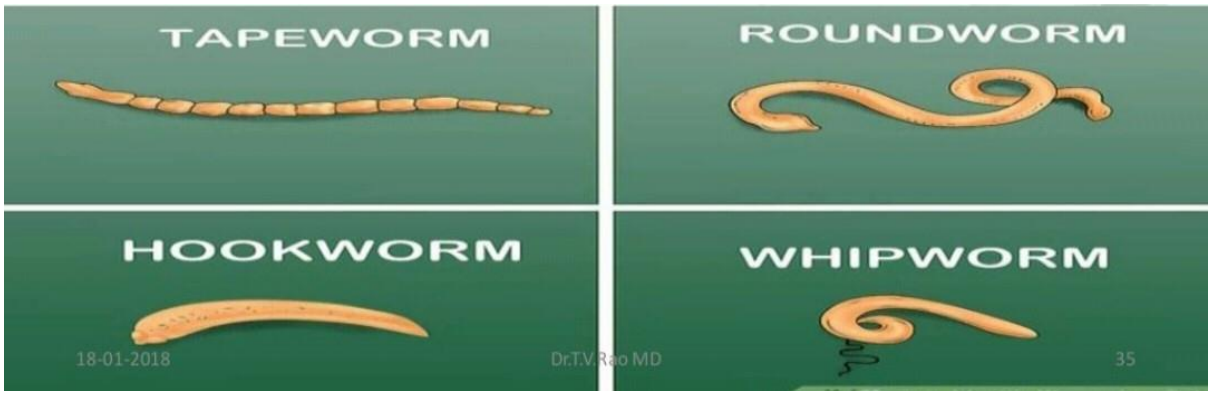


Figure 2.7: Types of endoparasites

Normal Values

- Undigested food materials – None to small amount
- Starch - None
- Eggs, Cysts, Parasitic fragments – None
- Yeasts – None
- Leukocytes – None

QBC Method is used in

- The QBC Malaria method is the simplest and most sensitive method for diagnosing the following diseases.
- Malaria
- Babesiosis
- Trypanosomiasis (Chagas disease, Sleeping Sickness)
- Filariasis (Elephantiasis, Loa-Loa) Relapsing Fever (Borreliosis)

How to read the QBC results.....

- When the operator looks through the wall of the tube, the nucleus of the parasite fluoresces bright green, and the cytoplasm shows up as yellow-orange. The shape and colours are quite characteristic, and since the parasites are concentrated up to 1000X, there are usually a large number of them in any field of view in this area of the tube.

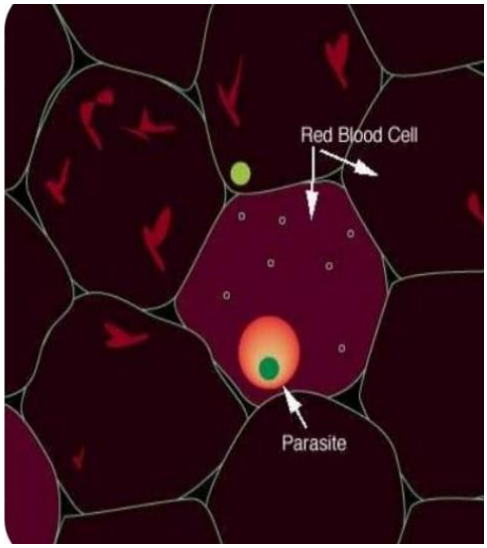
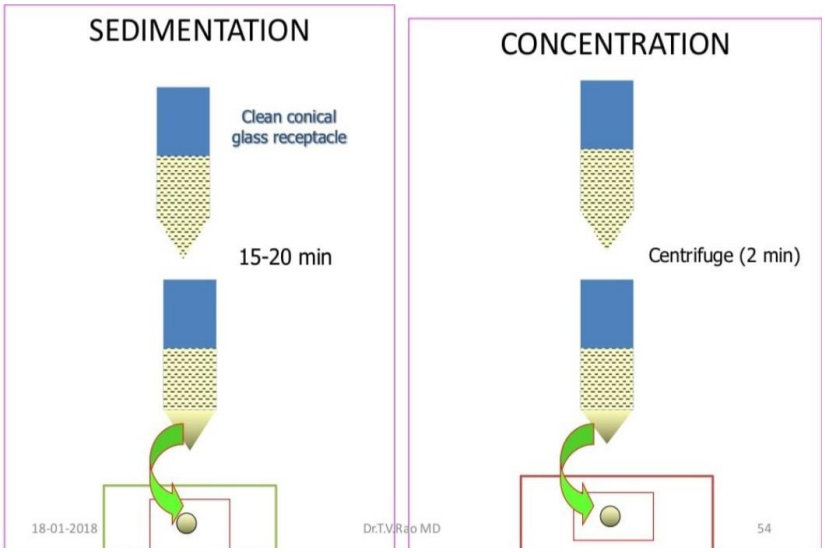


Figure 2.8: QBC results

Urine Examination



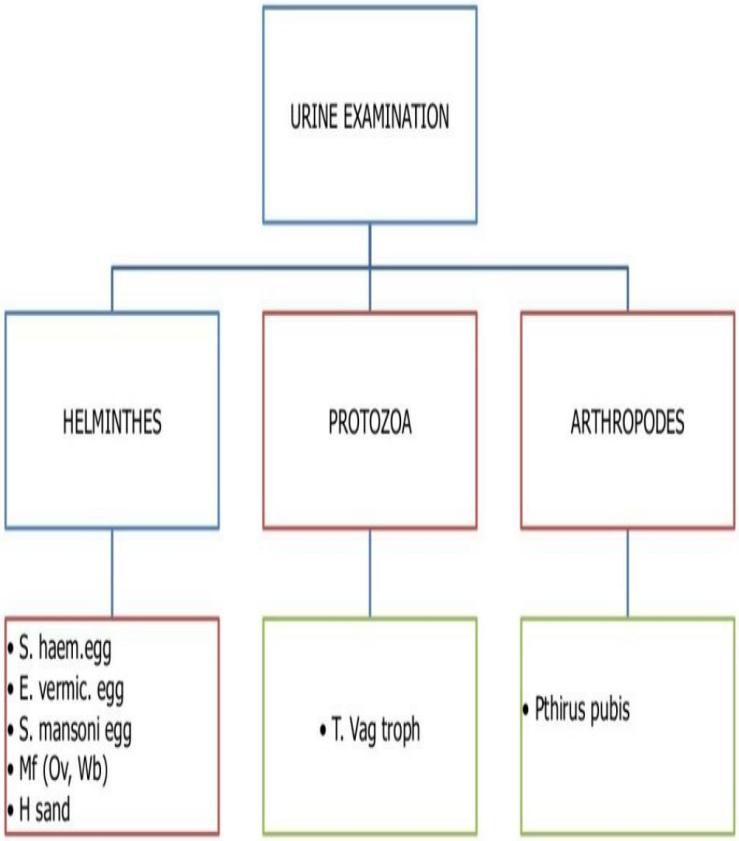


Figure 2.9: Urine Examination

Indirect immunological diagnosis

- Serology – All tests available
    - IHA
    - ELISA
- More useful in
    - Amoebiasis
    - Leishmaniasis
    - Malaria
    - Toxoplasmosis
    - Trichinosis
    - Filariasis
    - Echinococcosis
- Skin Tests – Specificity low, cross reactions common
    - Casoni's test
    - Leishmanin test

Laboratory Diagnosis of Parasitic infection

Specimens

- Faeces
- Blood
- Bone marrow
- Urine
- Biopsy material from spleen, liver, lymph nodes.

Collection of the specimen

- Collect in wide mouth clean container without contamination of urine, water or disinfectants.
- Collection of three faecal specimen to make the diagnosis of intestinal parasitic diseases.
- Two specimen obtained successive days during normal bowel movement
- 3rd is after magnesium purge.
- Liquid specimen should be examined within 30 min
- Semi formed stools within 60 min after collection for suspected infection like E. Histolytica, Giardia laiblia.
- Blood sample: finger prick, lobe of an ear or anti coagulated blood can be used

Preservatives

- Formalin solution.
- Polyvinyl alcohol
- Merthiolate – iodine – formalin solution (MIF)
- Schaudinn's solution.

Macroscopic examination of faeces

- Consistency
- Colour
- Odour
- Presence of blood or mucus
- If the presence of blood and mucus in stool (suggestive of amoebic dysentery)

Microscopic examination of faeces

- Saline wet mount
- lodine wet mount
- Smear after concentration
- Stained smear

Saline wet mount

- Small quantity of faeces is diluted with normal saline (0.9%) placed on clean glass slide, and cover with cover slip.
- Smear is examined under microscope.
- It is used to detect tropozoites and cysts of tropozoa and larvae helminthes.
- It detect live motility of E. Histolytica, Giardia lamblia and Balantidium coli.

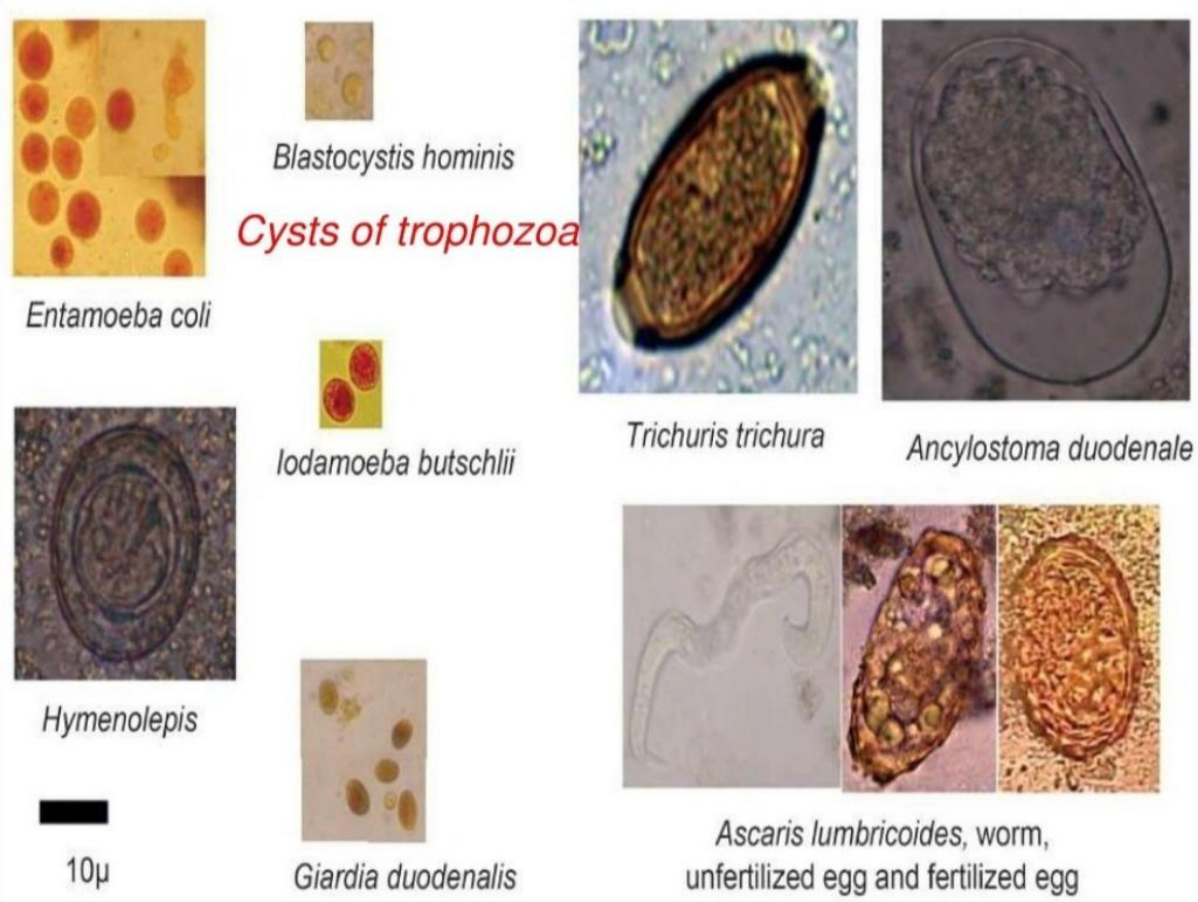


Figure 2.10: Microscopic images of parasite ova and cysts

Iodine wet mount

- Stool is emulsified in a drop of five times diluted solution of Lugol's iodine on a clean glass slide.
- Covered with cover slip and examined under microscope.
- It is used to identify the nuclear character of cysts and tropozoites.
- Lugol's iodine:
- 10 gm potassium iodide
- 100ml D/W
- 5gm iodine crystal

Wet preparation

- A drop of anti coagulated blood can be placed on a clean glass Slide.
- Cover the cover slip and examined under microscopically for large, motile, exo-erythrocytic parasites, such as: trypanosomes and microfilariae.

Concentration method

Floatation Techniques

- Saturated salt floatation technique Zinc sulphate centrifugal floatation technique
- Sedimentation technique
- Simple sedimentation
- Formalin ether sedimentation

Saturated salt flotation technique

- Take 1 gm of faeces is placed in a flat bottomed vial and add 5 drop of saturated salt solution are added.
- Stirred with a glass rod to make an emusolutio
- More salt solution is added so that the container is nearly full, mix solution.
- A glass slide is carefully laid on the top of the container and stand for 20-30 min.
- After which the glass slide is quickly lifted, turned over smoothly and examine under microscope
- It has been observed all the eggs except unfertilized of A. Lumbricoides, eggs of Taenia solium, T. Saginata float in saturated salt solution.

Zinc sulphate centrifugal floatation Technique

- About 1 gm of facces is thoroughly mixed in 10 ml of lukewarm distilled water.
- The filtrate is poured in to a 15 ml conical centrifugal tube and centrifuged at 2,500 RPM for 1 min.
- The clear supernatant is poured off and 3-4 ml of zinc
- Sulphate is added to sediment up to the top of tube.
- Again centrifuge at 2,500 RPM for 1 min.
- With the platinum wire loop sample is taken from the surface on to a glass slide and put cover slip observe under microscope.
- For protozoal cysts, one drop of iodine solution is added before the cover slip is put on.
- This technique effectively concentrates cysts of protozoa, eggs of nematodes, and small tapeworms.

Eggs of nematodes

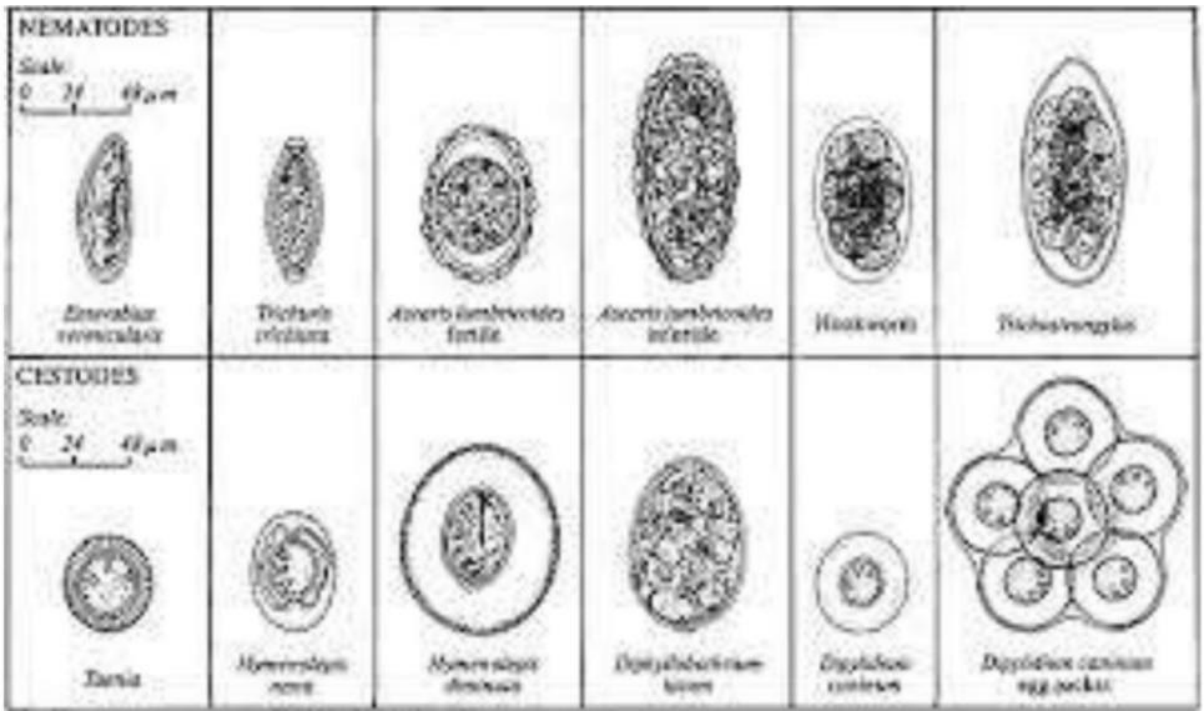


Figure 2.11: Eggs of nematodes

Simple Sedimentation

- A sufficient amount of faeces is thoroughly mixed with 10 20 time its volume of tap water.
- Allow to settle in a cone – shaped flask for an hour or two hrs.
- This process is repeat till supernatant fluid is clear.
- Taken the sediment at the bottom examined under microscope for the eggs.

Formalin – ether sedimentation

- Half teaspoon if faeces is thoroughly mixed in 10 ml of water and strained through two layers of gauze of funnel.
- The filtrate is centrifuged at 2,000 RPM for 2 min
- The supernatant is discarded and the sediment is resuspended
- In 10 ml of saline. Again centrifuge and discard the supernatant and add 7 ml of formalin saline and stand for 10 min.
- To this is add 3ml of ether the tube is stopperde and shaken vigorously to mix and centrifuge fortig min.
- The four layers are visible, the top layer is consist of ether, 2nd plug of debris, 3rd is clear layer formalin saline and 4th is sediment.
- Take a sediment on clean glass slide and examine under microscope.

Quantification of worm burden

There are two methods;

- Direct smear egg count
- Stoll's method

Direct smear egg count

- Two mg of faeces is mixed in a small drop of saline on a slide and cover the cover slip.
- Examined under low power of the microscope and count the eggs and calculate the number of eggs / gm of faeces.

Stoll's method

- Commonly used method for determination of helminthic eggs in faeces.
- 4gm of faeces is mixed with 56 ml of N/10 NaOH in a
- Flask to make a uniform suspension.
- This is facilitated by adding the glass beads and closing the mouth with a rubber and shake vigorously.
- 0.075 ml of emulsion is removed with pipette and placed on a glass slide and put cover slip.
- Count the eggs in low power of the microscope
- Calculate the egg/gm of faeces multiplied by 200.

Anal scraping and swabs

- Amoebiasis cutis of the perianal area may be diagnosed by demonstrating motile tropozoites of E histolytica in material scraped from ulcer and examined in saline suspension on a slide under cover slip.
- E. vermicularis infection is usually diagnosed by demonstrating the presence of egg on the perianal and perineal skin. Following methods are;
- Scotch cellulose adhesive tape method
- NIH swab.

Scotch cellulose adhesive tape Method

- A3 inch of the tape is held adhesive – side-out on the end of a wooden tongue blade by the thumb and index finger.
- The adhesive surface of the tape is then pressed against the perineal skin at several places.
- Then placed the adhesive – side-down on the Examinatplace
- A drop of toluene may be placed between the tape and slide.
- The toluene clear essentially everything except eggs and hair.

NIH Swab

- Eggs are deposited in large number on the perianal and perineal skin at night can be demonstrated by scraping this area by NIH swab in the morning before taking bath.
- Spread over glass slide and examined microscopically.
- This procedure should be repeated on three successive days

Figure 2.12: NIH Swab

Examination of urine

- The specimen is collected in a sedimentation of glass, and the eggs are allowed to sink to the bottom.
- A drop of sediment is placed on the glass slide and examine under microscope for Trichomonas Vaginalis.

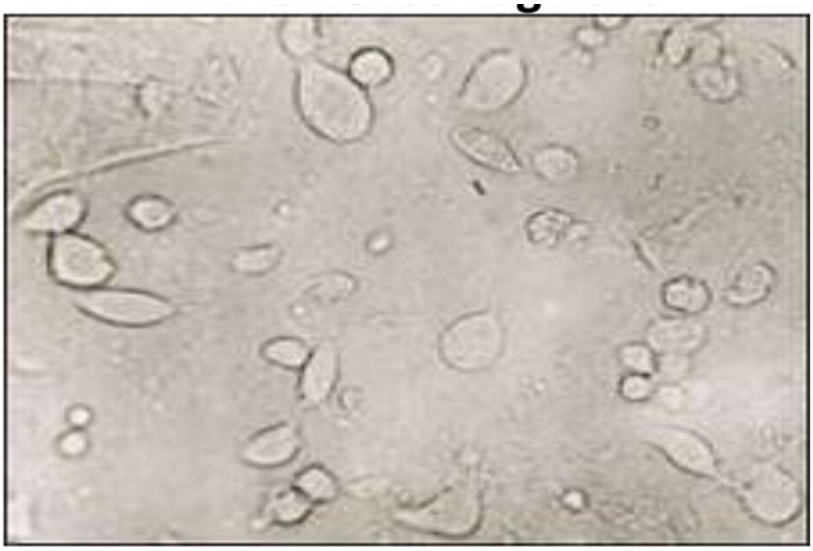


Figure 2.13: Trichomonas vaginalis

Trichomonas vaginalis

Examination of Sputum

- The sputum is spread in a Petri dish and material suspected of bearing eggs or pus and Charcot – Leyden crystals is placed on a glass slide.
- Cover the cover slip and examined under microscope for paragonimus westemani.

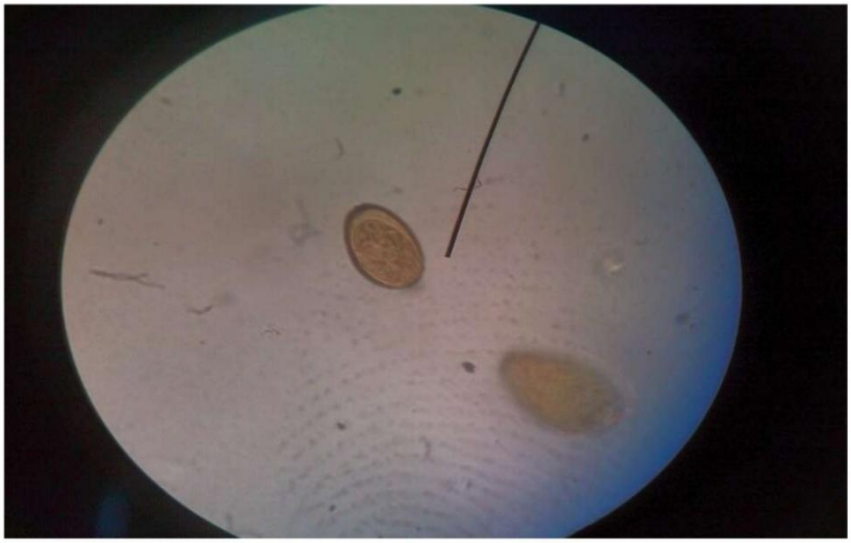


Figure 2.14: Examination of Sputum

Permanent stained

- Iron-haematoxylin stain
- Trichrome stain
- Modified acid – fast stain.

Iron-haematoxylin stain

- A thin smear of faeces is made on a clean, glass slide.
- Keep the slide in Schaudinn's solution for 15 min for fixation of the smear.
- The smear is immersed in 70% and 50% alcohol, 2 – 5 min in each.
- Wash in tap water for 2-10 min, immersed in 2% aqueous
- Ferric ammonium sulphate solution for 5-15 min.
- Wash in running tap water for 3-5 min.
- Then stained in 0.5% aqueous haematoxylin for 5-15 min and wash in running tap water for 2-5 min.
- Then differentiated in saturated aqueous solution of picric acid for 10-15 min.
- Dehydrated by immersion for 2-5 min each in 50%, 70%, 80% and 95% alcohol and 5 min each in two changes of absolute alcohol.
- Stained smear is then cleared in two changes of xylol for 3-5 min each, mount in Canada balsam and cover the cover slip.

Trichrome Stain

- Fixation is same as Iron-haematoxylin stain.
- Then it is stained with Trichrome solution for 10 min and differentiated in acid alcohol for 2-3 seconds.

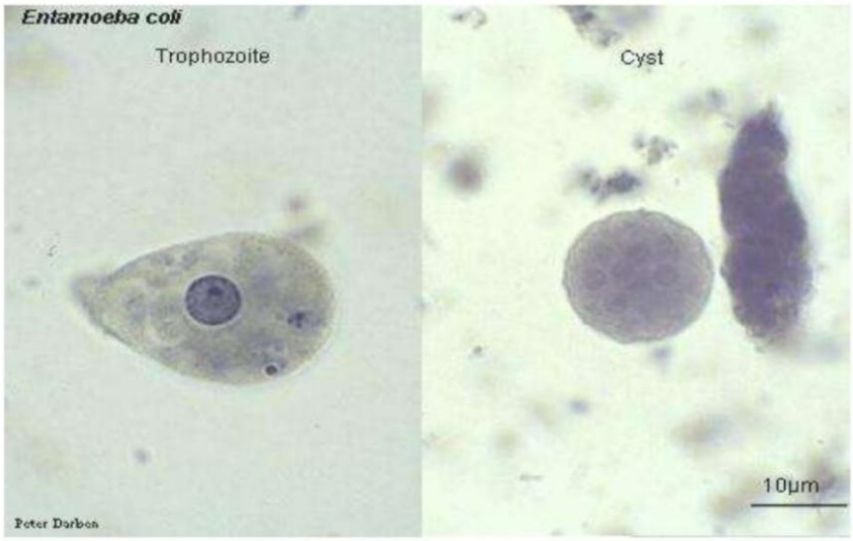


Figure 2.15: Trichome Stain

- Rinse in absolute alcohol in several alcohol for 2-5 min each.
- Stained smear is then cleared in two changes of xylol for 2-5 min each, mount in Canada balsam and cover with cover slip.

Modified acid – fast stain

- It is detection and identification of Cryptosporidium parvam and Isospora belli.
- Thin smear of faeces is made on a clean glass slide and fix by heat at 70°C for 10 min.
- Keep the slide on staining and add carbol fuchsine and heat the stain till stars steaming allow for 9 min.
- Wash it in tap water and decolorized with 5% sulphuric acid for 30 seconds.
- Wash the slide and add counter stain with methylene blue for 1 min.
- Wash it in tap water, dried, mount in Canada balsam and cover the cover slip.
- The acid fast Cryptosporidium oocyst stain red with carbol fuchsin and non acid fast background is blue.

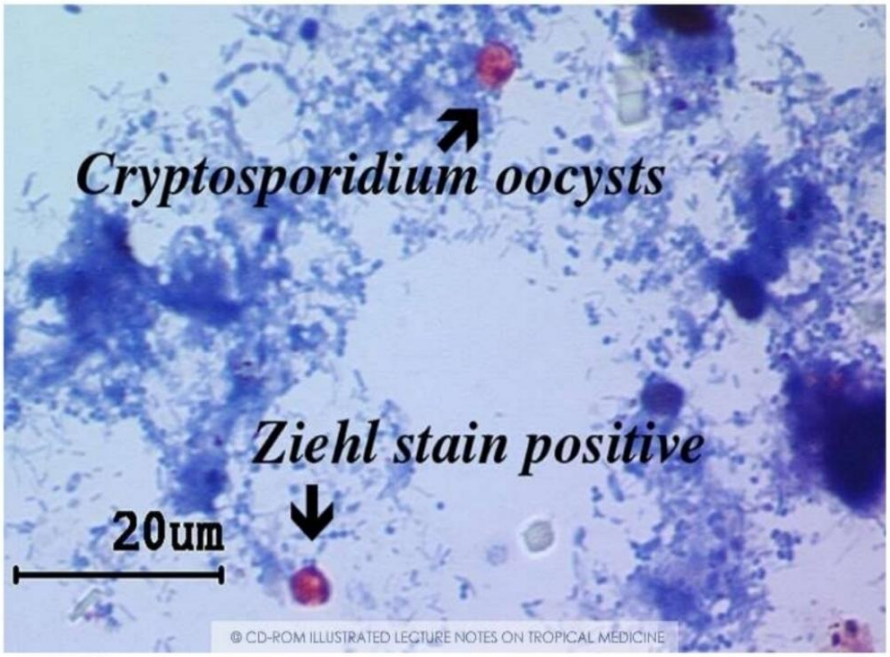


Figure 2.16: Modified acid – fast stain

Isospora belli

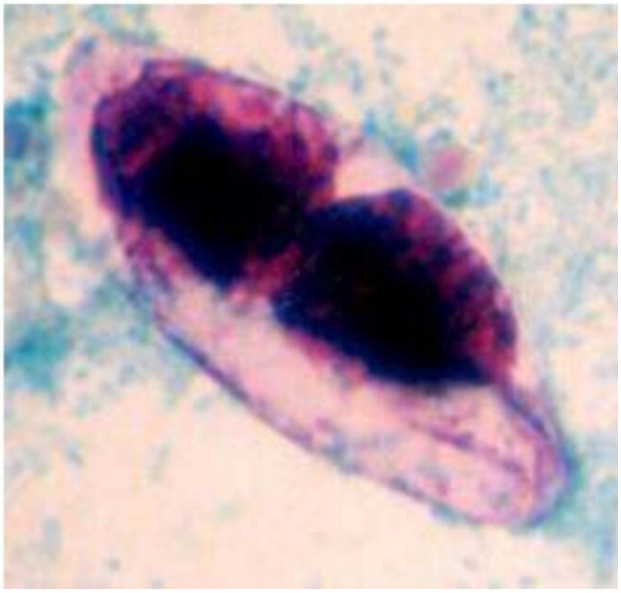


Figure 2.17: Isopora belli

EXAMINATION OF BLOOD FOR PARASITES

Examination of thin blood film:

Preparation:

- Prick the finger or ear lobe with surgical cutting needle under aseptic condition.
- Take a drop of blood not larger than a pin's head on a clean glass slide.
- Make smear with the help of spreider.
- Dry the smear and labeled the smear.

STAINS: with Leishman's stain

- Pour the stain on smear and allow it for 2 min.
- Dilute the stain with twice its volume of D/W which should be neutral.
- Allow the diluted stain to remain on the slide for 10-15 min.
- Wash the slide under running tap water, and dry the smear.
- The stained smear is observed under pil – immersion lens.

Examination of Thick Blood

Preparation:

- A big drop of blood is taken on a glass slide and spread with a needle or corner of another slide.
- The thickness of the film should be such as to allow a newsprint to be read through the preparation.
- Dry the smear in horizontal position and kept by cover by Petri dish.
- Drying may be accelerated by putting the slide inside an incubator.

STAINING:

- By Leishman's or Field' stain,

DEHAEMOGLOBINISATION:

- The film is flooded with the mixture of glacial acetic acid and tartaric acid.

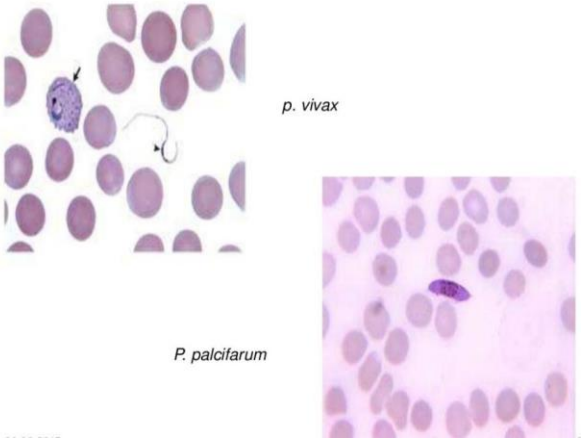


Figure 2.18: Malaria parasite in red blood cells, ring form stage of plasmodium

- Then fixed with methyl alcohol for 3-5 min and wash in D/W.
- After dehaemoglobinisation stain the smear in Leishman's stain same as the thin smear.

Examination of Blood for Microfilaria

- A thick film of blood is prepared and kept covered. Next morning it is, dehaemoglobinised by putting the slide in water.
- Then dry and fixed in methyl alcohol, then stain with Leishman's stain.
- Occasionally the microfilaria may be detected even in thin smear.

MICROFILARIA COUNT: The total number of microfilariae in the thick smear multiplied by 50 will give the no per ml of blood.

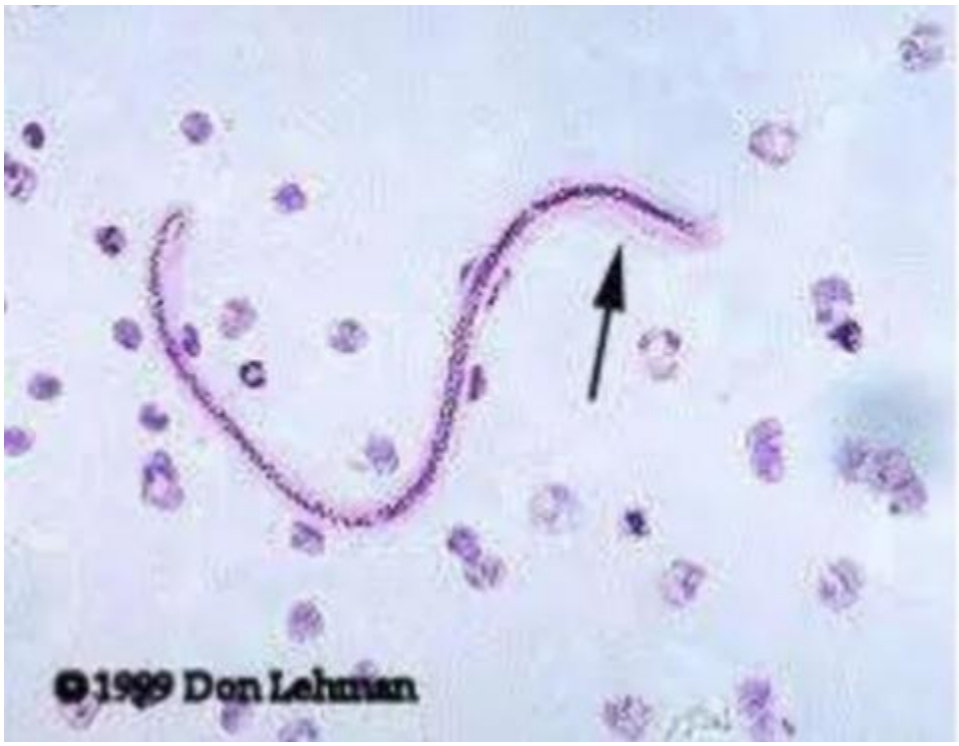


Figure 2.19: Microfilaria Count

Microfilaria

J.S.B. Staining

For thin smear:

- Fix the smear in methyl alcohol for 3-5 min and allow to dry.
- Immersed in solution I for 30 sec wash in tap water.
- Stain with solution II for 1 sec wash again in tap water for 4 sec.

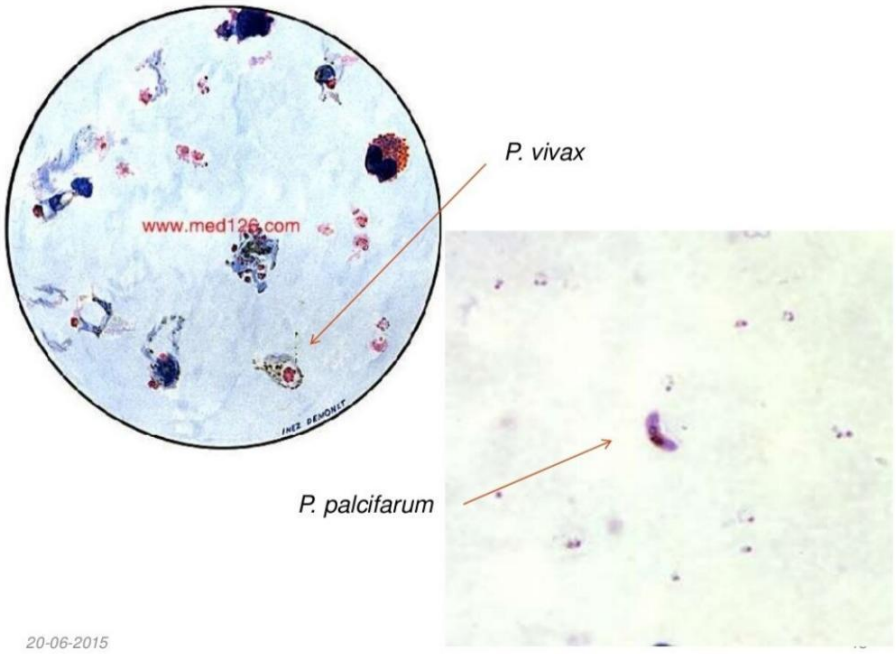


Figure 2.19: J.S.B Staining

- Immersed in solution I again for 30 sec, wash in Tap water for 10 sec, dried and examine under microscope.

Blood concentration methods

- Microhaematocrite centrifugation.
- Triple centrifugation.
- Buffy coat concentration.
- Knott concentration.
- Membrane filtration.
- Gradient centrifugation.

Culture

NNN medium

- NNN medium for the diagnosis of leishmaniasis.
- It consist two part of salt agar and one part of defibrinated rabbit blood.
- Blood, aspirates or small biopsy sample from spleen, liver or bone marrow inoculated in to water of condensation of the medium and incubated at 22-25°C.

- The amastigote form change in to promastigote form which multiplied rapidly to produce a large number of flagellates in bottom of the tube.

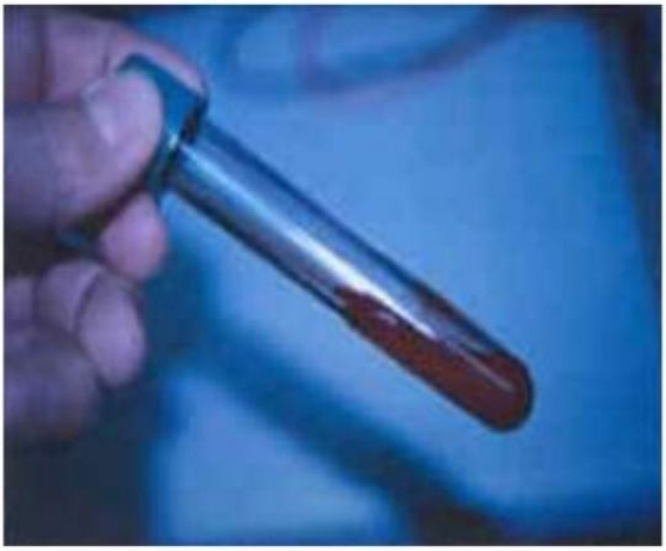


Figure 2.20: NNN medium

Hockmeyer's medium

- This medium consists of Schneider's commercially prepared insect cell culture medium.
- In addition of 30% heat inactivated foetal calf serum and 100 IU penicillin and 100 micro g streptomycin /ml.
- The medium is inoculated with specimen incubated at 22 - 25°C and examine the promastigote by microscopically.
- G. Lambiancan be grow axenically in Diamonds medium, used For axenic cultivation of E. Histolytica

Serologic Diagnosis

test	Disease
Indirect haemagglutination test (IHA)	Amoebiasis, cystcercosis, echinococcosis, filariasis, stronglyoidiasis, fascioliasis, changas' disease.
Fluorescent antibody test (FAT)	African trypanosomiasis
Indirect fluorescent antibody test (IFAT)	Leishmaniasis, malaria, schistosomiasis, toxoplasmosis.
Enzyme linked immunosorbent assay (ELISA)	Toxoplasmosis, toxocariasis, ascariasis.
Compliment fixation test (CFT)	Chagas' disease, paragonimiasis, leishmaniasis.
Latex agglutination test	Echinococcosis
Bentonite floculation test	Trichinellosis, echinococcosis. 53

Table 2: Test and Disease

Molecular assays

- DNA probe; used for diagnosis of malaria and filariasis.
- Polymerase chain reaction (PCR); used for Leishmaniasis, toxoplasmosis, Chagas' disease, onchocerciasis.

Stool Examination

Definition

- Human feces is called as STOOL.
- Faeces / Feces is plural of latin term faex meaning RESIDUE.
- It is the waste residue of indigestible materials of an animal's digestive tract expelled through the anus during defecation.
- Meconium is newborn's first feces.
- SCATOLOGY or CAPROLOGY is the study of feces.

Composition

- 34 Water, ¼4 Solid
- E Undigested and Unabsorbed food
- Intestinal secretions, Mucous
- Bile pigments and Salts
- Bacteria and Inorganic material

- Epithelial cells, Leukocytes

Collection

- Universal Precautions
- Stool should be collected in a dry, sterilized, wide mouthed container.
- It should be uncontaminated with Urine or any other body secretions.
- Properly named and always a fresh sample
- Should be tested.

Macroscopic examination

- Volume <200gms/day
- Colour
- Consistency
- Odour
- Parts of parasite and Adult Parasite
- Blood, Mucous

Colour of stool

Human fecal matter is normally yellowish brown in colour which results from a combination of bile and bilirubin.

VARIATIONS

- White
- Pale greasy
- Bright Red/Maroon
- Blood streak
- Yellow
- Green
- Black
- Blue
- Tan/Clay

Colour of feces in Infants

- Exclusively breast fed infants pass loose and green or pasty and yellow stools.
- Infants fed on cows' milk preparations pass stools of a paler yellow colour and of a much firmer consistency.
- Babies fed on newer modified cows' milk preparations have clay coloured or greenish stools.
- Some healthy children may pass frequent, loose stools containing undigested vegetable matter called as Toddler's diarrhoea.

Consistency of the stool

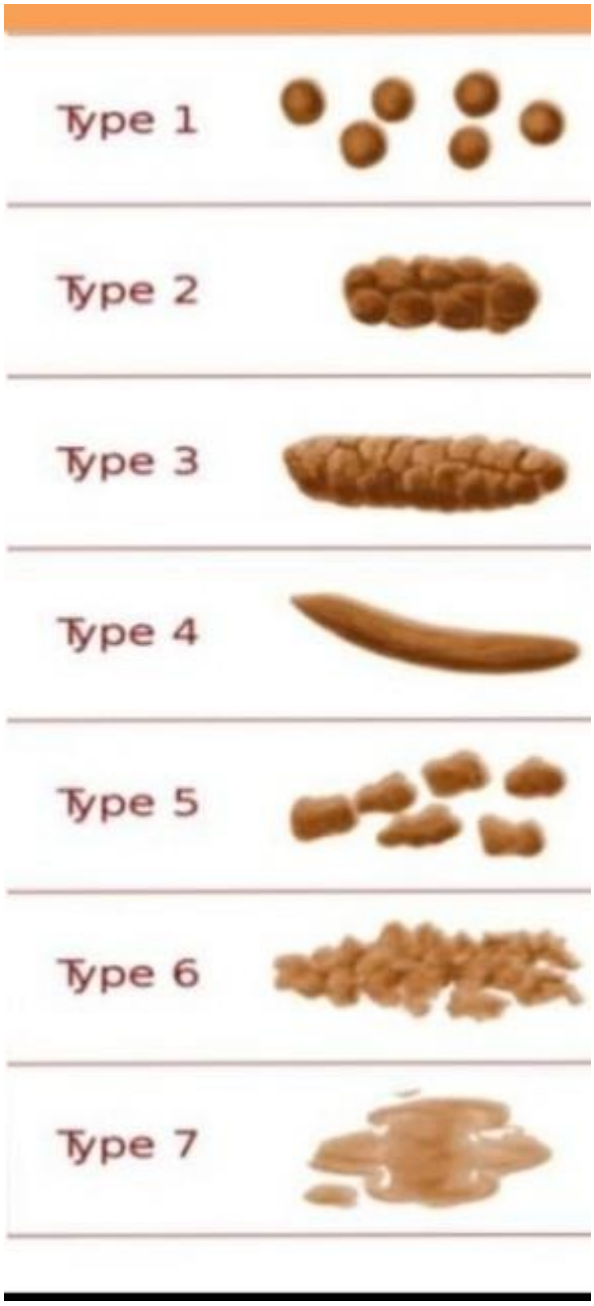


Figure 2.21: Consistency of the stool

- Type 1- Separate hard lumps, like nuts (hard to pass).
- Type 2- Sausage-shaped but lumpy.
- Type 3- Like a sausage but with cracks on the surface.
- Type 4- Like a sausage or snake, smooth and soft.
- Type 5- Soft blobs with clear-cut edges.
- Type 6- Fluffy pieces with ragged edges.
- Type 7- Watery, no solid pieces. Entirely Liquid.

Odour of stool

- Basically depends on the pH of the stool and INDOLE and SKETOLE are the substances that produce normal odour formed by Intestinal bacterial fermentation and putrefaction.
- A foul odour is caused by degradation of undigested protein and excessive carbohydrate intake.
- Sickly sweet odour is produced by undigested lactose
- Diarrhoea mixed with mucous and Blood is suggestive of Typhoid, Amoebiasis, Typhus, Large bowel Carcinoma.
- Diarrhoea mixed with mucous and Pus is suggestive of Ulcerative Collitis, Regional Enteritis, Shigellosis, Salmonellosis, Acute diverticullitis, Intestinal TB.
- Pasty stool with high fat content is suggestive of CBD Obstruction, Cystic fibrosis-butter stool.
- Translucent gelatinous mucous clinging to the surface of the formed stool is found in Spastic Constipation, Excessive straining, Mucous,Collitis.
- Rice water stools which is colourless and almost devoid of odour is suggestive of Cholera.
- Stools may look like Redcurrant jelly in Intussusception.

Parasite

- Round worm
- Hook worm
- Tape worm
- Pin worm
- Whipworm

Microscopic Examination

Materials

- Microscope slides
- Cover slips
- Sodium chloride solution
- Lugol's Iodine Solution
- Wooden applicator
- Fresh stool
- Gloves

Slide Preparation

Slides

- Saline Specimen preparation
- Iodine Specimen preparation

CONCENTRATION METHOD to detect Ova.

- A drop of warm Saline or Lugol's Iodine is placed over a clean microscopic slide.
- About 2mg of stool sample should be taken and mixed with soln placed over the slide.
- Coverslip is placed avoiding air bubbles.
- Examined under Microscope.

Pin Worm egg collection

Eggs of Pin worm – Enterobius vermicularis rarely appear in stools. These are usually collected in the folds of skin in perianal region.

Collection

Cotton swab / Plaster patch – Anus especially in early morning – Dipped in Saline – Observed.

Examination of parasites

- Warm stools are best for detecting Ova or parasites.
- Do not refrigerate the specimen.
- Because of cyclic life cycle of parasites, three separate random stool specimens are recommended for examination.

Normal values

- Undigested food materials – None to small amount
- Eggs, Cysts, Parasitic fragments – None
- Yeasts – None
- Starch – None
- Leukocytes – None

Leukocytes in stool

- **Large amounts of leukocytes** is suggestive of Chronic Ulceratice Collitis, Chronic Bacillary Dysentry, Localised Abscess, Fistulas.
- **Mononuclear Leukocytes** appear in Typhoid.
- **Polymorphonuclear Leukocytes** appear in Shigellosis, Salmonellosis, Invasice E. Coli diarrhoea, Ulceratice Collitis.
- **Absent Leukocytes** in Cholera, Viral diarrhoea,Non-specific diarrhoea, Amoebic Collitis, Giardiasis.

Hook worm

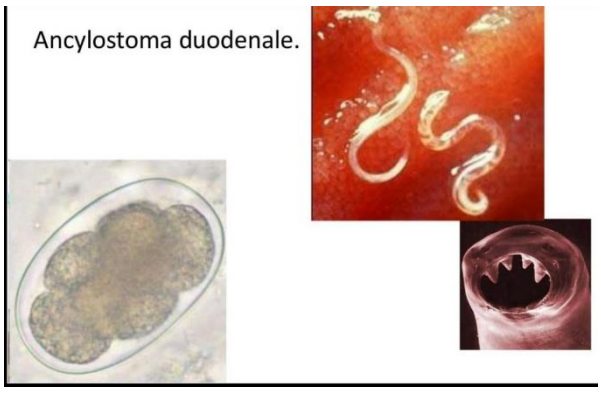


Figure 2.22: Hookworm

Round Worm

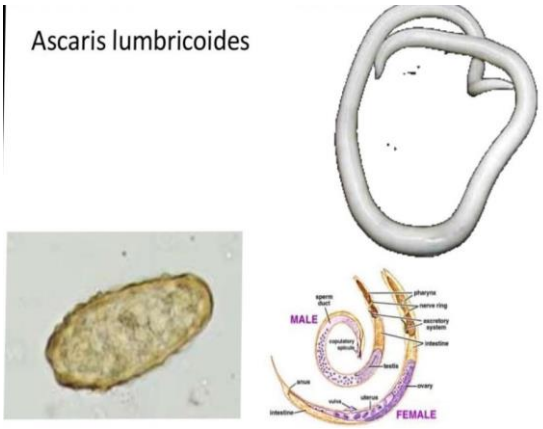


Figure 2.23: Roundworm

Tape worm

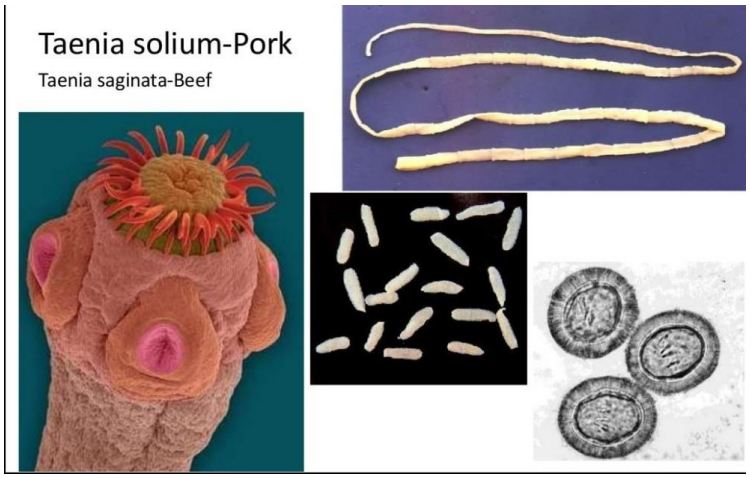


Figure 2.24: Tapeworm

Whip worm

Figure 2.25: Whipworm

Pin worm

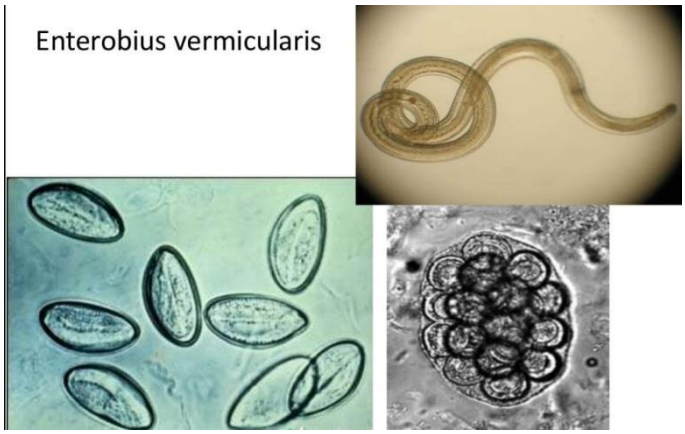


Figure 2.26: Pin worm

Entamoeba

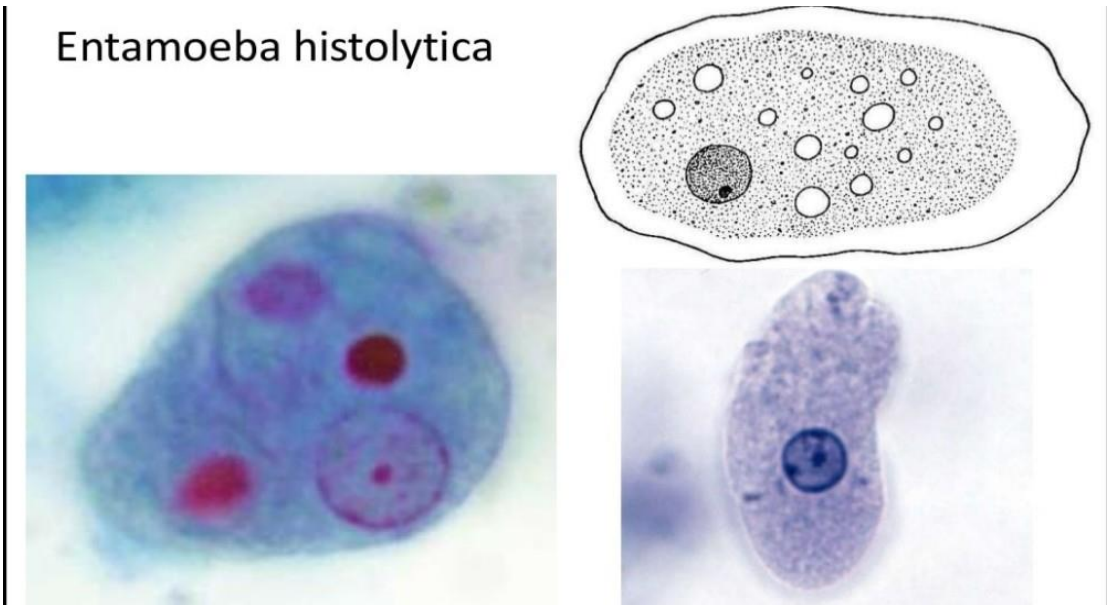


Figure 2.27: Entamoeba

Giadiasis

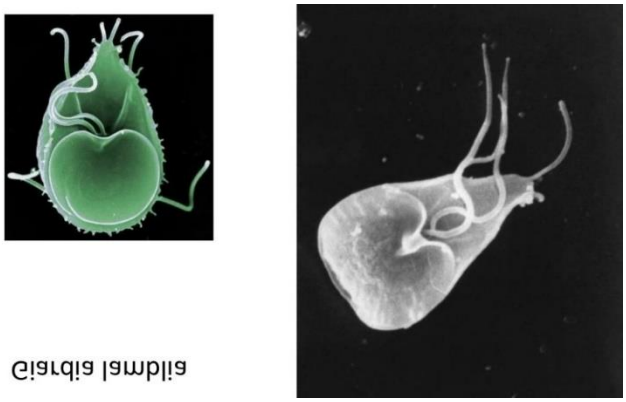


Figure 2.28: Giadiasis

Stool Culture

- Normal Microbial flora of GI tract contains following organisms.
- Gram-ve- E. Coli, Enterobacter, Proteus, Pseudomonas aeruginosa, Bacteroides.
- Gram +ve-Clostridia, Lactobacilli, Enterococci, Anaerobic streptococci.
- Human feces contain approximately 10'11 organisms per gram wet weight as normal flora. Whereas gut bacterial pathogens rarely exceed 10'5 organisms per gram.

Culture medias

Culture media usually used is of AGAR and is done aerobically.

- XLD Agar media – Salmonella, Shigella.
- TCBS Agar media- Cholera.
- MacConkey media – Yersinia enterocolitica
- Campylobacter culture media for Campylobacter species.

The mainstay of diagnosis of bacterial infections of the gut is by culture.

Hanging drop test

- Place a drop stool in the centre of a coverslip.
- Place a drop of water / vaseline at each corner of the coverslip.
- Invert a slide with a central depression over the coverslip
- The coverslip will stick to the slide and when the slide is inverted the drop of bacterial culture will be suspended in the central depression of the slide.
- Examine microscopically (X100) for motile organisms.

Chemical examination

Normalcy

- Water – Upto 75%
- pH-5.8 to 7.5
- Occult blood, RS – Negative
- Bile – Negative in Adults
  Positive in Children
- Sodium – 5.8 to 9.8 mEq/24hrs
- Chlorides – 2.5 to 3.9 mEq/24hrs
- Potassium – 15.7 to 20.7 mEq/24hrs
- Lipids / Fatty acids -0 to 6 gms/24hrs
- Nitrogen - <2.5g/24hrs

Ph

Increased pH-ALKALINE

- Colitis
- Antibiotic use
- Villous adenoma
- Excess Protein in diet.

Decreased pH-ACIDIC

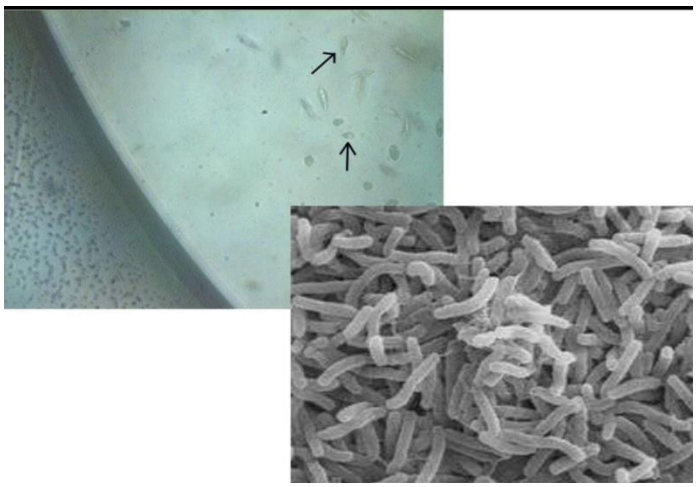


Figure 2.29: Optical micrographs

- Carbohydrate Malabsorption
- Fat Malabsorption
- Disaccharidase deficiency

Occult Blood

- PRINCIPLE- BENZIDINE TEST

Perioxidase action of hemoglobin in blood converts hydrogen peroxide to water and nascent oxygen. This oxygen oxidises benzidine in acid medium to form green to blue coloured complex.

- METHOD

Benzidine – Glacial acetic acid – Hydrogen peroxide – Over stool in slide – Colour change.

- GUAIAC TEST – gFOBT
- Found in Ulcers, Diverticullitis, Diaphragmatic Hernia, CA Colon, Gastrium ,Adenoma.

Fats in stool

Increased Fats is associated with Malabsorption Syndromes

- Obstructive Jaundice
- Non tropical sprue/Coeliac Sprue
- Crohn's disease
- Cystic Fibrosis
- Whipple's disease
- Enteritis and Pancreatic diseases
- Surgical removal of section of Intestine.

Reducing substances

- Tested for RS especially in infants with Chronic diarrhea to rule out Lactose intolerance.
- Stool will be positive for RS in variety of conditions especially in Rota viral Infection in Infants.

Mala / Purisha

- Mala – 7 Anjali Pramana
- One among ASHTA Sthana Pariksha
- Aama / Pakwa Purisha
- Tila Pishtha Nibha Varchas.....
- Purishaja Krimi
- Purisha Virajaneeya Dravyas
- Mala in Rajayakshma.
- Mala in Lakshanas of diseases.
- Mala in asadhyavastha of diseases.

Urine Analysis

- Urine analysis, also called Urinalysis – one of the oldest laboratory procedures in the practice of medicine.
- Also knows as Urine R&M (routine & microscopy)
- Is an array of tests performed on urine
- General evaluation of health
- Diagnosis of disease or disorders of the kidneys or urinary tract
- Diagnosis of other systemic disease that affect kidney function
- Monitoring of patients with diabetes
- Screening for drug abuse (eg. Sulfonamide or aminoglycosides)

Collection of urine Specimens

- Improper collection---- may invalidate the results

- Containers for collection of urine should be wide mouthed, clean and dry.
- Analysed within 2 hours of collection else requires refrigeration.

Figure 2.30: Sterile Specimen Cup

Types of urine sample

a.Clean catch urine collection method

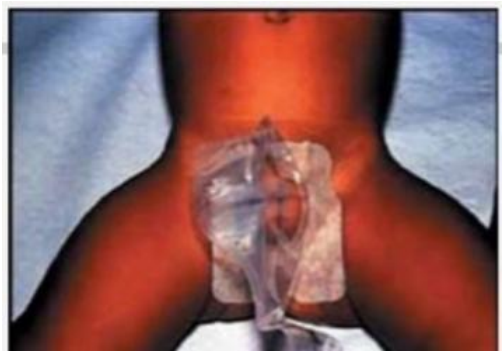


Figure 2.31: Clean catch urine collection method

b.Suprapubic aspiration of urine

Sample type	Sampling	Purpose
Random specimen	No specific time most common, taken anytime of day	Routine screening, chemical & FEME
Morning sample	First urine in the morning, most concentrated	Pregnancy test, microscopic test
Clean catch midstream	Discard first few ml, collect the rest	Culture
24 hours	All the urine passed during the day and night and next day Ist sample is collected.	used for quantitative and qualitative analysis of substances
Postprandial	2 hours after meal	Determine glucose in diabetic monitoring
Supra-pubic aspired	Needle aspiration	Obtaining sterile urine

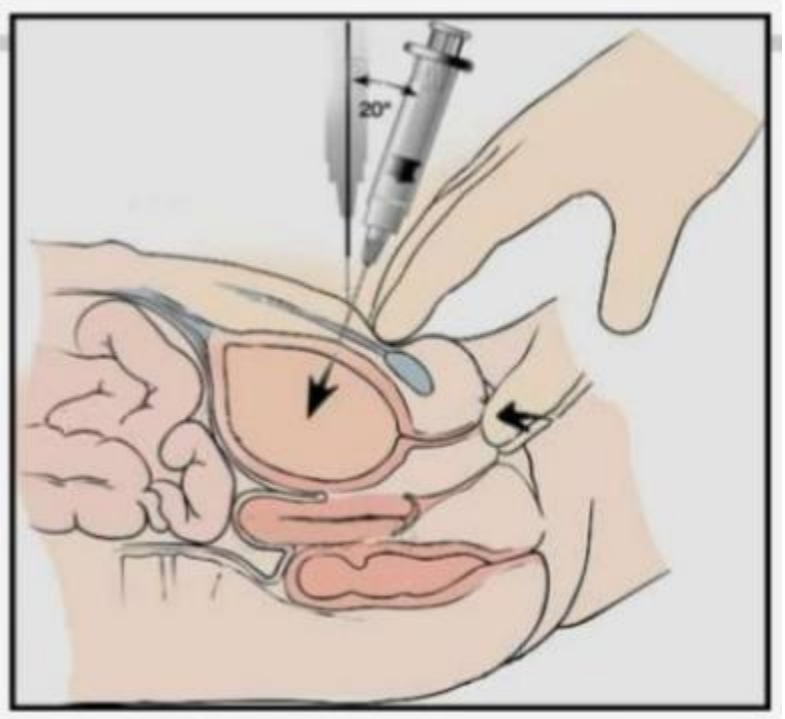


Figure 2.32: Suprapubic aspiration

c.Urine storage and transportation kit

Figure 2.33: Urine storage and transportation kit

Urinalysis

- Urinalysis consists of the following measurements:
    - Macroscopic or physical examination
    - Chemical examination
    - Microscopic examination of the sediment

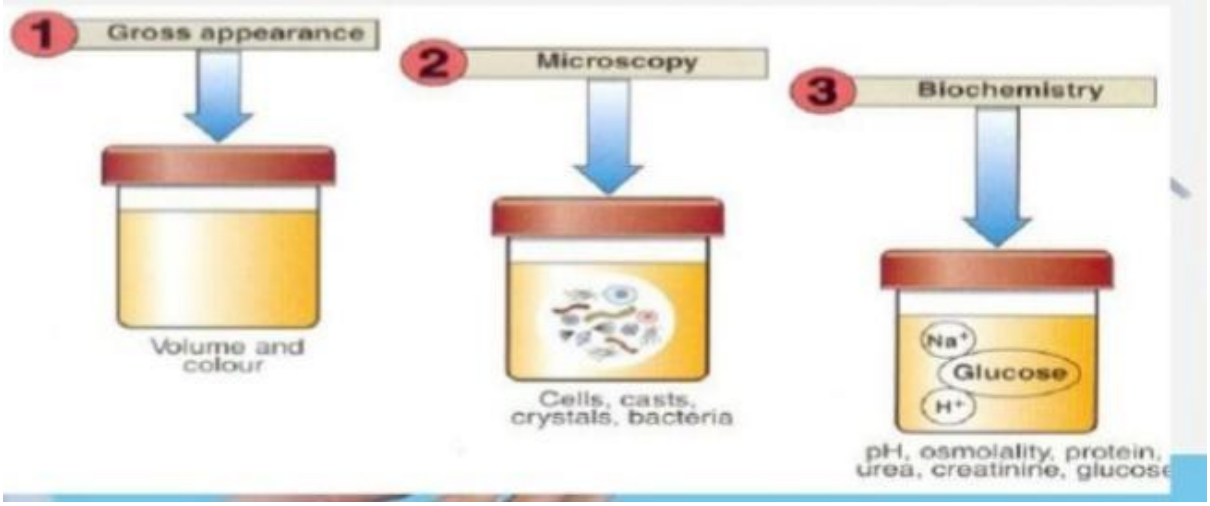


Figure 2.34: The place of biochemical testing in urinalysis

Physical Examination of Urine

Examination of physical Characteristics:

- Volume
- Colour
- Odour
- pH and
- Specific gravity
    - The refractometer or a reagent strip is used to measure specific gravity

Volume

- Normal- 1-2.5 L/day
- Oliguria- Urine Output < 400ml/day Seen in
    - Dehydration
    - Shock
    - Acute glomerulonephritis
    - Renal Failure
- Polyuria- Urine Output > 2.5 L/day Seen in
    - Increased water ingestion
    - Diabetes mellitus and insipidus.
- Anuria- Urine output < 100ml/day Seen in renal shut down

Colour

- Normal- pale yellow in color due to pigments urochrome, urobilin and uroerythrin.
- Cloudiness may be caused by excessive cellular material or protein, crystallization or precipitation of non pathological salts upon standing at room temperature or in the refrigerator.
- Colour of urine depending upon it's constituents.
- Abnormal colours: Deep yellow, Colour less

Figure 2.35: Urine Colour

Odour

- Normal – aromatic due to the volatile fatty acids
- On long standing – ammonical (decomposition of urea forming ammonia which gives a strong ammonical smell)
- Foul, offensive – pus or inflammation
- Sweet - Diabetes
- Fruity – Ketonuria
- Maple syrup-like – Maple Syrup Urine Disease
- Rancid – Tyrosinaemia
- Characteristic "rotten egg" odor

pH

- Reflects ability of kidney to maintain normal hydrogen ion concentration in plasma & ECF
- Urine pH ranges from 4.5 to 8
- Normally it is slightly acidic lying between 6-6.5.
- Tested by:
    - Litmus paper
    - pH paper
    - dipsticks
- Acidic Urine-Ketosis (diabetes, starvation, fever) systemic acidosis, UTI- E.coli, Acidification therapy

Specific Gravity

- It is measurement of urine density which reflects the ability of the kidney to concentrate or dilute the urine relative to the plasma from which it is filtered.

Figure 2.36: Urinalysis

- Decrease in Specific Gravity – Absence of ADH.
- Renal Tubular damage.
- Fixed specific gravity (isosthenuria)=1.010

Microscopic examination of urine

- A sample of well-mixed urine (usually 10-15 ml) is centrifuged in a test tube at relatively low speed (about 2000-3,000 rpm) for 5-10 minutes which produces a concentration of sediment (cellular matter) at the bottom of the tube.
- A drop of sediment is poured onto a glass slide, a thin slice of glass (a coverslip) is place over it ond observed under microscope
- A variety of normal and abnormal cellular elements may be seen in urine sediment such as:
  - Red blood cells
  - White blood cells
  - Mucus
  - Various epithelial cells

S.G	Osmolality (mosm/kg)
1.001	100
1.010	300
1.020	800
1.025	1000
1.030	1200
1.040	1400

  - Various crystals

- Bacteria
- Casts

Abnormal Findings

- Per High Power Field (HPF) (400x)
  - >3 erythrocytes
  - > 5 leukocytes
  - >2 renal tubular cells
  - > 10 bacteria
- Per Low Power Field (LPF) (200x)
  - >3 hyaline casts or> 1 granular cast
  - > 10 squamous cells (indicative of contaminated specimen)
  - Any other cast (RBCs, WBCs)
- Presence of
  - Fungal hyphae or yeast, parasite, viral inclusion
  - Pathological crystals (cystine, leucine, tyrosine)
  - Large number of uric acid or calcium oxalate
- Hematuria is the presence of abnormal numbers of red cells in urine due to any of several possible causes.
  - glomerular damage
  - tumors which erode the urinary tract anywhere along its length
  - kidney trauma
  - urinary tract stones
  - acute tubular necrosis
  - upper and lower urinary tract infections
  - nephrotoxins
- WBC in high numbers indicate inflammation or infection somewhere along the urinary tract

Red blood cells in urine appears as refractile disks

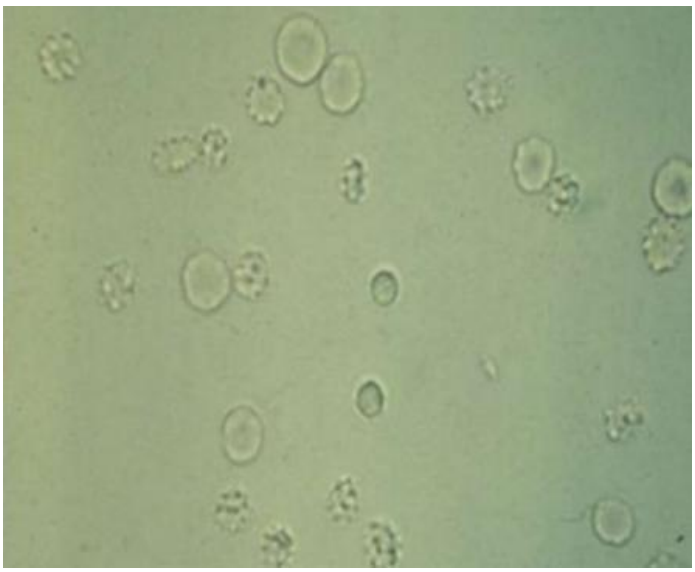


Figure 2.37: RBC in Urine sediment

White blood cells in urine

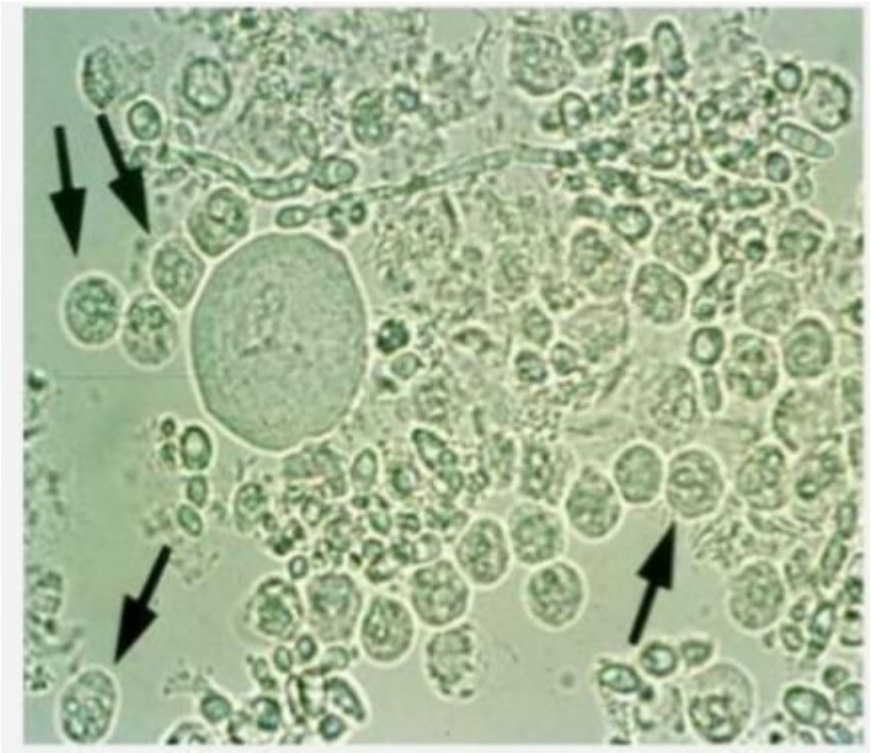
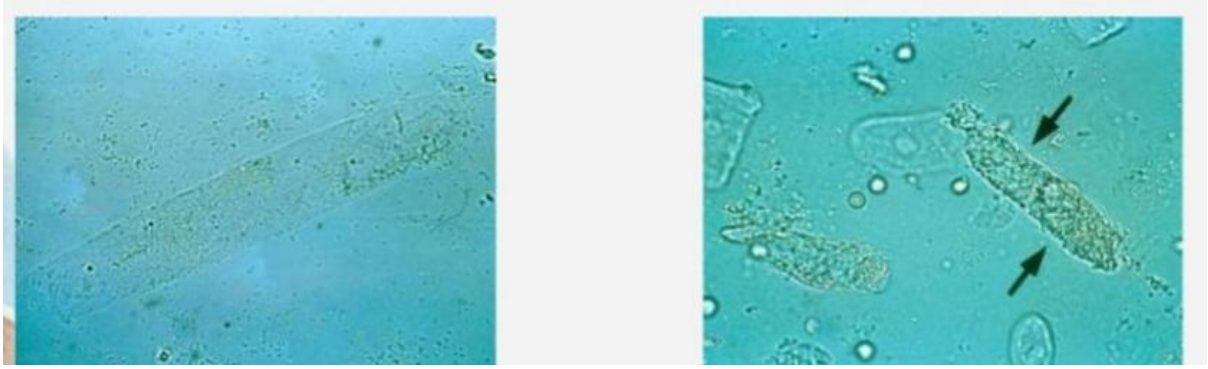


Figure 2.38: WBC in Urine sediment

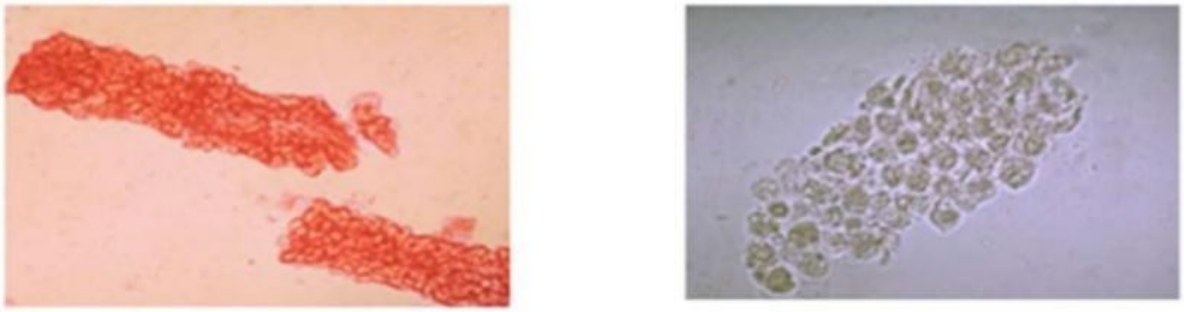
Casts

- Urinary casts are cylindrical aggregations of particles that form in the distal nephron, dislodge, and pass into the urine. In urinalysis the indicate kidney disease.
- They form via precipitation of Tamm Horsfall mucoprotein which is secreted by renal tubule cells.
- Types of cast seen :
  - **Acellular cast**: Hyaline casts, Granular casts, Waxy casts, Fatty casts, Pigment casts, Crystal casts.
  - **Cellular cast**: Red cell casts, White cell casts, Epithelial cell cast
- The most common type of cast- hyaline casts are solidified Tamm-Horsfall mucoprotein secreted from the tubular epithelial cells and seen in fever, strenuous exercisée damage to the glomerular capillary.
- Red blood cells may stick together and form red blood cell casts. Such casts are indicative of glomerulonephritis, with leakage of RBC's from glomeruli, or severe tubular damage
- White blood cell casts are most typical for acute pyelonephritis, but they may also be present



Hyaline Cast

Granular Cast



Red blood cell cast

In urine

White blood cell cast

In urine

Figure 2.39: Urinary Cast

- A variety of normal and abnormal crystals may be present in the urine sediment

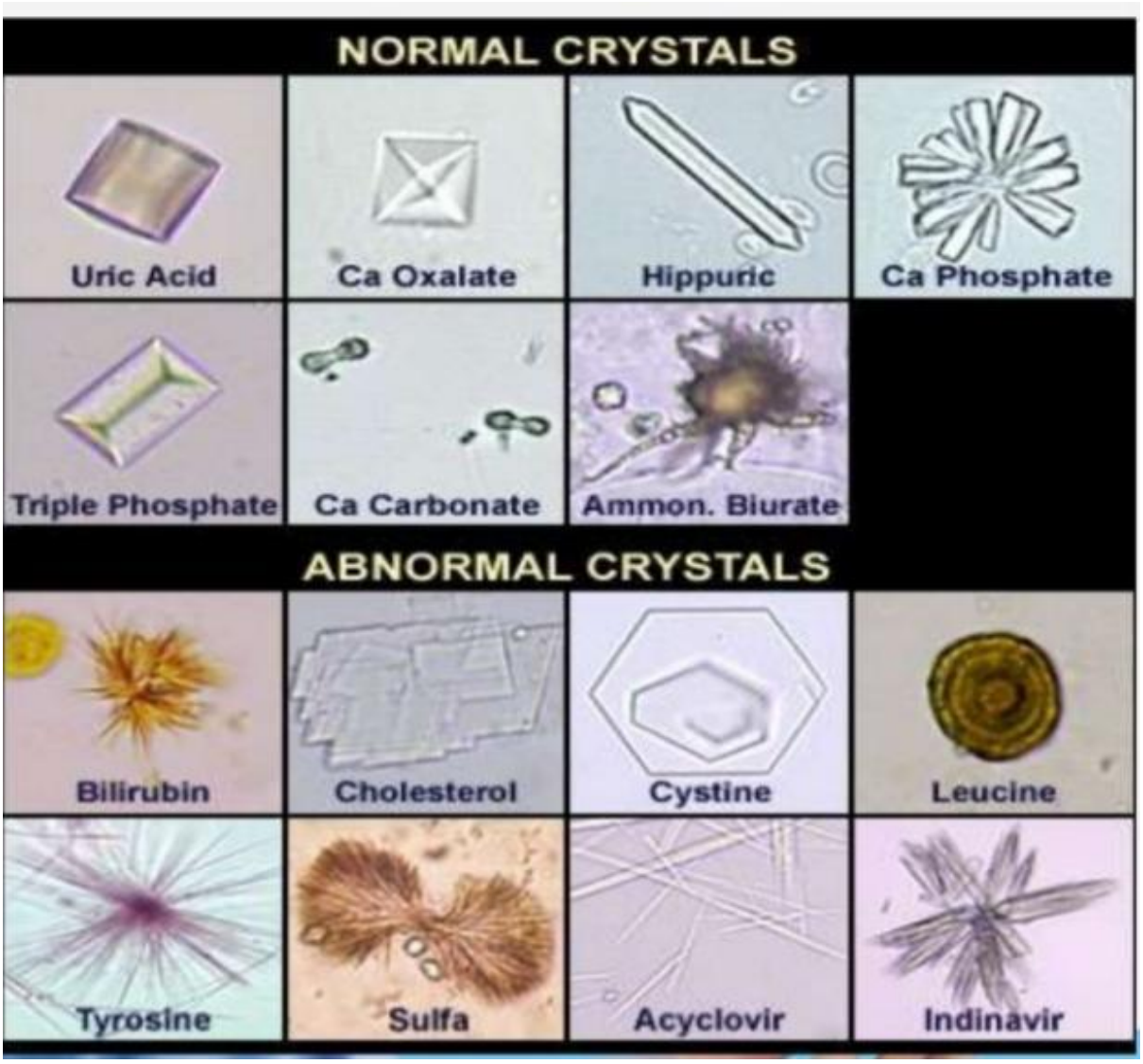


Figure 2.40: Normal Crystals and Abnormal Crystals

Chemical analysis of urine

- The chemical analysis of urine us undertaken to evaluate the levels of the following componen:
  - -Protein
  - -Glucose
  - -Ketones
  - -Occult blood
  - -Billirubin
  - -Urobilinogen
  - -Bile salts
- The presence of normal and abnormal chemical elements in the urine are detected using dry reagent strips called dipsticks.

- When the test strip is dipped in urine the reagents are activated and a chemical reaction occurs.
- The chemical reaction results in a specific color change.
- After a specific amount of time has elapse,This color change is compared against å reference colour chart provided by the analysis

The dipstick method of chemical analysis of urine

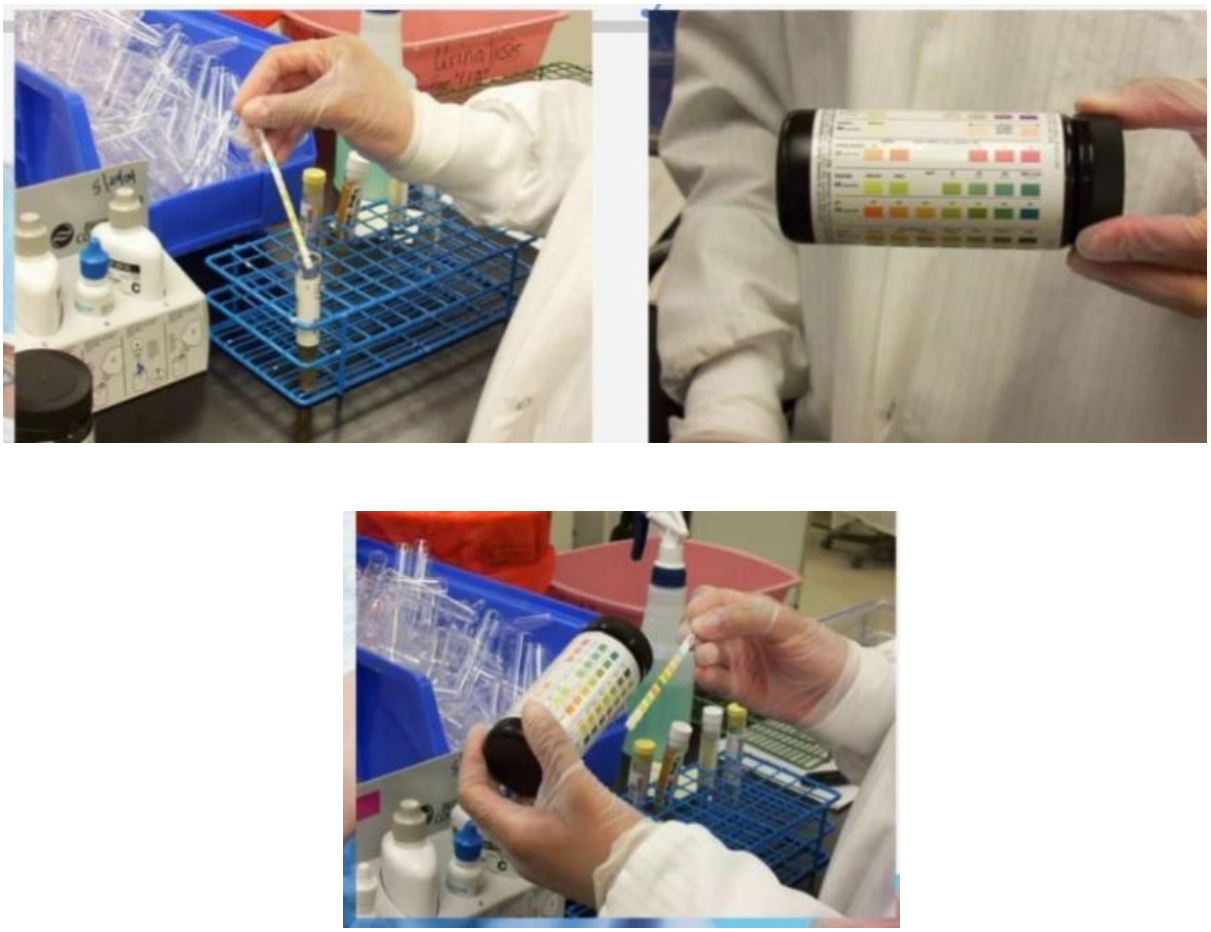


Figure 2.41: Dipstick method

Protein in urine:

- Detected by heat coagulation or dipstick method
- Urine proteins come from plasma protein and Tomm-Horsfall (T-H) glycoprotein
- Healthy individuals excrete <150 mg/d of total protein and <30 nig/d of albumin.
- Plasma cell dyscrasias (multiple myeloma)Can be associated with large amounts of excreted light chains in the urine, which may not be detected by dipstick. The light chains produced from these disorders

Chemical analysis of urine

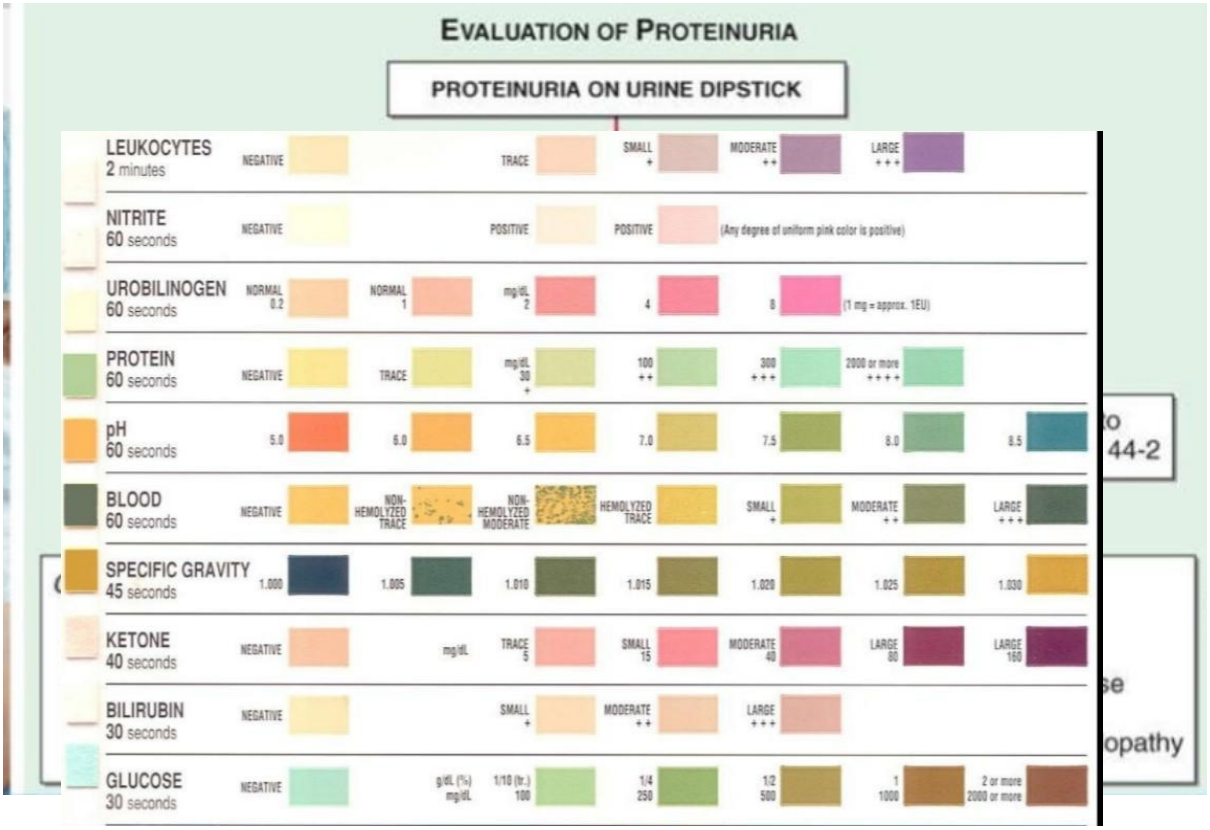


Figure 2.42: Chemical analysis of urine

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DEPARTMENT OF BIOTECHNOLOGY

UNIT – 3 – PROTOZOA – SMB 3201

UNIT 3

PROTOZOA

IDENTIFICATION OF INTESTINAL PROTOZOA:

AMOEBA:

Entamoeba Histolytica

Clinical Manifestations:

Patients have acute or chronic diarrhea, which may progress to dysentery. Extraintestinal disease may be present as a complication or as a primary problem (e.g., liver, lung or brain abscess, or skin or perianal infection).

Structure:

The trophozoite is 10 to 60 μm in diameter, ameboid, actively motile, and often erythrophagocytic. In stained specimens, the nucleus has a central karyosome with finely beaded peripheral chromatin. The cyst form is rounded, 10 to 20 μm in diameter, with one to four nuclei showing the characteristic appearance. A chromatoidal bar with rounded or square ends may be seen.

Classification and Antigenic Types:

Pathogenic strains can be grown at 37° C but not at room temperature and fall into specific enzyme assay groups.

Multiplication in the host occurs by binary fission. Nuclear replication produces four nuclei during cyst maturation. During excystation the cyst divides to form four cells which immediately divide again to yield eight tiny amebae.

Pathogenesis:

The colon may be colonized without invasion of mucosa. The critical factor determining colonization is the ability of the ameba to adhere to colonic mucosal lining cells. Invasion of the mucosa produces ulcers that sometimes progress by direct extension or by metastasis. Metastatic infection first involves the liver. Extension or metastasis from the liver may involve the lung, brain, or other viscera.

Host Defenses:

Gastric acid and rapid intestinal transit are nonspecific defenses. Humoral antibody and cell-mediated immunity play limited roles in preventing dissemination.

Epidemiology:

Fecal-oral transmission of cysts involves contaminated food or water. Amebas can be transmitted directly by sexual contact involving the anus.

Diagnosis:

Acute diarrhea is the usual presentation of symptomatic disease. Ulceration is associated with occult or gross blood in stool and/or with a visceral abscess. The condition may be confirmed by identification of *E histolytica* in the stool or in abscess aspirates. The ameba in abscesses line the wall of the abscess cavity

and thus will be found in the last material aspirated from the abscess. Ameba can be cultured. Positive serologic tests, particularly tests showing rising antibody levels, may provide indirect evidence of infection.

Detection:

detection of Entamoeba histolytica is performed by **microscopic examination for characteristic cysts and/or trophozoites in fecal preparations**. Differentiation of E. histolytica cysts and those of nonpathogenic amoebic species is made on the basis of the appearance and the size of the cysts.

Control

Prevention is largely a matter of personal and public hygiene. There are no effective immunizations or prophylaxis.

Various drugs are used to treat different clinical syndromes.

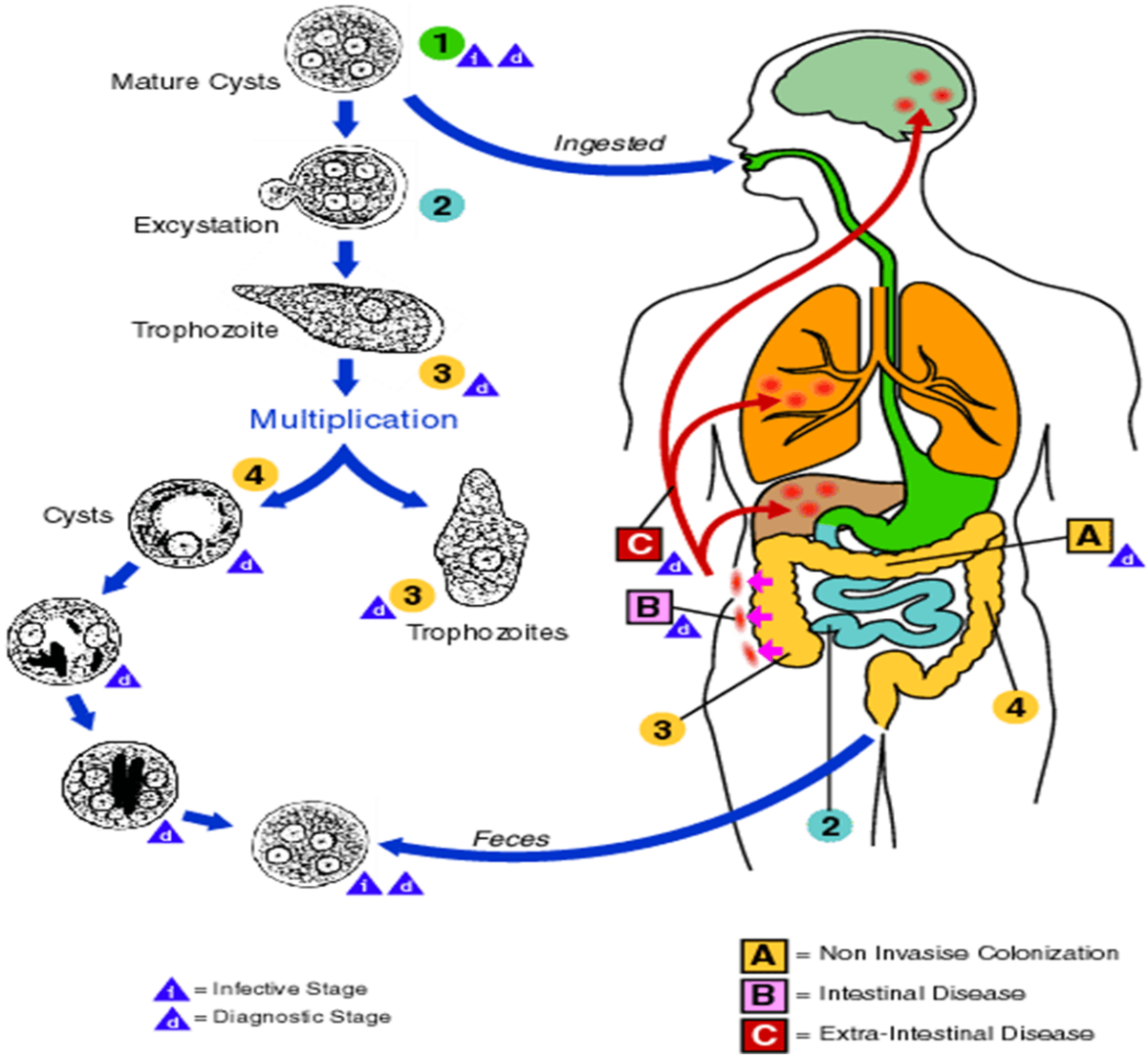

Figure 3: Life Cycle of Entamoeba histolytica

BLOOD PROTOZOA:

MALARIA:

Malaria is a disease caused by a parasite. The parasite is spread to humans through the bites of infected mosquitoes. People who have malaria usually feel very sick with a high fever and shaking chills.

Symptoms:

Signs and symptoms of malaria may include:

- Fever
- Chills
- General feeling of discomfort
- Headache
- Nausea and vomiting
- Diarrhea
- Abdominal pain
- Muscle or joint pain
- Fatigue
- Rapid breathing
- Rapid heart rate
- Cough

Some people who have malaria experience cycles of malaria "attacks." An attack usually starts with shivering and chills, followed by a high fever, followed by sweating and a return to normal temperature.

Malaria signs and symptoms typically begin within a few weeks after being bitten by an infected mosquito. However, some types of malaria parasites can lie dormant in your body for up to a year.

Causes:

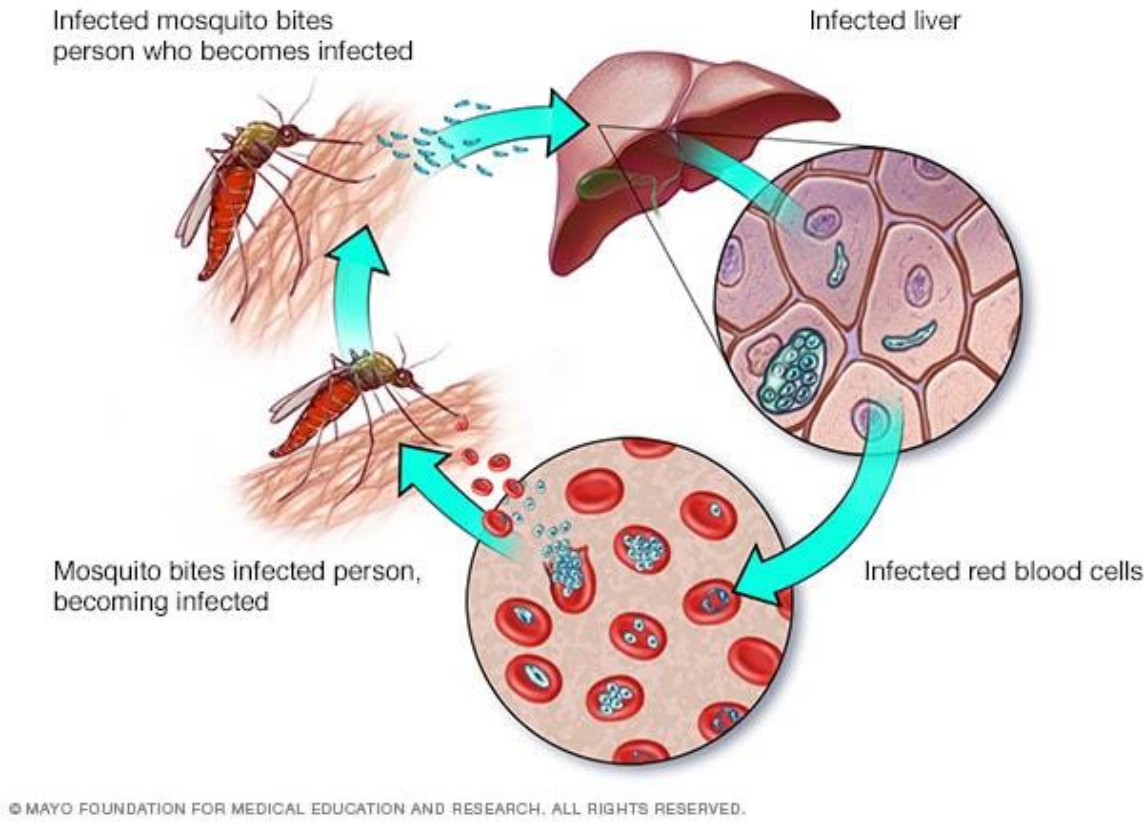

Figure 3.1: Malaria

Malaria is caused by a single-celled parasite of the genus plasmodium. The parasite is transmitted to humans most commonly through mosquito bites.

Mosquito transmission cycle

- **Uninfected mosquito.** A mosquito becomes infected by feeding on a person who has malaria.
- **Transmission of parasite.** If this mosquito bites you in the future, it can transmit malaria parasites to you.
- **In the liver.** Once the parasites enter your body, they travel to your liver — where some types can lie dormant for as long as a year.
- **Into the bloodstream.** When the parasites mature, they leave the liver and infect your red blood cells. This is when people typically develop malaria symptoms.
- **On to the next person.** If an uninfected mosquito bites you at this point in the cycle, it will become infected with your malaria parasites and can spread them to the other people it bites.

IDENTIFICATION:

Malaria parasites can be identified by **examining under the microscope a drop of the patient's blood**, spread out as a “blood smear” on a microscope slide. Prior to examination, the specimen is stained (most often with the Giemsa stain) to give the parasites a distinctive appearance.

Antigen Detection

Various test kits are available to detect antigens derived from malaria parasites. Such immunologic ("immunochromatographic") tests most often use a dipstick or cassette format, and provide results in 2-15 minutes. These "Rapid Diagnostic Tests" (RDTs) offer a useful alternative to microscopy in situations where reliable microscopic diagnosis is not available. Malaria RDTs are currently used in some clinical settings and programs.

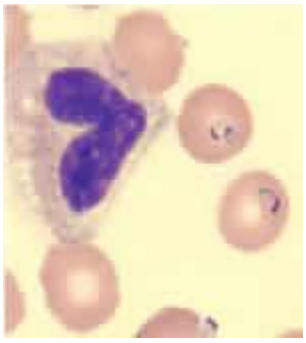

Figure 3.2: Antigen Detection

INTESTINAL HELMINTHES:

ASCARIS:

Ascaris is a genus of parasitic nematode worms known as the "small intestinal roundworms", which is a type of parasitic worm.

Their eggs are deposited in feces and soil. Plants with the eggs on them infect any organism that consumes them. *A. lumbricoides* is the largest intestinal roundworm and is the most common helminth infection of humans worldwide. Infestation can cause morbidity by compromising nutritional status, affecting cognitive processes, inducing tissue reactions such as granuloma to larval stages, and by causing intestinal obstruction, which can be fatal.

An estimated 807 million–1.2 billion people in the world are infected with *Ascaris lumbricoides* (sometimes called just *Ascaris* or ascariasis). *Ascaris*, hookworm, and whipworm are parasitic worms known as soil-transmitted helminths (STH). Together, they account for a major burden of parasitic disease worldwide.

People with ascariasis often show no symptoms. If symptoms occur they can be light. Symptoms include abdominal discomfort or pain. Heavy infections can block the intestines and slow growth in children. Other symptoms such as cough are due to migration of the worms through the body. Ascariasis is treatable with medication prescribed by your healthcare provider.

Humans can also be infected by pig roundworm (*Ascaris suum*). *Ascaris lumbricoides* (human roundworm) and *Ascaris suum* (pig roundworm) are hard to tell apart. It is unknown how many people worldwide are infected with *Ascaris suum.*

DIAGNOSIS — The diagnosis of ascariasis is usually made via **stool microscopy**. Other forms of diagnosis are through eosinophilia, imaging, ultrasound, or serology examination. q Microscopy — Characteristic eggs may be seen on direct examination of feces or following concentration techniques.

TEANIA:

Taeniasis in humans is a **parasitic infection caused by the tapeworm species Taenia** saginata (beef tapeworm), Taenia solium (pork tapeworm), and Taenia asiatica (Asian tapeworm). Humans can become infected with these tapeworms by eating raw or undercooked beef (T. saginata) or pork .

SYMPTOMS:

Most people who have taeniasis don't have any symptoms. If signs and symptoms are present, they may include:

- pain
- *unexplained weight loss*
- *blockage of the intestine*
- digestive problems

Some people with taeniasis may also experience irritation in the perianal area, which is the area around the anus. Worm segments or eggs being expelled in the stool cause this irritation.

People often become aware that they have a tapeworm when they see worm segments or eggs in their stool.

Infections can take between 8 and 14 weeks to develop.

DIAGNOSIS:

Since it is difficult to diagnose using eggs alone, **looking at the scolex or the gravid proglottids** can help identify it as Taenia saginata. Proglottids sometimes trickle down the thighs of infected humans and are visible with unaided eye, so can aid with identification.

ENTEROBIUS(PINWORM):

Figure 3.3: ENTEROBIUS(PINWORM)

Pinworm infection is caused by a small, thin, white roundworm called Enterobius vermicularis. Although pinworm infection can affect all people, it most commonly occurs among children, institutionalized persons, and household members of persons with pinworm infection.

A pinworm ("threadworm") is a small, thin, white roundworm (nematode) called *Enterobius vermicularis* that sometimes lives in the colon and rectum of humans. Pinworms are about the length of a staple. While an infected person sleeps, female pinworms leave the intestine through the anus and deposit their eggs on the surrounding skin.

SYMPTOMS:

Pinworm infection (called enterobiasis or oxyuriasis) causes itching around the anus which can lead to difficulty sleeping and restlessness. Symptoms are caused by the female pinworm laying her eggs. Symptoms of pinworm infection usually are mild and some infected people have no symptoms.

SPREAD:

Pinworm infection is spread by the fecal-oral route, that is by the transfer of infective pinworm eggs from the anus to someone's mouth, either directly by hand or indirectly through contaminated clothing, bedding, food, or other articles.

Pinworm eggs become infective within a few hours after being deposited on the skin around the anus and can survive for 2 to 3 weeks on clothing, bedding, or other objects. People become infected, usually unknowingly, by swallowing (ingesting) infective pinworm eggs that are on fingers, under fingernails, or on clothing, bedding, and other contaminated objects and surfaces. Because of their small size, pinworm eggs sometimes can become airborne and ingested while breathing.

DIAGNOSIS:

Itching during the night in a child's perianal area strongly suggests pinworm infection. Diagnosis is made by identifying the worm or its eggs. Worms can sometimes be seen on the skin near the anus or on underclothing, pajamas, or sheets about 2 to 3 hours after falling asleep.

Pinworm eggs can be collected and examined using the “tape test” as soon as the person wakes up. This “test” is done by firmly pressing the adhesive side of clear, transparent cellophane tape to the skin around the anus. The eggs stick to the tape and the tape can be placed on a slide and looked at under a microscope. Because washing/bathing or having a bowel movement can remove eggs from the skin, this test should be done as soon as the person wakes up in the morning before they wash, bathe, go to the toilet, or get dressed. The “tape test” should be done on three consecutive mornings to increase the chance of finding pinworm eggs.

Because itching and scratching of the anal area is common in pinworm infection, samples taken from under the fingernails may also contain eggs. Pinworm eggs rarely are found in routine stool or urine samples.

BLOOD HELMINTHES:

WUCHERERIA BANCROFTI:

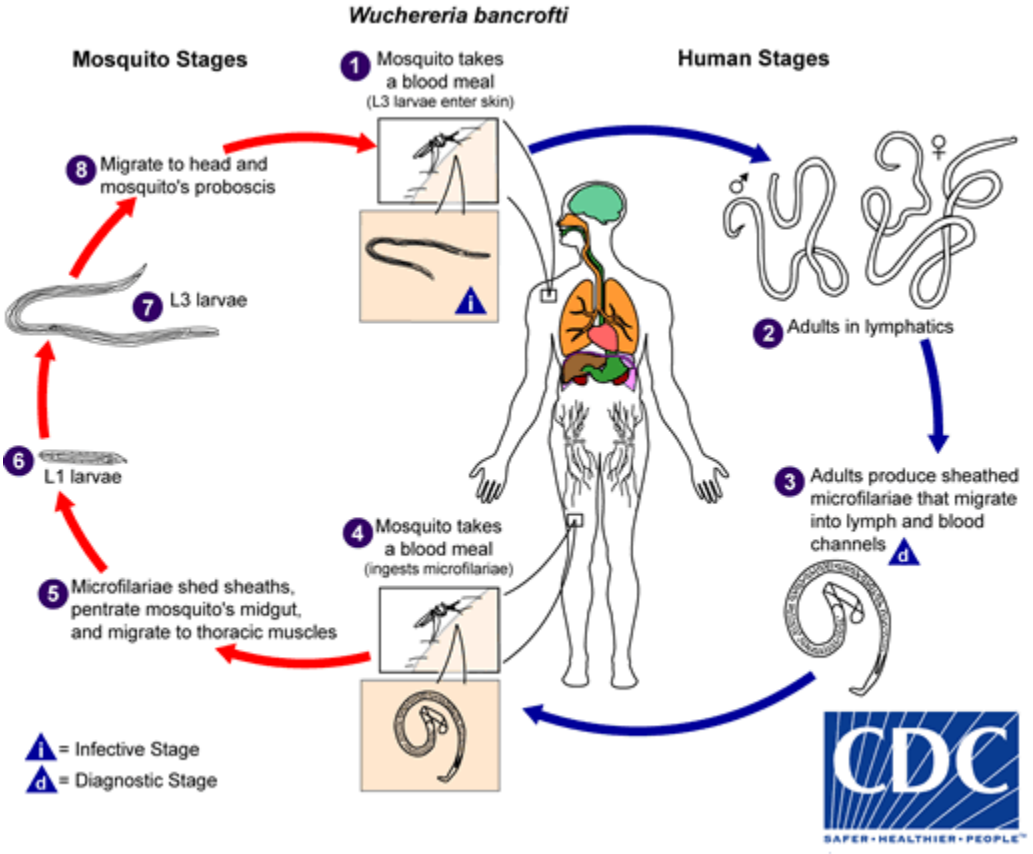

Figure 3.4: Life cycle of Lymphatic filariasis in human and mosquito

Different species of the following genera of mosquitoes are vectors of *W. bancrofti* filariasis depending on geographical distribution. Among them are: *Culex* (*C. annulirostris*, *C. bitaeniorhynchus*, *C. quinquefasciatus*, and *C. pipiens*); *Anopheles* (*A. arabinensis*, *A. bancroftii*, *A. farauti*, *A. funestus*, *A. gambiae*, *A. koliensis*, *A. melas*, *A. merus*, *A.*

punctulatus and *A. wellcomei*); *Aedes* (*A. aegypti*, *A. aquasalis*, *A. bellator*, *A. cooki*, *A. darlingi*, *A. kochi*, *A. polynesiensis*, *A. pseudoscutellaris*, *A. rotumae*, *A. scapularis*, and *A. vigilax*); *Mansonia* (*M. pseudotitillans*, *M. uniformis*); *Coquillettidia* (*C. juxtamansonia*). During a blood meal, an infected mosquito introduces third-stage filarial larvae onto the skin of the human host, where they penetrate into the bite wound ❶. They develop in adults that commonly reside in the lymphatics ❷. The female worms measure 80 to 100 mm in length and 0.24 to 0.30 mm in diameter, while the males measure about 40 mm by .1 mm. Adults produce microfilariae measuring 244 to 296 µm by 7.5 to 10 µm, which are sheathed and have nocturnal periodicity, except the South Pacific microfilariae which have the absence of marked periodicity. The microfilariae migrate into lymph and blood channels moving actively through lymph and blood ❸. A mosquito ingests the microfilariae during a blood meal ❹. After ingestion, the microfilariae lose their sheaths and some of them work their way through the wall of the proventriculus and cardiac portion of the mosquito's midgut and reach the thoracic muscles ❺. There the microfilariae develop into first-stage larvae ❻ and subsequently into third-stage infective larvae ❼. The third-stage infective larvae migrate through the hemocoel to the mosquito's prosboscis ❽ and can infect another human when the mosquito takes a blood meal ❶.

DISEASE AND SYMPTOMS:

Although the parasite damages the lymph system , most infected people have no symptoms and will never develop clinical symptoms. These people do not know they have lymphatic filariasis unless tested. A small percentage of persons will develop lymphedema. This is caused by fluid collection because of improper functioning of the lymph system resulting in swelling. This mostly affects the legs, but can also occur in the arms, breasts, and genitalia. Most people develop these symptoms years after being infected.

The swelling and the decreased function of the lymph system make it difficult for the body to fight germs and infections. These people will have more bacterial infections in the skin and lymph system. This causes hardening and thickening of the skin, which is called elephantiasis. Many of these bacterial infections can be prevented with appropriate skin hygiene as well as skin and wound care .

Men can develop hydrocele or swelling of the scrotum due to infection with one of the parasites that causes LF specifically *W. bancrofti.*

DIAGNOSIS:

The standard method for diagnosing active infection is the identification of microfilariae in a blood smear by microscopic examination. The microfilariae that cause lymphatic filariasis circulate in the blood at night (called nocturnal periodicity). Blood collection should be done at night to coincide with the appearance of the microfilariae, and a thick smear should be made and stained with Giemsa or hematoxylin and eosin. For increased sensitivity, concentration techniques can be used.

Serologic techniques provide an alternative to microscopic detection of microfilariae for the diagnosis of lymphatic filariasis. Patients with active filarial infection typically have elevated levels of antifilarial IgG4 in the blood and these can be detected using routine assays.

Because lymphedema may develop many years after infection, lab tests are most likely to be negative with these patients.

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DEPARTMENT OF BIOTECHNOLOGY

UNIT – 4 –VIROLOGY- SMB 3201

UNIT – 4

VIROLOGY

LABORATORY METHODS IN BASIC VIROLOGY

It is difficult to identify a virus in relation to the disease observed, or when conducting a retrospective study of a population to determine exposure to a virus,or when measuring the response of an individual to a vaccine. In these cases, indirect methods of measure are needed, such as measuring antibody response to the virus of interest. Several methods exist for this purpose. A few of the most commonly used methods include:

- Virus neutralization (VN)
- Hemagglutination inhibition (HI)
- Enzyme linked immunosorbent assay (ELISA)
- Indirect fluorescent antibody (IFA)
- Complement fixation (CF)
- Agar-gel immunodiffusion (AGID)
- Agar-gel precipitin (AGP)
- Latex agglutination (LA).

The principles of these assays are fundamentally the same, they depend upon antibody-antigen interactions and consist of a known virus or viral protein, a patient sample (usually serum), and an indicator. If antibodies are present in the patient's serum, they will bind to the virus. If no antibodies are present, no binding will occur. The indicator is observed to determine whether the sample is positive or negative for antibodies.

VirusNeutralization:

In the virus neutralization (VN) test, the sample of interest is incubated with the target virus and changes in cell culture are observed (called cytopathic effect, CPE). If the sample contains antibodies, it will prevent the virus from growing in the cell culture and no CPE will be observed. If no antibodies are present in the sample, the virus will grow and CPE will be observed.

HemagglutinationInhibition :

Certain viruses have a protein on their surface that interacts with red blood cells and is able to attach to them. This property is called hemagglutination and the surface protein of the virus is hemagglutinin. The inhibition or blocking of this activity is the basis of the hemagglutination inhibition (HI) test. The most well known virus with this property is the influenza virus. Like the virus neutralization (VN) test, the patient's serum sample is incubated with the virus of interest

but instead of growing the virus in cells, red blood cells are added to the virus-serum mix. If anti-bodies are present, the hemagglutination activity will be blocked; if no antibodies are present the virus will agglutinate (bind together). In this case the red blood cells are the indicator.

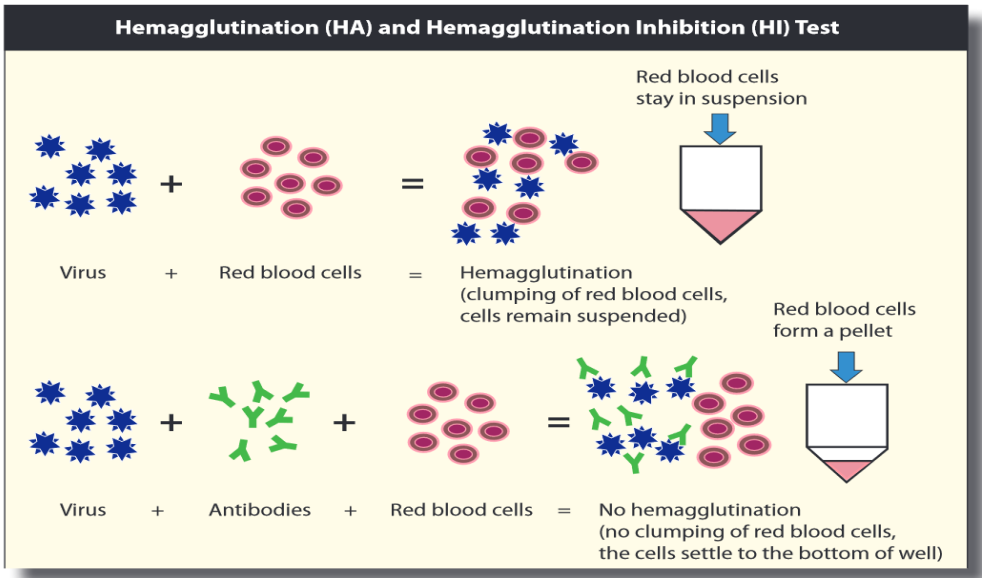

Figure 4: HA and HI test

Enzyme-Linked Immunosorbent Assay:
The enzyme-linked immunosorbent assay ELISA is a very popular technique due to the ease of use and low cost. The ELISA consists of plastic wells coated with either the antigen (virus) of interest or a protein specific to the antigen (virus) of interest. The unknown sample (serum) is allowed to bind to the coated well,an antibody labeled with an enzyme is applied, an indicator is added, and then a colour change is observed.The presence of colour indicates the presence of antibodies and the absence of colour indicates the absence of antibodies.

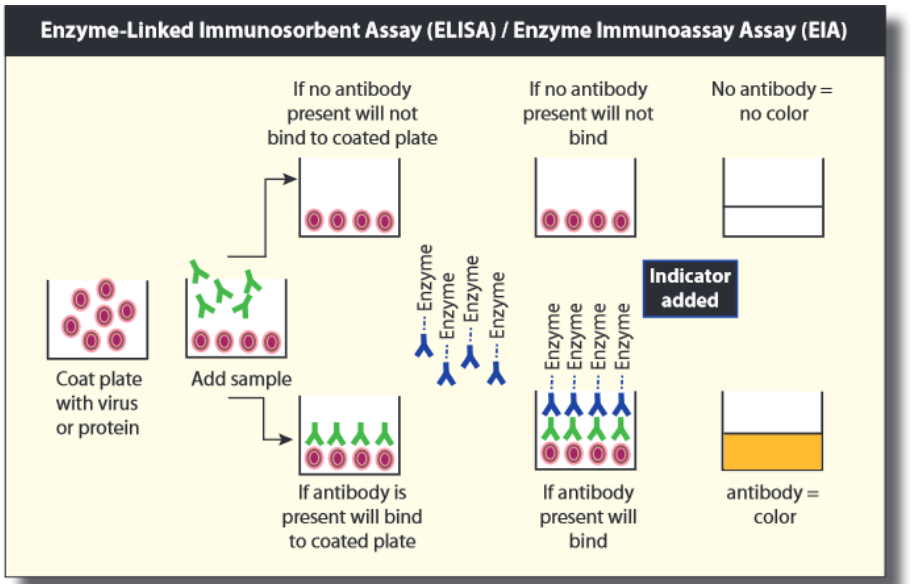

Figure 4.1: ELISA

Agar-Gel Immunodiffusions (AGID):

The agar-gel immunodiffusion (AGID), also referred to as an agar gel precipitin (AGP) test, involves the diffusion of virus and antibody through an agar (gelatin-like substance), which will form a line of identity where the antigen-antibody complexes form.

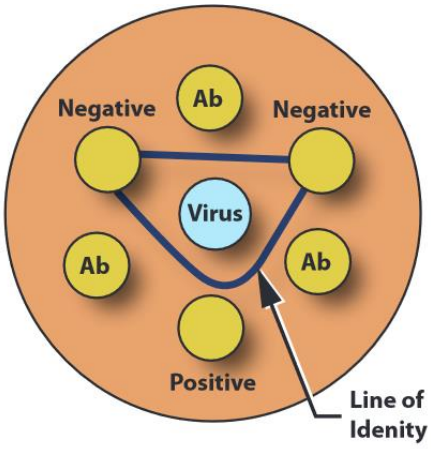

Figure 4.2: Schematic of an agar gel immunodiffusion (AGID) or agar gel precipitin (AGP) test. "Ab" represents a know antibody to the known virus in the middle

DETECTION OF VIRAL ANTIGEN

FLUROSCENT ANTIBODY:

A **direct fluorescent antibody** (**DFA** or **dFA**), also known as "**direct immunofluorescence**",[1] is an antibody that has been tagged in a **direct fluorescent antibody test**. Its name derives from the fact that it directly tests the presence of an antigen with the tagged antibody, unlike western blotting, which uses an indirect method of detection, where the primary antibody binds the target antigen, with a secondary antibody directed against the primary, and a tag attached to the secondary antibody.

Commercial DFA testing kits are available, which contain fluorescently labelled antibodies, designed to specifically target unique antigens present in the bacteria or virus, but not present in mammals (Eukaryotes). This technique can be used to quickly determine if a subject has a specific viral or bacterial infection.

In the case of respiratory viruses, many of which have similar broad symptoms, detection can be carried out using nasal wash samples from the subject with the suspected infection. Although shedding cells in the respiratory tract can be obtained, it is often in low numbers, and so an alternative method can be adopted where compatible cell culture can be exposed to infected nasal

wash samples, so if the virus is present it can be grown up to a larger quantity, which can then give a clearer positive or negative reading.

As with all types of fluorescence microscopy, the correct absorption wavelength needs to be determined in order to excite the fluorophore tag attached to the antibody, and detect the fluorescence given off, which indicates which cells are positive for the presence of the virus or bacteria being detected.

Direct immunofluorescence can be used to detect deposits of immunoglobulins and complement proteins in biopsies of skin, kidney and other organs. Their presence is indicative of an autoimmune disease. When skin not exposed to the sun is tested, a positive direct IF (the so-called Lupus band test) is an evidence of systemic lupus erythematosus.[2] Direct fluorescent antibody can also be used to detect parasitic infections, as was pioneered by Sadun .

SOLID PHASE IMMUNOASSAY:

The simplest type of solid-phase immunoassay that can be used, with peptides, is one in which the synthetic peptide is allowed to react, with antibodies, while still attached to the resin support used for its synthesis. By immunological testing at each step of the synthesis, it is possible to assess the contribution of each successive amino acid to the antigenic reactivity of the growing peptide. Solid-phase immunoassays can be used to compare the anti-genicity of a series of peptide analogs, for instance in experiments designed to assess the contribution of individual amino acids to the antigenic reactivity of the peptide. Solid-phase immunoassays, such as enzyme-linked immunosorbent assay (ELISA) and solid-phase radioimmunoassay (RIA) have become increasingly popular, and these assays are now commonly used for measuring the antigenic activity of synthetic peptides.

VIRAL SEROLOGY:

HEPATITIS

Viral hepatitis is liver inflammation due to a viral infection.[1][2] It may present in acute form as a recent infection with relatively rapid onset, or in chronic form.

The most common causes of viral hepatitis are the five unrelated hepatotropic viruses hepatitis A, B, C, D, and E. Other viruses can also cause liver inflammation, including cytomegalovirus, Epstein-Barr virus, and yellow fever. There also have been scores of recorded cases of viral hepatitis caused by herpes simplex virus.

Mode Of Transmission

Viral hepatitis is either transmitted through contaminated food or water (A, E) or via blood and body fluids (B, C). The viruses which get transmitted through water and food are mostly self-limited resulting in acute illness with full resolution. The blood borne viruses (B, C) can cause both acute and chronic liver disease and can be transmitted from mother to child during birth, through contact with body fluids during sex, unsafe injections and through unscreened blood transfusions.

The most common types of hepatitis can be prevented or treated. Hepatitis A and hepatitis B can be prevented by vaccination. Effective treatments for hepatitis C are available but costly.

In 2013, about 1.5 million people died from viral hepatitis, most commonly due to hepatitis B and C. East Asia, in particular Mongolia, is the region most affected

Hepatitis viruses

The most common cause of hepatitis is viral. Although the effects of various viruses are all classified under the disease hepatitis, these viruses are not all related.

Hepatitis viruses						
	Hepatitis A virus (HAV)	**Hepatitis B virus (HBV)**	**Hepatitis C virus (HCV)**	**Hepatitis D virus (HDV)**	**Hepatitis E virus (HEV)**	**Hepatitis G virus (HGV)**
Viral species	*Hepatovirus A*	*Hepatitis B virus*	*Hepacivirus C*	*Hepatitis delta virus*	*Orthohepevirus A*	*Also known as Human Pegvirus (HPgV) and as GB virus C (GBV-C)*
Viral family	*Picornaviridae*	*Hepadnaviridae*	*Flaviviridae*	*Incertae sedis*	*Hepeviridae*	*Flaviviridae*
Genome	(+)ssRNA	dsDNA-RT	(+)ssRNA	(−)ssRNA	(+)ssRNA	(+)ssRNA
Antigens		HBsAg, HBeAg	Core antigen	Delta antigen		
Transmission	Enteral	Parenteral	Parenteral	Parenteral	Enteral	Parenteral
Incubation period	20–40 days	45–160 days	15–150 days	30–60 days	15–60 days	14-20 days
everity/Chronicity[6]	Mild; acute	Occasionally severe; 5–10% chronic	Subclinical; 70% chronic	Exacerbates symptoms of HBV; chronic with HBV	Mild in normal patients; severe in pregnant women; acute	
Vaccine	2 injections; at least 20 years of protection[7]	3 injections; lifetime protection	None available	None available, but not considered necessary; Hep B vaccine	Investigational (approved in China)	

				provides protection[8]		

Table 4: Hepatitis Virus

SEROLOGY:

Hepatitis serology (HepA, HepB, HepC) is a relatively complex set of tests to determine past infection, current infection, immunity and infectivity relating to the Hepatitis viruses. These viruses all affect the liver, but behave quite differently from each other.

Hepatitis serology is a blood test which requires a few millilitres of blood from a vein. Any of the three common hepatitis viruses can be tested for individually – for example only Hepatitis B serology – depending on the reasons for the test.

Hepatitis serology may be requested by your doctor for a number of reasons, including:

- Screening test to determine hepatitis status, for example prior to childbirth, dialysis or a surgical procedure.
- Investigation of jaundice (yellow skin or eyes) or abnormal Liver Function Tests (LFTs).
- Checking immunity to Hepatitis B, for healthcare workers or those with occupational (or non-occupational) exposure to body fluids with infective potential – eg a needlestick injury.

Hepatitis serology results will usually give an indication of previous infection, as well as current infection, and give an indication of immunity (antibodies) to future exposure, in the case of Hepatitis B.

Interpretation of these results can be quite complex, but the laboratory performing the test usually gives a short comment or explanation on the report.

LIFECYCLE:

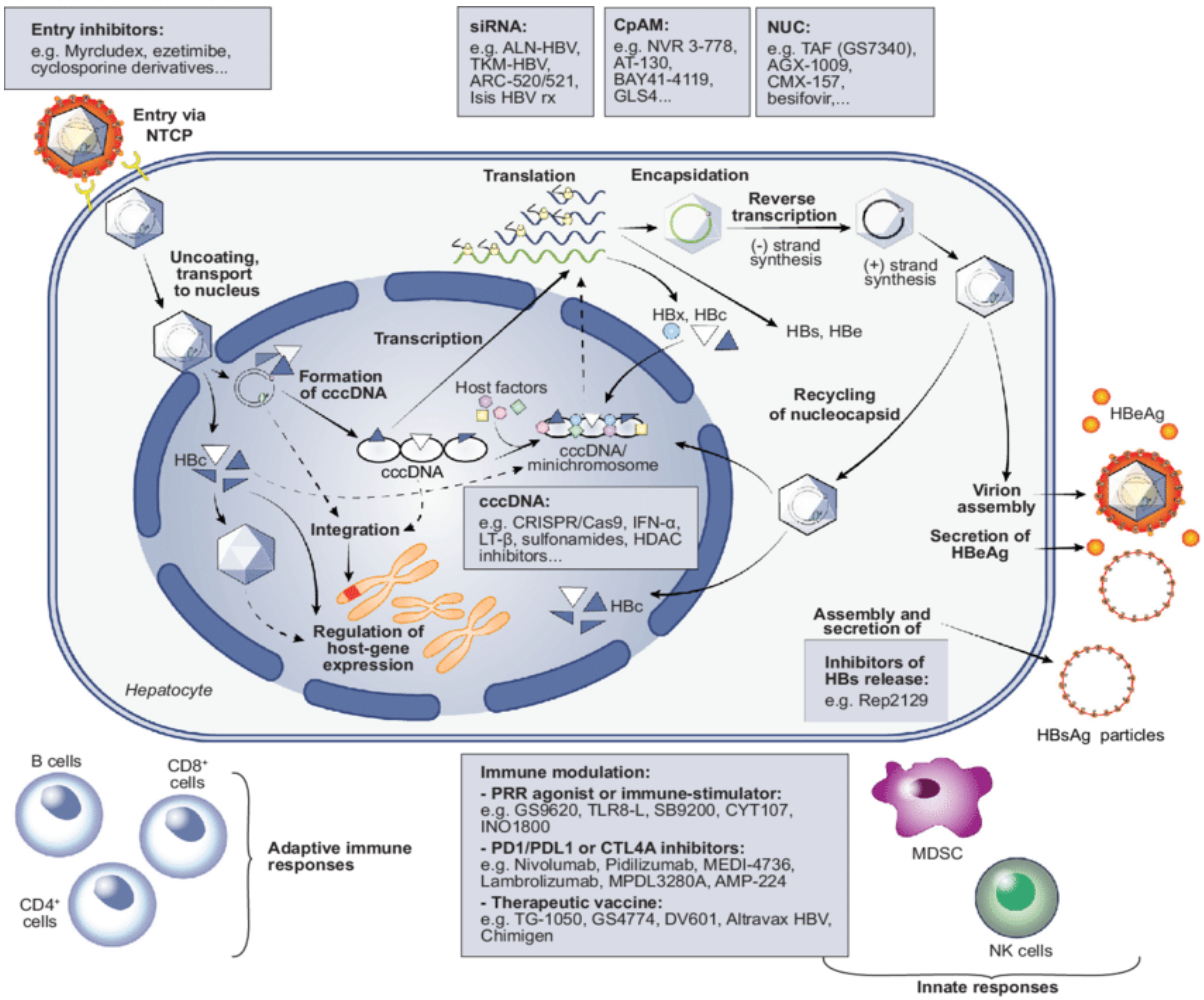

Figure 4.3: HBV life cycle and main classes of antivirals in development

AIDS:

Human immunodeficiency virus infection and acquired immunodeficiency syndrome (**HIV/AIDS**) is a spectrum of conditions caused by infection with the human immunodeficiency virus (HIV), a retrovirus. Following initial infection an individual may not notice any symptoms, or may experience a brief period of influenza-like illness. Typically, this is followed by a prolonged incubation period with no symptoms. If the infection progresses, it interferes more with the immune system, increasing the risk of developing common infections such as tuberculosis, as well as other opportunistic infections, and tumors which are otherwise rare in people who have normal immune function. These late symptoms of infection are referred to as acquired immunodeficiency syndrome (AIDS). This stage is often also associated with unintended weight loss.

HIV is spread primarily by unprotected sex (including anal and oral sex), contaminated blood transfusions, hypodermic needles, and from mother to child during pregnancy, delivery, or breastfeeding. Some bodily fluids, such as saliva, sweat and tears, do not transmit the virus.

Methods of prevention include safe sex, needle exchange programs, treating those who are infected, as well as both pre- and post-exposure prophylaxis. Disease in a baby can often be prevented by giving both the mother and child antiretroviral medication. Known as the Berlin Patient and the London Patient, two individuals have been reported cured of AIDS and the NIH and Gates Foundation pledged $200 million focused on developing a global cure for AIDS. While there is not yet a broadly available cure or vaccine, antiretroviral treatment can slow the course of the disease and may lead to a near-normal life expectancy. Treatment is recommended as soon as the diagnosis is made. Without treatment, the average survival time after infection is 11 years.

In 2020, about 37 million people worldwide were living with HIV and 680,000 deaths had occurred in that year. An estimated 20.6 million of these live in eastern and southern Africa. Between the time that AIDS was identified (in the early 1980s) and 2020, the disease has caused an estimated 36 million deaths worldwide. HIV/AIDS is considered a pandemic—a disease outbreak which is present over a large area and is actively spreading.

HIV made the jump from other primates to humans in west-central Africa in the early-to-mid 20th century. AIDS was first recognized by the United States' Centers for Disease Control and Prevention (CDC) in 1981 and its cause—HIV infection—was identified in the early part of the decade.

HIV/AIDS has had a large impact on society, both as an illness and as a source of discrimination. The disease also has large economic impacts. There are many misconceptions about HIV/AIDS, such as the belief that it can be transmitted by casual non-sexual contact. The disease has become subject to many controversies involving religion, including the Catholic Church's position not to support condom use as prevention. It has attracted international medical and political attention as well as large-scale funding since it was identified in the 1980s.

SEROLOGY:

HIV/AIDS is diagnosed via laboratory testing and then staged based on the presence of certain signs or symptoms. HIV screening is recommended by the United States Preventive Services Task Force for all people 15 years to 65 years of age, including all pregnant women. Additionally, testing is recommended for those at high risk, which includes anyone diagnosed with a sexually transmitted illness. In many areas of the world, a third of HIV carriers only discover they are infected at an advanced stage of the disease when AIDS or severe immunodeficiency has become apparent.

HIV testing

Figure 4.4: HIV Rapid Test being administered

Most people infected with HIV develop specific antibodies (i.e. seroconvert) within three to twelve weeks after the initial infection. Diagnosis of primary HIV before seroconversion is done by measuring HIV-RNA or p24 antigen. Positive results obtained by antibody or PCR testing are confirmed either by a different antibody or by PCR.

Classifications

Two main clinical staging systems are used to classify HIV and HIV-related disease for surveillance purposes: the WHO disease staging system for HIV infection and disease,[1] and the CDC classification system for HIV infection. The CDC's classification system is more frequently adopted in developed countries. Since the WHO's staging system does not require laboratory tests, it is suited to the resource-restricted conditions encountered in developing countries, where it can also be used to help guide clinical management. Despite their differences, the two systems allow a comparison for statistical purposes.

The World Health Organization first proposed a definition for AIDS in 1986. Since then, the WHO classification has been updated and expanded several times, with the most recent version being published in 2007. The WHO system uses the following categories:

- Primary HIV infection: May be either asymptomatic or associated with acute retroviral syndrome
- Stage I: HIV infection is asymptomatic with a $CD4^+$ T cell count (also known as CD4 count) greater than 500 per microlitre (µl or cubic mm) of blood. May include generalized lymph node enlargement.

- Stage II: Mild symptoms, which may include minor mucocutaneous manifestations and recurrent upper respiratory tract infections. A CD4 count of less than 500/µl.
- Stage III: Advanced symptoms, which may include unexplained chronic diarrhea for longer than a month, severe bacterial infections including tuberculosis of the lung, and a CD4 count of less than 350/µl
- Stage IV or AIDS: severe symptoms, which include toxoplasmosis of the brain, candidiasis of the esophagus, trachea, bronchi, or lungs, and Kaposi's sarcoma. A CD4 count of less than 200/µl[27]

The United States Center for Disease Control and Prevention also created a classification system for HIV, and updated it in 2008 and 2014. This system classifies HIV infections based on CD4 count and clinical symptoms, and describes the infection in five groupsIn those greater than six years of age it is:

Stage 0: the time between a negative or indeterminate HIV test followed less than 180 days by a positive test.

- Stage 1: CD4 count ≥ 500 cells/µl and no AIDS-defining conditions.
- Stage 2: CD4 count 200 to 500 cells/µl and no AIDS-defining conditions.
- Stage 3: CD4 count ≤ 200 cells/µl or AIDS-defining conditions.
- Unknown: if insufficient information is available to make any of the above classifications.

For surveillance purposes, the AIDS diagnosis still stands even if, after treatment, the $CD4^+$ T cell count rises to above 200 per µL of blood or other AIDS-defining illnesses are cured.

LIFE CYCLE:

The seven stages of the HIV life cycle are: 1) binding, 2) fusion, 3) reverse transcription, 4) integration, 5) replication, 6) assembly, and 7) budding.

To understand each stage in the HIV life cycle, it helps to first imagine what HIV looks like.

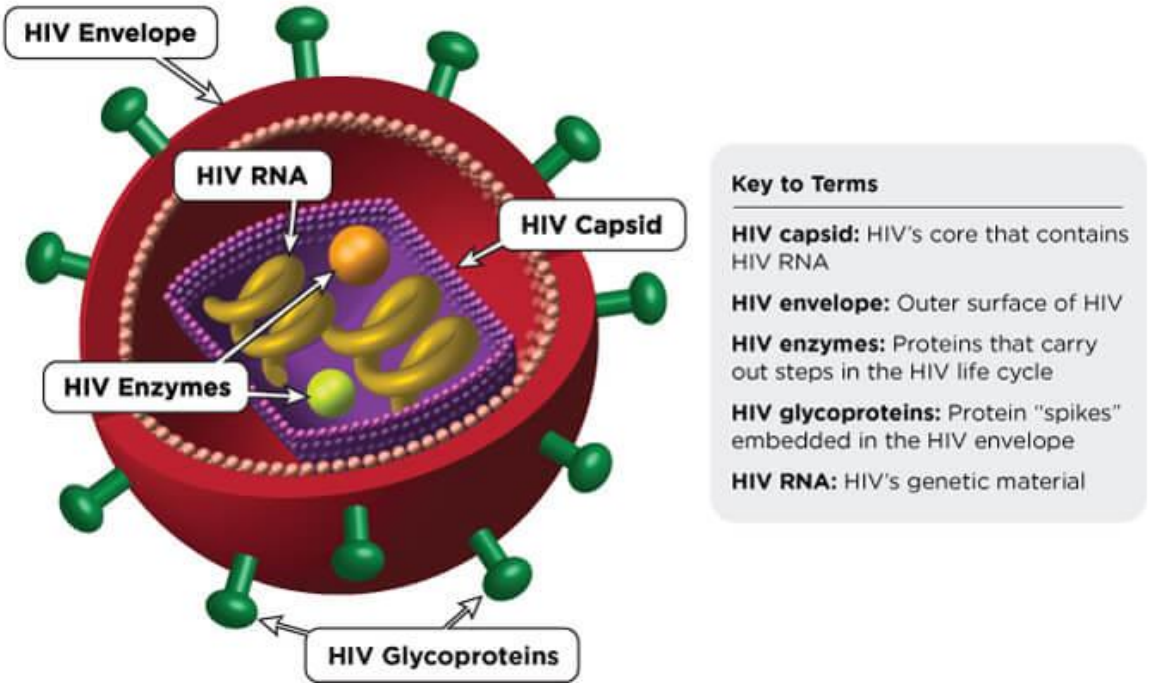

Figure 4.5: HIV Structured Labelled

Now, follow each stage in the HIV life cycle as HIV attacks a CD4 cell and uses the machinery of the cell to multiply.

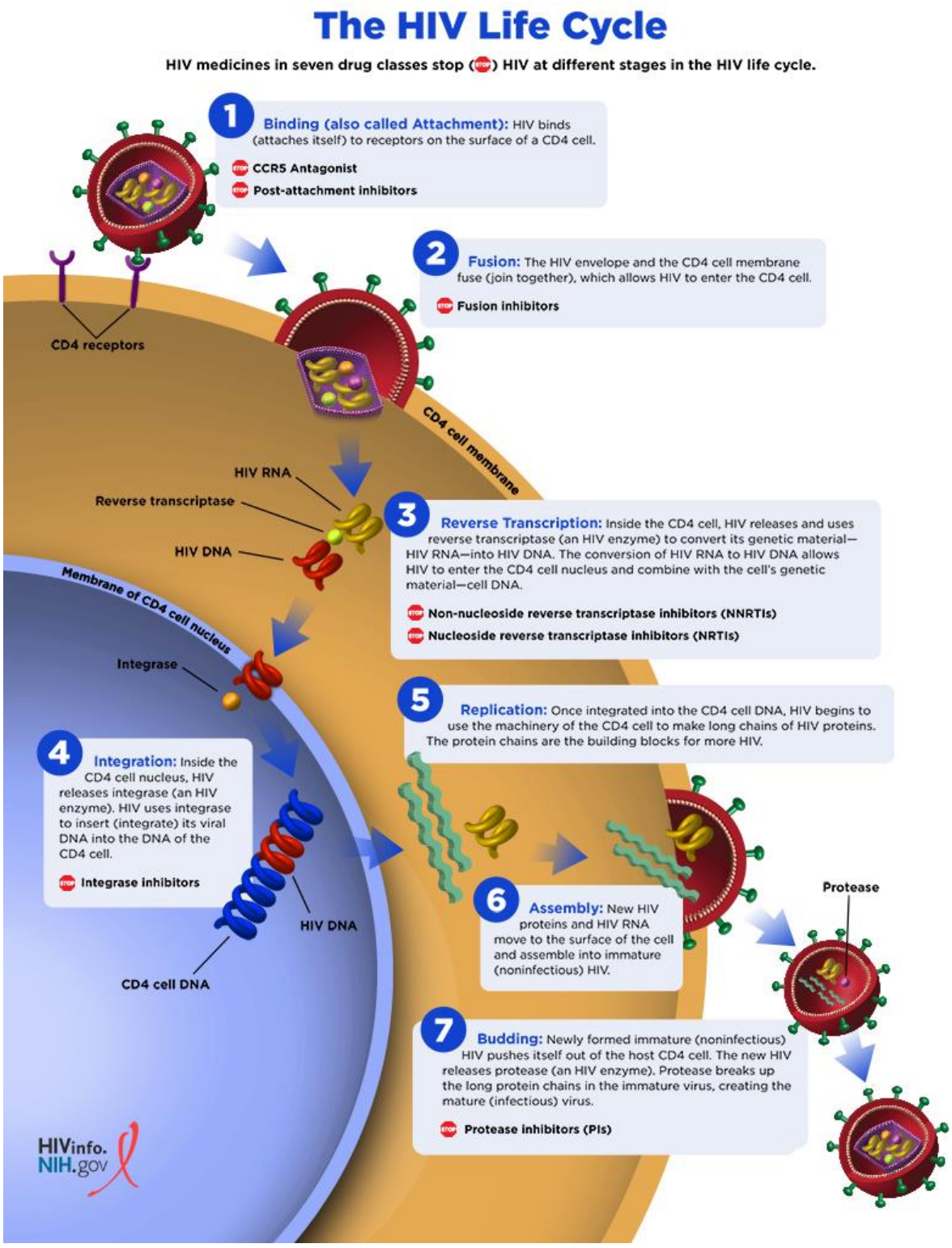

Figure 4.6: HIV Life cycle

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DEPARTMENT OF BIOTECHNOLOGY

UNIT – 5 – VIRAL CULTURE – SMB 3201

Unit 5 viral culture

Virus

- Viruses are extremely small infect agents that invade cells of all types.
- Viruses are obligate intracellular parasites so they depend on host for their survival.
- They cannot be grown in non -living culture media or on agar plates alone, they must require living cells to support the replication.

PURPOSE OF VIRUS CULTIVATION

The primary purposes of viral cultivation are:

1. To isolate and identify viruses in clinical specimens
2. To prepare viruses for vaccines
3. To do detailed research on viral structure, cycles, genetics, and effects on host cells.

VIRUS CULTIVATION SYSTEMS

- Tissue culture system
- Embryonated eggs system
- Whole animal systems

a) Natural host
b) Experimental animals
c) Transgenic

TISSUE CULTURE SYSTEM

Use isolated cell from animal that are cultured *invitro.*
It is the preferred type of growth medium for viruses.

- Three discoveries greatly enhanced the usefulness of cell cultures for virologist and scientists

1. The discovery and use of antibiotics made it possible to prevent bacterial and fungal contamination
2. The discovery of proteolytic enzymes (e.g. trypsin) can free animal cells from surrounding tissues without injuring freed cells
3. This technique has also become possible by the development of growth media for animal cells.

STEPS IN TISSUE CULTURE TECHNIQUE

Cultivating animal viruses using tissue culture technique involves following three main steps:

1. Monolayer preparation
2. Clonal cell line preparation
3. Infection with virus

The first two steps are summarised with the notes on cell culture in the next slides.

Monolayer and clonal cell line preparation

CELL CULTURE

Cell culture refers to the removal of cells from an animal or plant and their subsequent growth in a favourable artificial environment.

The cells may be removed from the tissue directly and disaggregated by enzymatic or mechanical means before cultivation, or they may be derived from a cell line or cell strain that has already been already established.

Can be classified under the following cell lines.

i) Primary culture
ii) Diploid cell lines
iii) Continuous cell lines

PRIMARY CULTURE

Primary culture refers to the stage of the culture after the cells are isolated from the tissue and proliferated under the appropriate conditions until they occupy all of the available substrate (i.e., reach confluence) (e.g. Primary monkey kidney, mice fibroblasts)

Heterogeneous – many cell types
-Technical hassle
-5 to 20 cell divisions
-Normal chromosome number
-Contact inhibition
-need constant source
-Closest to animal

DIPLOID CELL LINES

After the first subculture, the primary culture becomes known as a Diploid cell line or subclone.
E.g(human fetal lung)

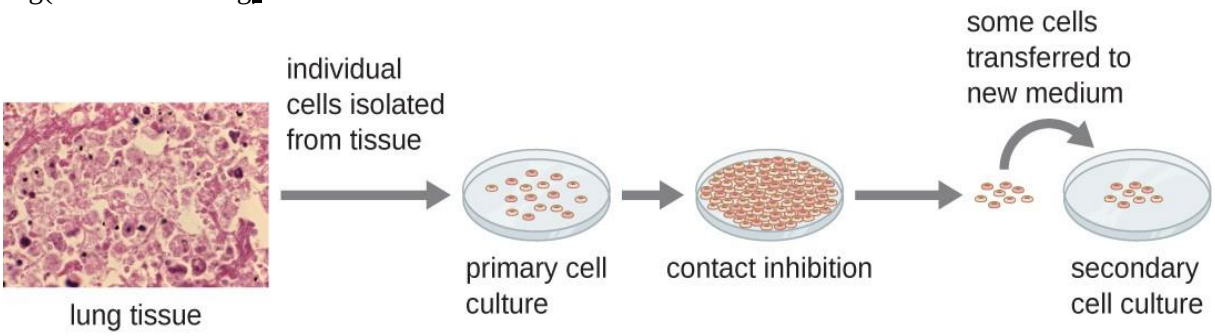

Figure 5: Primary cell cultures grow attached to the surface of the culture container. Contact inhibition slows the growth of the cells once they become too dense and begin touching each other. At this point, growth can only be sustained by making a secondary culture.

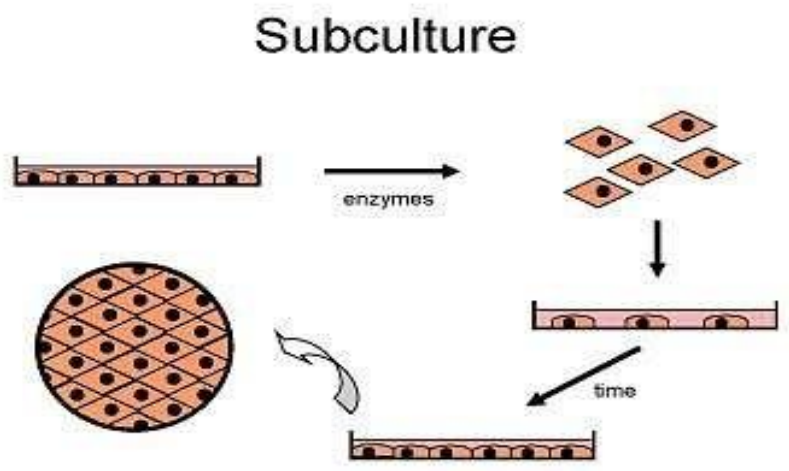

Figure 5.1: Subculture

CONTINUOUS CELL LINES

- When a finite cell line undergoes transformation and acquires the ability to divide indefinitely, it becomes a continuous cell line.
- Become immortal through a process called transformation.
- Can occur spontaneously or can be chemically or virally induced.

-Immortal
-Most homogeneous
-Genetically weird – Different from animal
-Hassle free
-Suspension or monolayer
-Aneuploid- abnormal in chromosome morphology and number, Grow rapidly.
(e.g.various types of cancer cells - HeLa cells, Hep 2 cells, or human amnion cells, continual monkey kidney cell line, dog kidney cell line, etc.).

- continuous cell lines, usually derived from transformed cells or tumors, are often able to be subcultured many times or even grown indefinitely (in which case they are called immortal).
- Continuous cell lines may not exhibit anchorage dependency (they will grow in suspension) and may have lost their contact inhibition.
- As a result, continuous cell lines can grow in piles or lumps resembling small tumor growth

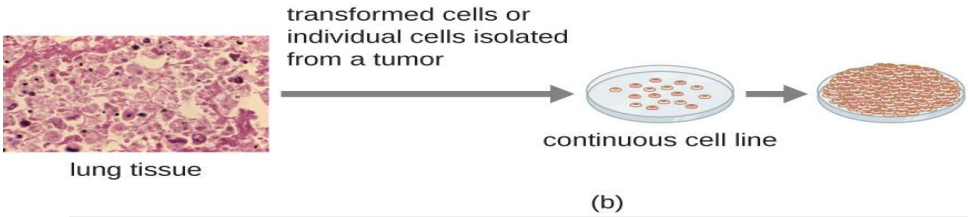

Figure 5.2: Continuous cell cultures are not affected by contact inhibition. They continue to grow regardless of cell density. (credit "micrographs": modification of work by Centers for Disease Control and Prevention)

Infection with virus

The clonal cell lines suspended in suitable media are infected with any desired virus which replicates inside the multiplying cells. If the virus is virulent, they cause lysis of cells and virus particles are released in the surrounding medium.

These newly produced virus particles (virions) infect the adjacent cells. As a result localized areas of cellular destruction and lysis (called plaques) often are formed

CULTURE CONDITIONS

Culture conditions vary widely for each cell type.

The artificial media invariably consist of a substrate or medium that supplies the essential nutrients (amino acids, carbohydrates, vitamins, minerals), growth factors, hormones, and gases (O2, CO2).

- It also regulates the physico chemical environment (pH, osmotic pressure, temperature).
- Most cells are anchorage dependent and must be cultured while attached to a solid or semi-solid substrate(adherent or monolayer culture),

- Others can be
- grown floating in the culture (suspension culture)

DISADVANTAGES OF CELL CULTURES

- Long period (up to 4 weeks) required for result.
- Often very poor sensitivity, sensitivity depends on a large extent on the condition of the specimen.
- Susceptible to bacterial contamination
- Many viruses will not grow in cell culture e.g. Hepatitis B, Diarrhoeal viruses, parvovirus, papillomavirus

EMBRYONATED EGGS

- The Embryonated hen's egg was first used for cultivation of viruses by Good Pasteur and Burnet (1931).
- Cultivation of viruses in organized tissues like chick embryo necessitates a different type of approach..
- For all practical purposes they all themselves behave as tissue cultures.
- The process of cultivation of viruses in embryonated eggs depend on the type of egg which is used.
- The egg used for cultivation must be sterile and the shell should be intact and healthy.
- Use embryonated chicken, duck or turkey for inoculation of viral suspension
- Are used especially for the influenza viruses isolation.
  7 - 10 days old embryonated eggs are used.
- The egg must be cleaned, the shell decontaminated with a disinfectant and checked in ovoscope if it is alive
  Ovoscope is the equipment used for candling

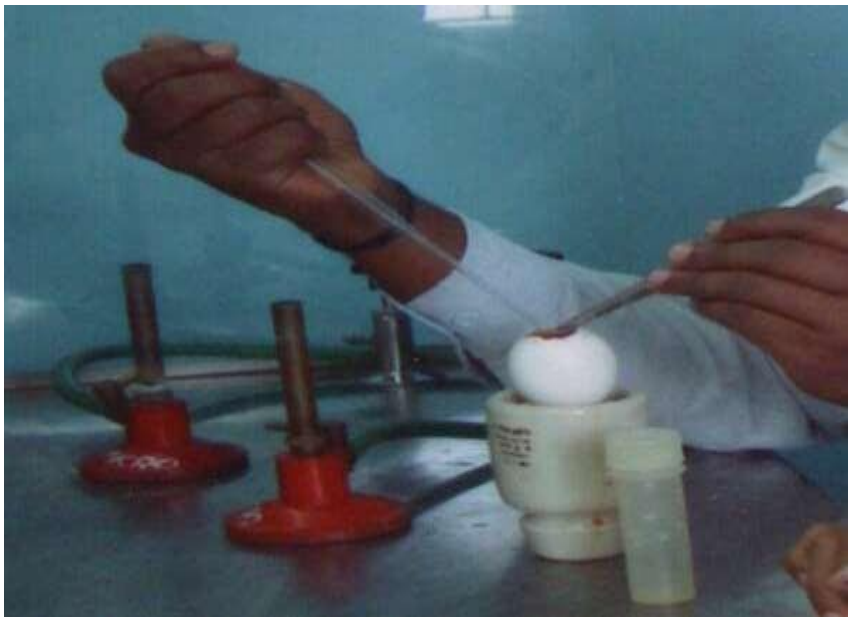

Figure 5.3: Egg Candling Device

DETECTION OF VIRAL GROWTH

The signs of viral growth include

I) death of the animal

I) defects in animal development.

The infected animal tissue can be prepared for examination with an electron microscope

VIRAL QUANTIFICATION

Virus quantification involves counting the number of viruses in a specific volume to determine the virus concentration.

It is utilized in both (R&D) in commercial and academic laboratories as well as production situations where the quantity of virus at various steps is an important variable

The methods used include but not limited to:

i) Hemagglutination assay

ii) Plaque assay

iii) iii)$TCID_{50}$

HEMAGGLUTINATION ASSAY

A direct method to titre virus.

Based on the ability of some viruses to agglutinate RBCs

Virus is tittered by making serial two fold dilutions of the virus and determining the highest dilution of virus that causes agglutination of RBCs.

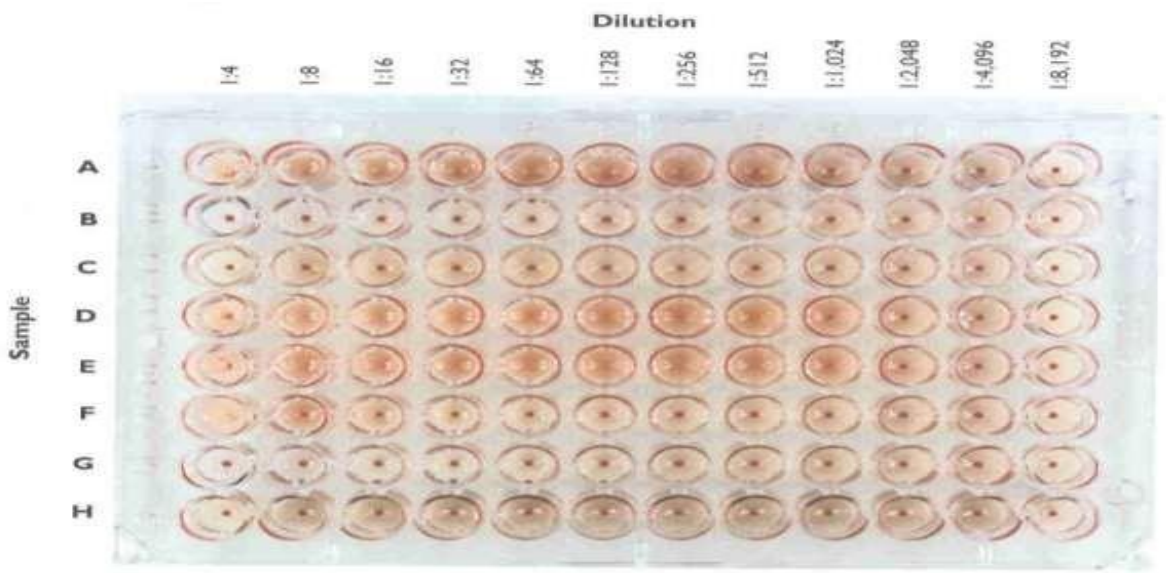

Figure 5.4: Two-fold dilutions of samples of different influenza viruses (A-H)

PLAQUE ASSAY

When cells grow as monolayers, they can be used to quantify the number of viruses using plaque assay.

- The virus is serially diluted in a liquid medium.
- For each dilution a set amount is added to separate plate containing monolayer of tissue culture cells and the viruses in that solution are allowed to attach to the tissue culture cells.
- After attachment has been allowed to occur, a semi solid medium is added to restrict the movement of new viruses produced so that only adjacent cells will be infected.

$TCID_{50}$ (Tissue Culture Infectious Dose)

$TCID_{50}$ is the measure of infectious virus titer.

- This endpoint dilution assay quantifies the amount of virus required to kill 50% of infected hosts or to produce a cytopathic effect in 50% of inoculated tissue culture cells
- This assay may be more common in clinical research application

where the lethal dose of virus must be determined or if the virus does not form plaques.

- When used in the context of tissue culture, host cells are plated and

serial dilutions of the virus are added. After incubation, the percentage of cell death (i.e. infected cells) is manually observed and recorded for each virus dilution, and results are used to mathematically calculate a $TCID_{50}$ result.

Isolation of Viruses

- Viruses require a living host cell for replication. Infected host cells (eukaryotic or prokaryotic) can be cultured and grown, and then the growth medium can be harvested as a source of virus.
- Virions in the liquid medium can be separated from the host cells by either centrifugation or filtration.
- Filters can physically remove anything present in the solution that is larger than the virions; the viruses can then be collected in the filtrate

ELISA

INTRODUCTION

- ELISA, or enzyme -linked immunosorbent assay,are quantitative immunological producers in which the Ag-Ab reaction is monitored by enzyme measurements
- The term ELISA was first used by Engvall & perlma in 1971
- The term ELISA test, or the enzyme immunosorbent (ELA),was the first screening test commonly employed for HIV. It has a high sensitivity

Principle of ELISA

- use an enzyme to detect the binding of antigen (Ag) antibody (Ab)
- The enzyme converts a colourless substrate (chromogen)to coloured product, indicating the presence Ag :Ab binding
- An ELISA can be used to detect either the presence of antigens or antibodies in a sample depending how the test is designed
- ELISA was developed in 1970 and become rapidly accepted

Materials needed

- Testing sample
- Antibody (1,2)/ antigen
- Polystyrene microtiter plate
- Blocking buffer

- Washing buffer
- Substrate
- Enzyme

ANTIGEN (Ag)

- Any molecule that induces production of antibodies in the body of an animal is called antigen
- Any "thing " ,foreign to the immune system .e.g. bacteria, virus ,(or their parts ,pollen ,etc .
- protein molecule
- carbohydrate molecule
- microorganisms
- Allergens
- Virus etc

Antibody (Ab)

proteins produced by the immune system which help defend against antigen

specimen sample for ELISA

- serum
- CSF
- Sputum
- Urine
- Semen
- Supernatant of culture
- Stool

Enzymes used in ELISA

- Horseradish peroxidase (most commonly used)
- Alkaline phosphatase
- Beta –galactosidase
- Lactoperoxidase
- Tetra Methyl
- In case of peroxidase the substrate hydrogen peroxidase is is converted into water and o2 in the presence of electron donors
- Oxidation of diaminobenzidine produces dark brown colour while that of 4-chloronaphthol which themselves oxidized in the reaction)
- Oxidation of diaminobenzidine produces dark brown color while that of 4-chloromaphthol yields purple color which is the basis of ELISA

ENZYME SUBSTRATE

- Initially the substrate should be colourless
- After degradation by the enzyme it should be strongly colored or fluorescent

Types of ELISA

- Indirect ELISA
- Sandwhich ELISA
- Competetive ELISA

Indirect ELISA

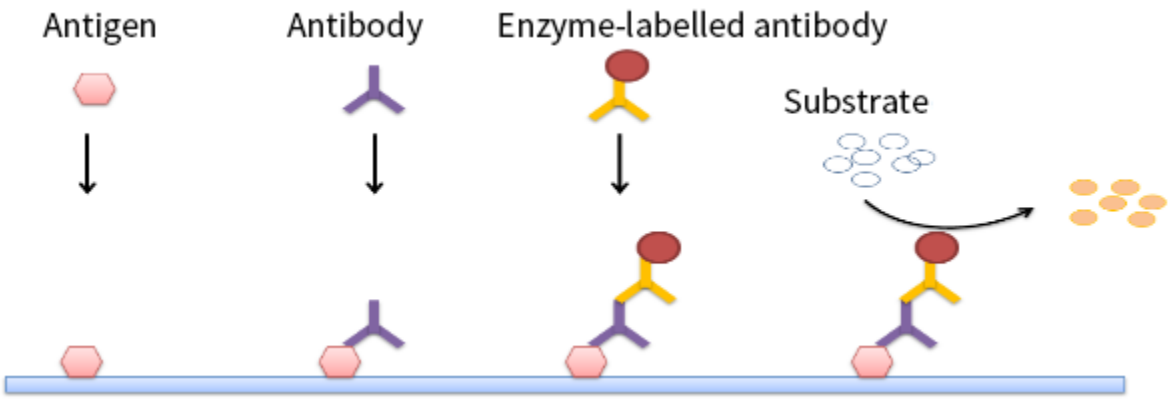

Figure 5.5: Indirect ELISA

Sandwich ELISA

- Antigen such as tumor markers ,hormones and serum proteins may be determined
- Antigen in the sample binds with the capture antibody on the microwell and becomes immobilized
- The antibody of the enzyme conjugate binds with the immobilized antigen to form a sandwich of antibody -antigen /enzyme bound to the microwell

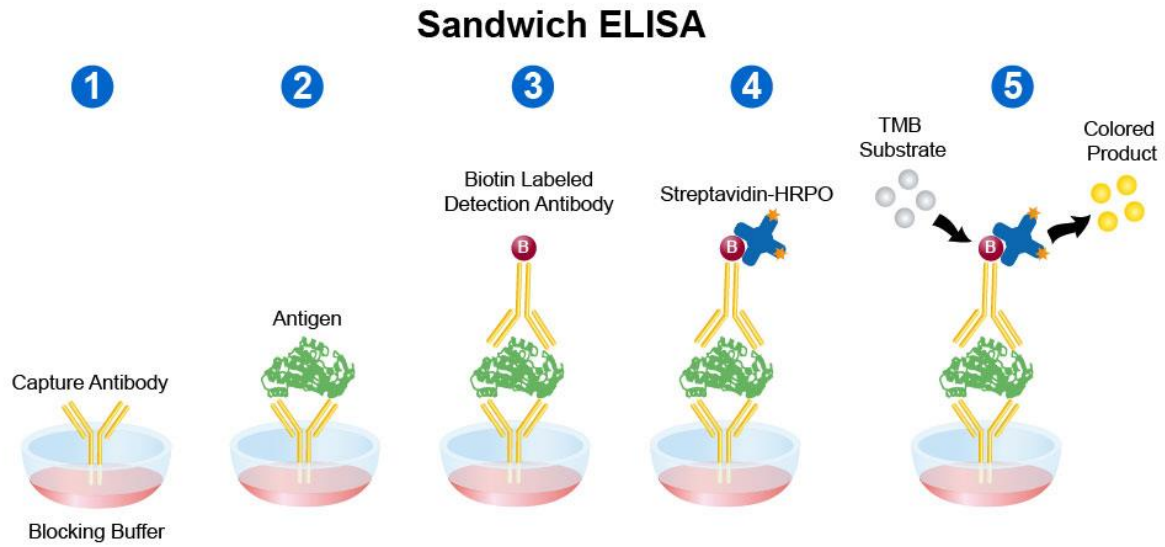

Figure 5.6: Sandwich ELISA

Competitive ELISA

- Used to determine small molecule antigen (T3,T4 progesterone etc)
- Antibody coated microwell
- Serum antigen labelled antigen added together -competition
- Antibody -antigen -enzyme complex bound is inversely related to the concentration of antigen present in the sample
- The bound enzyme conjugate reacts with the chromogenic substarte added to produce a color reaction

- Increased serum antigen results in reduced binding to the antigen -enzyme activity and color (yellow)formation
- Substrate serum antigen result in reduced binding of the antigen -enzyme conjugate with the capture antibody producing less enzyme activity and color (yellow) formation

Importance of incubation step

- During the test performance incubation time and mentioned temperature is must require For the proper binding with conjugate and color development of substrate .
- Importance of washing ; for the removal of any unbound antibody /antigen proper washing and taping is required other wise we get the incorrect result
- So incubation and washing is mush important for good result

So incubation and washing is much important for good result

ELISA plates

- Microtitre wells
- Generally 96 wells
- Marked on one side alphabetically
- Numerically on the other side
- Comes with the kit

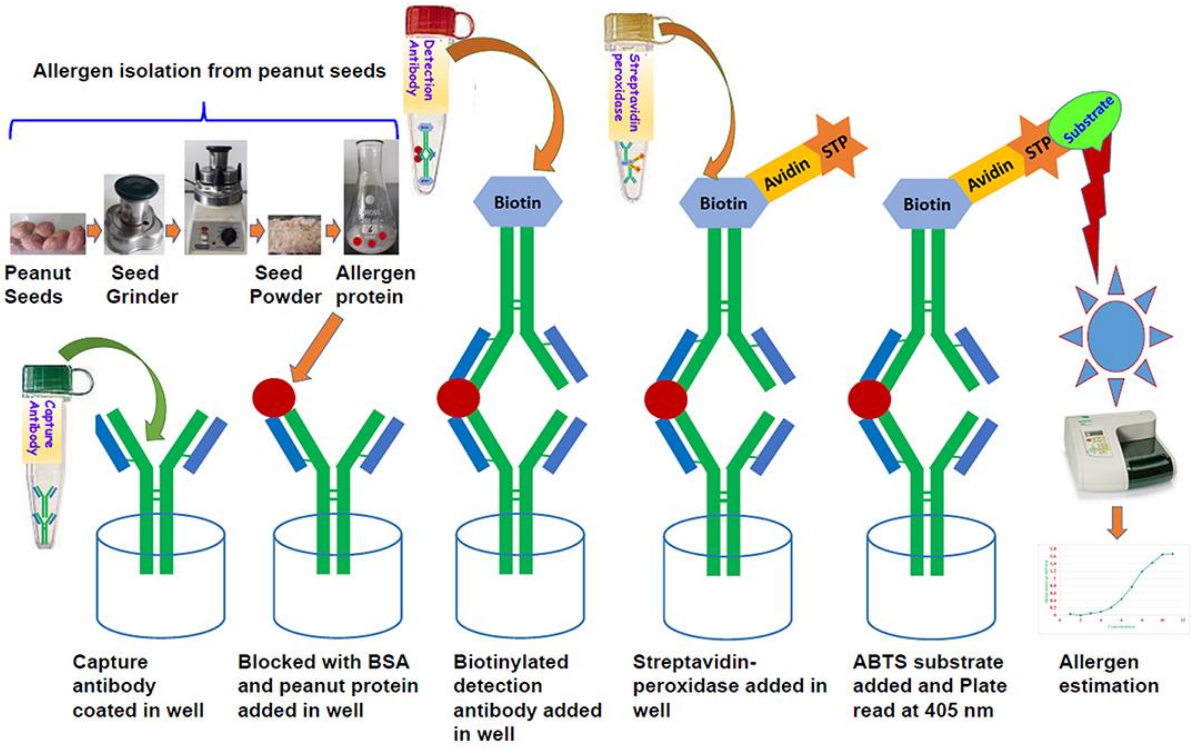

Figure 5.7: Schematic diagram showing the protocol for allergen estimation in peanut seed through sandwich ELISA

Advantages of ELISA

- Reagents are relatively cheap and have a long shelf life
- ELISA is highly specific and sensitive
- No radiation hazards occur during labelling or disposal of waste
- ELISA can be used variety of infections

Disadvantage of ELISA

- Measurement of enzyme activity can be more complex than measurement of activity of some type of radioisotopes
- Enzyme activity may be affected by plasma constituents
- Kits are commercially available but not cheap
- Very specific to a particular antigen

RIA

INTRODUCTION

- Radioimmunoassay is a very sensitive in vitro assay technique used to measure concentration of antigen by use of antibodies
- The RAST test is an example of radioimmunoassay .it is used to detect the causative allergen for an allergy
- To perform a radioimmunoassay ,a known quantity of an antigen is made radioactive ,frequently by labelling it with gamma radioactive isotopes of iodine attached to tyrosine
- To perform a radioimmunoassay, a known quantity of an antigen is made radioactive ,frequently by labelling it with gamma -radioactive isotopes of iodine attached to tyrosine

Principle of radioimmunoassay

- Uses an immune reaction (antigen -antibody reaction) to estimate

a ligand

- Unbound Ag* and Ag washed out
- Radioactivity of bound residue measured
- Ligand concentration is inversely related to radioactivity
  (Ag :ligand to be measured ;Ag*radiolabelled ligand)

Advantages and Disadvantage of RIA

- Highly specific :Immune reaction are specific
- High sensitivity : Immune reaction are sensitive

Disadvantages

- Radiation hazards uses radiolabelled reagents
- Requires specially trained persons
- Labs requires special license to handle radioactive

Requirements for RIA

- Preparation and characterisation of the antigen
- Radiolabelling of the antigen
- Preparation of the specific antibody
- Development of assay system

Preparation and Radiolabelling of the Antigen

- Antigens prepared by
  Synthesis of the molecule
  Isolation from natural sources
- Radiolabelling (Tagging procedure)
  Antigens are tagged
  Tagging should not affect antigen specificity

Preparation of the specific antibody

- Antigen injected intradermally into rabbits or guinea pigs -antibody production
- Antibodies recovered from the serum
- Some ligands are not antigenic

Hormone ,steroids ,drugs

Antibody types of labelling

- Radioisotopes
- Enzyme
- Fluorescent
- Chemi-luminescent
- Probes
- Metal tags

Advantages

- Flexibility
- Sensitivity
- Size

Disadvantages

- Toxicity
- Shelf life
- Disposal costs

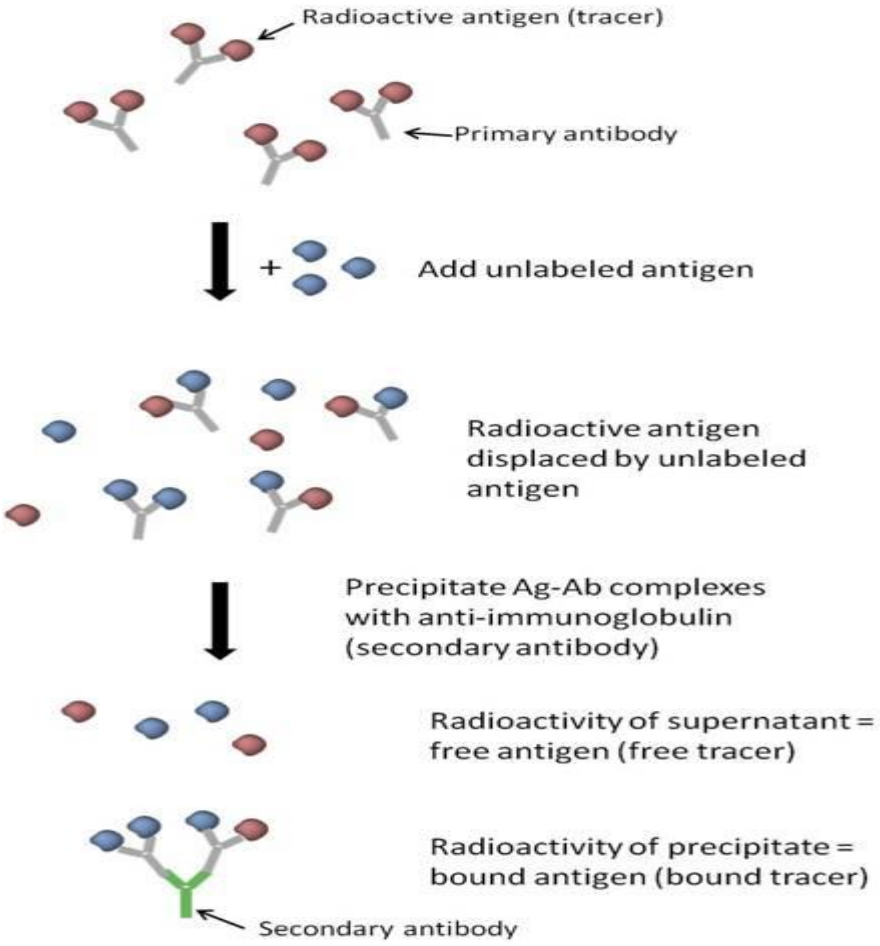

Figure 5.8: Radioimmunoassay

Nucleic acid hybridization

- A technique which has the ability of individual single stranded nucleic acid molecules to form double stranded molecules
- The principle of hybridization is the addition of a probe to a complex mixture of target DNA, The mixture is incubated under condition that promote the formation of hydrogen bonds between complementary strands

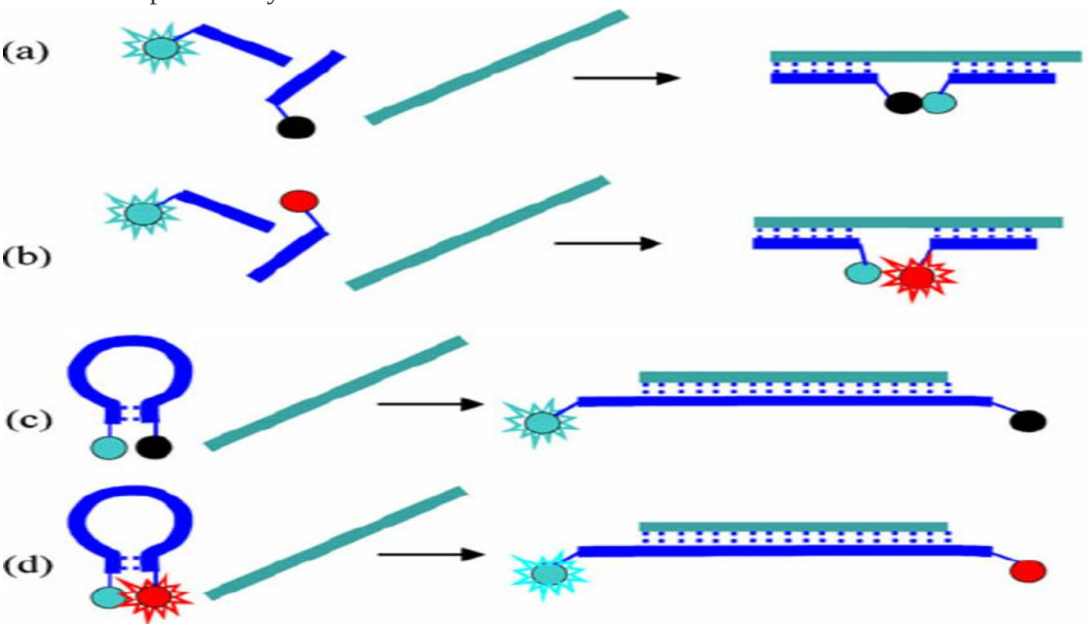

Figure 5.9: Schematic representation of nucleic acid hybridization probes: quenching-type binary probe (a), FRET-type binary probe (b), quenching-type molecular beacon (c), and FRET-type molecular beacon (d)

About DNA

- Watson and crick model of DNA was followed and it possess two strands
- According to Watson -crick base paring ,Adenine pairs with Thymine and guanine pairs with cytosine by hydrogen bonding
- The mechanism can be achieved by means of denaturation and annealing
- Since, Nucleic acid hybridization is a process used to identified specific DNA sequences .this is performed by means of DNA probes
- Probes used in hybridization reaction are usually chemically synthesized DNA or RNA that has been labelled with fluorescent dye or radioactive isotope
- Nucleic acid hybridiation reaction are usually chemically synthesized DNA or RNA that has been labelled with fluorescent dye or radioactive isotope
- Nucleic acid hybridization can be done in all combination :DNA-RNA,DNA-DNA or RNA-RNA, Insitu hybridization and FISH analysis

Factors affecting nucleic acid hybridization :

- Strand length
- Base composition
- Chemical environment

Hybridization probes

- It is a nucleic acid fragment that is complementary to another nucleic acid sequence and thus ,when labelled (with radioisotope ,fluorescent dye ,etc) can be used to identify complementary segments

- A probe actually hybridizes to single stranded nucleic acid (DNA/RNA) molecules because of complementarity between the probe and target
- Nucleic acid probes can be synthesized in the laboratory, as single and double stranded probes ,but a working nucleic acid should be a single stranded only to bind with complementary target
  Probes are of three types
  DNA probes: It is a short sequence of DNA labelled isotopically or chemically that is used for the detection of a complementary nucleotide sequence
  RNA probes :It is a short sequence of RNA labelled isotopically or chemically that is used for detection of a complementary nucleotide sequences
  Oligonucleotide probes :It is a short sequence of nucleotides synthesized to match a region where a mutation is known to occur and then used as a molecular probe to detect the mutation

Labelling of probes

Hybridization probes can be labelled by two methods:

- In vivo labelling :By supplying labelled nucleotides to the cultured cell
- In vitro labelling :An enzyme is used to incorporate a labelled nucleotide in the probe

TYPES OF HYBRIDIZATION :

There are mainly three techniques of hybridization .they are as follows :

1.southern hybridization
2.northern hybridization
3.colony hybridization

Southern hybridization :

- Southern blot is a techniques employed for detection of a specific DNA sequence in DNA samples that are complementary to a given RNA OR DNA sequence
- It was first given by E.M southern ,a british biologist .this method includes separation of restricted DNA fragments by electrophoresis and then transferred to a nitrocellulose or a nylon membrane ,followed by detection of the fragment using probe hybridization

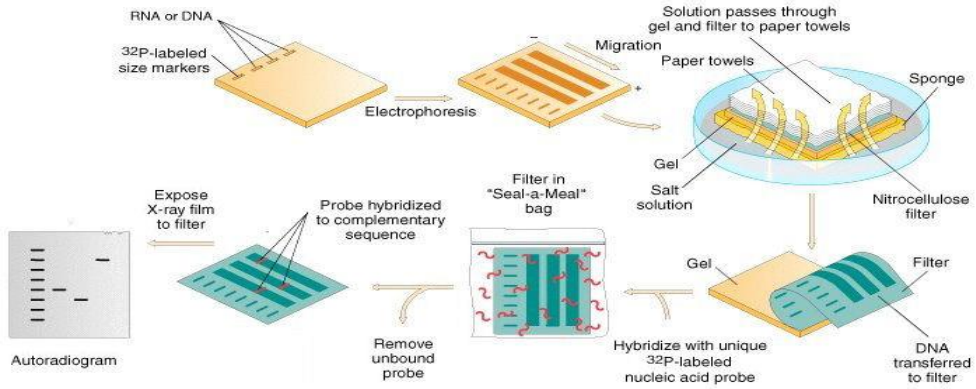

Figure 5.10: Southern Hybridization

APPLICATIONS :

- Southern blots are used in gene discovery ,mapping ,evolution and development studies
- To identify specific DNA in the sample
- To isolate desired DNA for construction of DNA
- used in phylogenic analysis
- used to make RFLP MAPS

Northern hybridization :

- Northern blotting was developed by James Alwine , George stark and David kemp. In this technique ,RNA is being analysed instead of DNA

- It is a technique by which RNA fragments are separated by electrophoresis and immobilized on a membrane .the identification of specific RNA is done by using nucleic acid probes .It help to study gene expression by detection of RNA

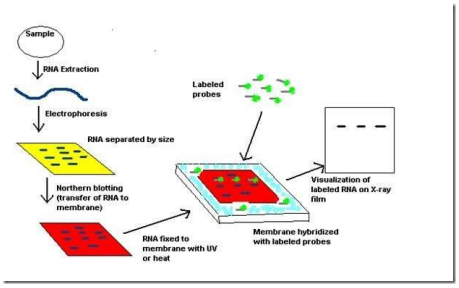

Figure 5.11: Northern hybridization

Colony hybridization :

- It is a rapid method of isolating a colony containing a plasmid harboring a particular sequence or a gene from a mixed population.
    - The colonies to be screened are first replica placed onto a nitrocellulose filter disc that has been place on the surface of an agar plate prior to inoculation

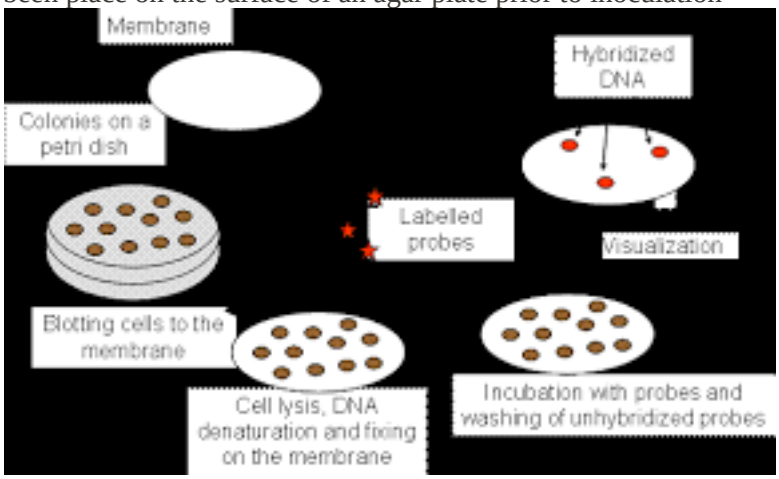

Figure 5.12: Colony hybridization

In situ hybridization :

It is a technique that employs a labelled complementary nucleotide strands for localizing specific DNA and RNA targets

- Chromogenic insitu hybridization
- Flourescence insitu hybridization

Application

- Library screening
- Southern blot
- Northern blot
- ASOs (allele -specific oligonucleotides)to detect mutation

PCR (polymerase chain reaction)

- Polymerase chain reaction is technique generating large quantities of a specified DNA
- PCR is a cell free amplification technique for synthesizing multiple identical copies of any DNA of interest

Principle of PCR

- The double -stranded DNA of interest is denatured to separate into 2 individual strands
- Each strand is allowed to hybridize with a primer (renaturation)
- The primer -template duplex is used for DNA synthesis (DNA polymerase)
- Denaturation ,renaturation and synthesis are repeated again and again to generate multiple forms of target DNA

Technique of PCR

- Requirements for PCR
- A target DNA (100-35,000bp in length)
- Two primers that are complementary to regions flanking the target DNA
- Four deoxyribonucleotides (d ATP ,d CTP ,d Gtp ,d Ttp)
- A DNA polymerase that can withstand at a temperature up to 95 degree
- PCR involves repeated cycles for amplification of target DNA
- Each cycle has 3stage
- Denaturation
- Renaturation or annealing
- Synthesis

Stages of PCR

- Denaturation :
- On increasing the temperature to about 95degree for 1 minute ,the DNA gets denatured and two strands separate
- Renaturation or annealing
- As the temperature of mixture is slowly cooled at about 55 degree c .the primers base pair

With complementary regions flanking targeting DNA stands

Synthesis

- The initiation of DNA synthesis occurs at 3' hydroxyl end of each primer
- The primers are extended by joining the bases complementary to DNA strands
- The primers are extended by joining the bases complementary to DNA strands
- The synthetic process is comparable to DNA replication of leading strand
- Optimum temperature has to be maintained as required by DNA polymerases

Cycle 1

- The new DNA strand joined to each primer is beyond the sequence that is complementary to second primer
- New strands are referred as long template
- They will be used in second cycle

Cycle 2

- The DNA strands are denatured annealed with primers and subjected to DNA synthesis
- At the end of second round ,long templates and short templates and short templates are formed

Cycle 3

- The original DNA strands along with long and short templates are starting materials .
- Denaturation, renaturation and synthesis are repeated
- This process is repeated again and again for each cycle
- At the end of 33nd cycle of PCR ,about million fold target DNA is synthesized

Sources of DNA polymerase

- Kl enow fragment of E. coli DNA polymerase is used in original technique
- This enzyme gets denatured at higher temperature ,therefore fresh enzyme had to be added for each cycle
- Taq DNA polymerase is heat resistant ,not necessary to freshly add this enzyme for each cycle

Variation of PCR

- Inverse PCR
- Anchored PCR
- Reverse transcription PCR (RT-PCR)
- Nested PCR
- Asymmeteric PCR
- Real time quantitative PCR
- Random amplified polymorphic DNA(RAPD)
- Amplified fragment length polymorphism
- Rapid amplification of cDNA ends (RACE)

Application of PCR

- Prenatal diagnosis of inherited diseases
- PCR is used for prenatal diagnosis of various diseases by using chorionic villus samples or Cells from amniocentesis
- Sickle -cell anemia beta -thelassema and PKU can be detected
- Diagnosis of retroviral diseases
- Used for diagnosis of HIV infection

Blotting

Visualization of specific DNA,RNA and protein among many thousands of contaminating molecules requires the convergence of number of techniques which are collectively termed BLOT transfer

Types of blotting techniques

- Southern blotting (to detect DNA)
- Northern blotting (to detect RNA)
- Western blotting (to detect protein)

Southern blotting

Southern blotting is named after Edward M. Southern. This method is used for analysis of DNA sequences. It involves the following steps:

• Firstly, large weighted DNA is cut into small fragments by using Restriction endonucleases .

• Then, these fragments are electrophoresed on separating gel so that they can separate according to their size .

• If DNA fragments are much larger in size so firstly the gel should be treated with HCl, causes depurination of DNA fragments .

• After separating these fragments, placed a nitrocellulose sheet over the separating gel. Apply pressure over the membrane so that proper interaction can occur between these two.

• After that the membrane is exposed to ultraviolet radiation so that the fragments are permanently attached to the membrane .

• Then the membrane is exposed to hybridization probe. But the DNA probe is labeled so that it can easily detect, when the molecule is tagged with a chromogenic dye .

• After hybridization process, excess probe is washed away by using SSC buffer and it can be visualized on X-ray film with the help of autoradiography .

Applications

i) It is used in the technique called RFLP (Restriction fragment length polymorphism) mapping .

ii) Also used in phylogenetic analysis .
iii) To identify the gene rearrangements

2) Western blotting

Western blotting is named after W. Neal Burnette. This method is used for detection and analysis of protein in a given sample . It involves the following steps:

• Firstly, isolating the protein from particular sample.

After that beta- mercaptoethanol (BME) and Sodium dodecyl Sulfate (SDS) is added into the protein suspension .

• Then, protein- SDS complex is placed on top of the gel in the well. A molecular weight marker is also loaded in one of the well in order to determine the molecular weight of other proteins. After that the samples are added in the remaining wells .

• Once the samples and markers are loaded then current is passed across the gel. Protein is pulled down to the positive pole of the well because it is tightly bound to SDS which is negatively charged. Movement of protein is inversely proportional to its size .

• After this step, gel is placed against a membrane and current is passed across the gel so that all the proteins are transferred onto the membrane .

• Then Immunoblotting has to be done. In this method, firstly block the membrane with non-specific protein in order to prevent antibody from binding to the membrane where the protein is not present .

• After that primary antibody is added to the solution. These antibodies are responsible for recognizing a specific amino-acid sequence. Then wash it to remove unbound primary antibody and add secondary antibody .

• Now these antibodies are conjugated with an enzyme and recognize the primary antibody. Lastly, another wash is done to remove unbound secondary antibody .

• Here, chemiluminescent substrate is used for detection. The light is being emitted once the substrate has been added and can be detected with film imager .

Applications:

i) Used in clinical purposes.
ii) Used to detect specific protein in low quantity.
iii) Used to quantifying a gene product .

3) Northern blotting

Northern blotting is given by Alwine. This method is used to analyse and detection of RNA in a sample .

• Firstly, extraxt and purify mRNA from the cells .
• Separate these RNA on agarose gels containing formaldehyde as a denaturing agent for the RNA .
• This gel is immersed in depurination buffer for 5-10 minutes and washed with water .
• Then transfer these RNA fragments onto the carrier membrane i.e aminobenzyloxymethyl filter paper .
• After transferring the RNA,it is fixed to the membrane by using UV or heat.
• Add DNA labellled probe for hybridization .

Wash off the unbound probe and at the end mRNA-DNA hybrid are then detected by X-ray film .

Applications:

Used in screening .