

Diagnostic Bacteriology

CHAPTER I

COLLECTION, TRANSPORT AND EXAMINATION OF BACTERIOLOGICAL SPECIMENS

Learning objective

At the end of this chapter the student will be able to:

- Describe the general points **about collection** of bacteriological samples
- Describe collection, transport and examination of **sputum**
- Describe collection, transport, and examination of **throat and mouth specimen**
- Describe collection, transport, and examination of **nasopharyngeal aspirates and nasal swab**
- Describe collection, transport and examination of **ear discharges**
- Describe collection, transport, and examination of **eye specimens**

- Describe collection, transport and examination of **skin specimens**
- Describe collection, transport, and examination of **pus** from wounds, abscesses, burns, and sinuses
- Describe collections, transport, and examination of **urogenital specimens**
- Describe collection, transport, and examination of **faeces**
- Describe collection, transportation, and examination of **urine**
- Describe collection, transport, and examination of **cerebrospinal fluid** (CSF)
- Describe collection, transport, and examination of **blood** and **bone marrow**

Introduction

- If pathogens are to be isolated successfully the following points must be considered:

→ ***Type of specimen;***

- The correct type of specimen to collect will depend on the pathogens to be isolated

Ex - a **cervical** not a vaginal swab is required for the

most successful isolation of N.gonorrhoea from a woman

- Sputum not saliva is essential for the isolation of respiratory pathogen

➔ ***Time of collection:***

- Before collecting a specimen for **bacteriological examination**, the **right time** at which the organism proliferate maximum should be take in to account and before the pt take antimicrobial drug

Ex- Urine and sputum are best collected soon after a patient wakes (**early morning**)

- Blood for culture is usually best collected when **a patient's temp. begin to rise**

➔ ***Collection techniques:***

- the laboratory should issue written instructions to all those responsible for collecting specimens including the staff of wards, out patient clinics, and health centers .
- The following precautions apply to the collection of most microbiological specimen;-

□ **Use a collection technique that will ensure a specimen contains only those organism from the site where it was collected**

- A strictly sterile (aseptic) procedure is essential when collecting from sites that are normally sterile (Ex blood, CSF)
- Avoid contaminating discharges or ulcer material with skin commensals
- The **swab** used to collect the specimen must be sterile and free from any antibacterial substances

- Collect specimens in sterile, leak- proof, dry **containers**, free from all traces of disinfectant
- Containers must be clean but need not be sterile for the collection of sputum and stools
- Containers must be **easy** for patients **to use**
 - Patients must be **instructed** how to collect aseptically

- Macroscopic **feature of the sample** must be reported
 - Color
 - Presence of pus, blood, mucus or parasites
 - The appearance of urine, pus, vaginal discharge , faeces, CSF

❑ **Labeling of specimens and the sending of a request form**

- Each specimens must be clearly labeled with the date and time of collection
- The patients- name, number, Ward or Health center
- Slides with one end frosted should be used to label with a lead pencil

❖ Each specimen must be accompanied by a **request form** which gives:-

- the patient's name,
- Age,
- Pt number, and
- ward or health center
- type of specimen and the date and time of its collection
- Investigation requested
- Whether the patient has started any antimicrobial treatment

Specimens containing dangerous pathogens

- Those delivering, receiving, and examining specimens must be informed if a specimen is likely to contain highly infectious organisms.
- Such a specimen should be labeled **HIGH RISK**, and when ever possible carry a warning symbol such as a red dot, or star, which helps for easy recognition

- Specimens which should be labeled as **HIGH RISK** include:
 - sputum likely to contain M. tuberculosis
 - Fecal specimen that may contain V.cholerae or S.typhi
 - Fluid from ulcers or pustules that may contain anthrax bacilli or treponemes
 - Specimens from patients with suspected HIV infection, hepatitis, viral hemorrhagic fever, or plague
- After collection these samples should immediately sealed in a plastic bag or in a container with a tight- fitting lid.

Preservatives and transport media for microbiological specimen

- In general, specimens for microbiological investigations should be delivered to the laboratory as soon as possible. This will help to ensure that **pathogens are living when they reach the laboratory**
- If there is any delay use **chemical preservative**. This will help to prevent organisms from dying due to
 - Enzyme action
 - Change of PH
 - Lack of essential nutrients

- A **transport medium** is usually needed to preserve an aerobes
 - Amies transport medium- for specimens collected on swabs (especially N. gonorrhoea)
 - Cary-Blair medium for faeces specimen (especially which contains salmonella, shigella, campylobacter or vibrio species)

- Some of chemical preservatives are:
 - **Boric acid** → added in urine
 - **Cetylpyridium chloride sodium chloride**
(CPC- NaCl) → added to sputum
- chemical preservatives which contain formaldehyde should not be used for microbiological samples
 - Formaldehyde kill the bacteria
- Refrigeration at 4-10°C can help to preserve cells and reduce the multiplication of commensals in unpreserved specimen

1. COLLECTION, TRANSPORT AND EXAMINATION OF SPUTUM

- There are different pathogenic microorganisms that we can be present in sputum
 - Bacteria
 - *Streptococcus pneumoniae*
 - *Haemophilus influenzae*
 - Parasite
 - *Paragonimus* species
 - Fungi and Actinomycetes
 - *Blastomyces dermatitidis*
 - *Candida albicans*

- Sputum when collected **passes through the pharynx and mouth**, therefore becomes contaminated with small numbers of commensal organisms from the upper respiratory track and mouth

Collection and transportation of sputum In hospital Microbiology Laboratory:-

- Sputum for microbiological investigation is collected and transported as follows:-
 - Give the patient **a clean** (need not be sterile), dry, wide necked, leak proof container, and request him to cough deeply to produce a sputum specimen
- ***Important***
 - The specimen must be **sputum, not saliva**
 - Sputum is best collected in the **morning** soon after the patient wakes and before any mouth wash
 - **Gastric washings** can be used for the isolation of *M.tuberculosis* in sputum for young child

- Label the container
- If **pneumonia** or **bronchopneumonia** is suspected,
 - Deliver the sputum to the Lab with **a little delay** because *H.influenzae* and *S.pneumoniae* require culturing as soon as possible
- If the specimen is for the isolation of *M.tuberculosis*,
 - Delivered to the Lab with in 2 hours or kept at 4°C

- ❑ Specimens for the isolation of *S.pneumonia* and *H. influenzae* must **never** be refrigerated
- ❑ If **pneumonic plague** is suspected
 - ❑ Deliver the sputum to the Laboratory as soon as possible.
 - ❑ Make sure the specimen is marked
HIGH RISK

In a health center for dispatch to a microbiology laboratory:

- Collect the sputum in a container supplied by the Microbiology lab
- If pneumonia and broncho-pneumonia pathogens suspected:-
 - **Collect** a purulent part of the sputum on a cotton wool swab and insert in a container of **Amies transport medium**
 - **Label** the container using a lead pencil
 - **Make a smear** of a purulent part of the sputum on a slide for **Gram stain**

➤ **Fix** using heat or alcohol

➤ **Send the swab** and smear with a request form to reach the microbiology Lab. within 6 hrs

■ If ***M. tuberculosis*** is suspected:-

➤ Make a smear of the sputum on a slide for **AFB staining** using caseous particles and the most purulent material

- Let it air dry and fix it with alcohol
- Mix the sputum in its container with equal volume of **CPC- Nacl solution**

Note:

- CPC-Nacl solution **digests (liquefies) sputum** in about 24 hours and prevents the growth of other bacteria ***M. tuberculosis*** will remain viable in the sample for up to 8 days
 - Send the smear and CPC-Nacl treated sputum to microbiology lab. Within one week

- If **pneumonic plague** is suspected:
 - Send a swab of the sputum in **Carry-Blair transport medium** to the Microbiology Lab with in 4-6 hrs

Summary of Microbiological Examination of Sputum

Day 1

1 Describe Specimen	■ <i>Report whether specimen:</i> – purulent, mucopurulent, mucoid, salivary – contains blood	ADDITIONAL INVESTIGATIONS
2 Examine Microscopically	■ <i>Gram smear:</i> For pus cells and bacteria ■ <i>Zn smear:</i> For AFB	■ <i>Giemsa smear:</i> When pneumonic plague or histoplasmosis is suspected ■ <i>KOH preparation:</i> When <i>Aspergillus</i> infection is suspected ■ <i>Toluidine blue-O and Giemsa smears:</i> When <i>Pneumocystis</i> pneumonia is suspected ■ <i>Eosin preparation:</i> When an allergic condition requires investigation ■ <i>Saline preparation:</i> When paragonimiasis is suspected
3 Culture Specimen	■ <i>Blood agar</i> – Add an optochin disc – Incubate aerobically ■ <i>Chocolate agar</i> – Incubate in CO₂	■ <i>Culture for M. tuberculosis</i> (In Reference Laboratory) See text

Day 2 and Onwards

4 Examine and Report Cultures	■ <i>Blood and chocolate agar cultures</i> Report <i>significant</i> growth of: <i>S. pneumoniae</i> <i>H. influenzae</i> <i>S. aureus</i> Less commonly found pathogens: <i>K. pneumoniae</i>, <i>P. aeruginosa</i>, <i>M. catarrhalis</i>, <i>S. pyogenes</i>, <i>Proteus</i>, <i>C. albicans</i>	■ Test <i>H. influenzae</i> for beta-lactamase production ■ Antimicrobial susceptibility tests as required
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Key: Zn – Ziehl-Neelsen, KOH – Potassium hydroxide, CO₂ – Carbon dioxide

2. COLLECTION, TRANSPORT, AND EXAMINATION OF THROAT AND MOUTH SPECIMEN

■ Possible pathogens are:-

➤ *S.pyogenes*

➤ *C.diphtheriae*

➤ *C. ulcerans*

➤ *B. vincenti* etc

➤ Fungi- *Candida albicans*

- Pathogens in the **upper respiratory tract** such as *Bordetella pertussis*, *S. pneumoniae*, *N.meningitidis* are usually more successfully isolated from **pernasal and nasopharyngeal** specimens
- It should also be noted that there are plenty of throat and **mouth commensals**

collection and Transport of throat and mouth swabs

➔ In a hospital with a microbiology laboratory

- In a good light and using the handle of a spoon to **depress the tongue, examine** the inside of the mouth for **inflammation**, and the presence of **any membrane, exudates**, or **pus**.
 - A grayish- yellow membrane (later becoming grayish green black & smelly)- diphtheria
 - Patches of white exudates may be seen attached in places to the mucous membrane of the mouth- *C.albicans*

- Swab the affected area using a sterile cotton or wool swab.
- Return it to its sterile container
- Within two hours of collection, deliver the swab with a request form to the laboratory.

➔ In a health center for dispatch to a Microbiology Laboratory

- Using a sterile swab (supplied in a tube of silica gel). **Collect a specimen** from the infected area.
- Taking care not to contaminate the swab, **return it to** its tube.
- **Seal** with adhesive tape and label using a lead pencil.
- **Send** the swab with its request form to reach the microbiology Lab with in 3 days

Summary of Microbiological Examination of Throat and Mouth Swabs

Day 1

1 Culture Specimen	ADDITIONAL INVESTIGATIONS	
	■ <i>Blood agar</i> – Add a bacitracin disc – Incubate, preferably anaerobically (or in CO₂)	■ <i>MTM or TBA</i> : When diphtheria suspected
2 Examine Microscopically	■ <i>Gram smear</i> Look for: – Pus cells and Gram negative Vincent's organisms – Gram positive pleomorphic rods when diphtheria suspected – Gram positive yeast cells when thrush suspected	■ <i>Giemsa or Wayson's smear</i> : When diphtheria suspected

Day 2 and Onwards

3 Examine and Report Cultures	■ <i>Blood agar culture</i> Look for <i>beta</i> -haemolytic streptococci, sensitive to bacitracin. <i>Identify as S. pyogenes</i> Lancefield group PYR test	■ <i>MTM or TBA cultures</i> Examine for growth of <i>C. diphtheriae</i>
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Key: MTM – Modified Tinsdale medium, TBA – Tellurite blood agar

3. COLLECTION, TRANSPORT, AND EXAMINATION OF NASOPHARYNGEAL ASPIRATES AND NASAL SWAB

- The possible pathogens in nasopharyngeal aspirates and pernasal swabs include:-

- *S.pneumoniae*
- *C. diphtheriae*
- *H. Influenzae*
- ***Bordetella pertussis***
- *Klebsiella* species

- ***C. diphtheriae*** is usually isolated from throat swabs.
- In the anterior Nasal swabs examination possibly look for:-
 - *S. aureus*
 - *S.pyogenes*
 - *N.meningitides*
 - *H.influenzae*
- There are also so many commensals found in Nasopharyngeal Aspirates, pernasal and Anterior nasal swabs.

Collection and Transport of upper respiratory track specimens

→ In hospital with a microbiology laboratory

■ Nasopharyngeal aspirates;-

- Gently **pass a sterile catheter** through one nostril as for the nasopharynx
- Attach a sterile syringe to the catheter, and **aspirate a specimen of muco-pus**
- Dispense the specimen into a small sterile container
- Label, and deliver with a complete request form to the laboratory

- **Per-nasal swabs** for the culture of ***B.pertussis***:-
 - Using a sterile cotton wool swab attached to an easily bent piece of wire, gently pass the swab along the floor of one nostril **directing the swab down wards** and back wards as far as the nasopharynx
 - Taking care **not to contaminate the swab**, replace it in its sterile container
 - Label, and deliver immediately to the laboratory with a request form

■ **Anterior nasal swabs to detect carriers:-**

- Using a sterile cotton wool swab moistened with sterile **peptone water**, gently swab the inside surface of the nose
- Taking care not to contaminate the swab, replace it in its sterile container
- Label, and within 2 hrs deliver the swab with a request form to the laboratory

→ In a health center for dispatch to a microbiology laboratory

■ Nasopharyngeal aspirates:-

➤ Collect in the same way as collected in hospital and make a smear and dispatch as described for sputum

■ **Pernasal swabs for culture of *B.pertussis*:-**

- Swab in the same way as described for hospital
- Insert the swab in a bijou containing **special Bordetella transport medium** and seal the cap with adhesive tape.
- Send the swab to the microbiology laboratory to reach with in 2 days

■ **Anterior nasal swabs** to detect carriers:-

- Collection is similar with hospital collection
- Transport the swab in **Amies transport medium**.

Laboratory examination of upper respiratory track specimens

Day -1

- Culture the specimen (Blood agar and chocolate agar)
- To detect H.influenzae, N.meningitides and S.aureus carriers:-
 - Inoculate the swab on chocolate agar
 - Incubate the plate in CO₂ enriched atmosphere at 35-37°C/48hrs
 - Examine for growth after overnight incubation

- **Charcoal cephalalexin blood agar** if **whooping cough** is suspected
 - Inoculate the swab over the entire surface of a plate of charcoal cephalalexin blood agar
 - Incubate the plate **aerobically** in a moist atmosphere in a **plastic bag** or polythene bag or polythene container with a wet piece of cotton wool) at 35-37⁰C 16 days,
 - Examine for growth after about 48 hrs incubation

- Culture of a swab received in Bordetella transport Medium:-
 - Inoculate the swab on a plate of CCBA
 - Return the swab to its container and incubate it at 35-37°C
 - Incubate the plate
 - Examine the plate after 48 hrs incubation
- If no growth is seen,
 - inoculate a second plate of CCBA with the incubated swab
 - Re incubate the first plate for a further four nights
 - Examining for growth every 24 hrs.
 - Incubate the second plate up to 6 days

Day- 2 and onwards

- **Examine and report the cultures** (Blood agar and chocolate agar cultures)
- Look especially for colonies that could be:
 - *H.influenzae*
 - *S.aureus*
 - *S.pyogenes*
 - *N.meningitides*

4. COLLECTION, TRANSPORT AND EXAMINATION OF EAR DISCHARGES

→ Same of possible pathogens in ear discharges are :-

- *S. aureus*
- *H.influenzae*
- *S. pyogenes*
- *S.pneumonia*
- *P.aeruginosa*
- *Proteus spp* etc

- Fungal infection of the ear (**otomycosis**) can occur due to
 - *Aspergillus* spp.
 - *Candida* spp. etc
- The middle ear is **normally sterile**, but many commensals can be found in the external ear

1. collection and Transport of Ear discharges

■ In a hospital with a microbiology laboratory

- When ever possible, collect or aspirate a small amount of the discharge in **a sterile leak proof container**
- Label and send to the laboratory as soon as possible with the request form
- If an actual specimen container of the discharge can not be obtained, collect, a specimen on a sterile , dry, **cotton wool swab**
- If fungal infection is suspected do KOH test from the sample
- Deliver the swab and the sample as soon as possible

➔ **In a health center for dispatch to a microbiology Laboratory**

- collect a specimen of the discharge on a sterile cotton wool swab
- Place the swab in **Amies transport media** and tight the bottle cap
- Make a smear of the discharge on a slide (for Gram stain)
- Label and send the specimens to reach microbiology laboratory within 6 hrs

2. Laboratory Examination of Ear discharges

Day- 1

I. Culture the specimen

- Blood agar and MacConkey agar
 - Inoculate the sample on Blood agar and MacConkey agar
 - Incubate both plates aerobically at 35-37°C over night

- Chocolate agar **if the patient is a child**
 - Inoculate the specimen on chocolate agar for the isolation of *H. influenzae*
 - Incubate it in a CO₂ enriched atmosphere at 35-37°C/48 hrs
 - Examine for growth after overnight incubation
 - Blood agar (**kanamycin**) for anaerobic incubation

- **If infection** is chronic:-
 - Inoculate the specimen on blood agar, preferably that which contain kanamycin
 - Incubate the plate anaerobically for up to 48hrs
 - Check for growth after over night incubation
- If a **fungal infection** is suspected use **Sabouraud agar**
 - Inoculate the sample and incubate it at room temperature for up to 6 days

□ Gram smear

- Do gram stain of the smear and examine for pus cells and bacteria and fungi
- G+ve, cocci that could be **S.aureus**
- G+ve, streptococci or diplococci that could be **pathogenic streptococci**
- G-ve, rods that could be ,
 - *H.influenzae*
 - *P.aeruginosa*
 - *Klabsiella spp.*
 - *Proteus spp.*
 - *E.coli*
 - G+ve yeast cells that could be *candida spp.*

□ **KOH test** if fungal infection is suspected

- Mix a small amount of the specimen with a drop of KOH (20%w/v) on a slide, and cover with a cover glass
- After 10 minute examine the preparation microscopically using 10x and 40x objectives

3. Examine and report the cultures

On day-2

- Blood agar and MacConkey agar cultures
 - Look for colonies that could be:-
 - *S.aureus*
 - *S.pyogenes*
 - *P.aeruginosa*
 - *S.pneumoniae*
 - *E.coli*
 - *Proteus spp.*

- Chocolate agar culture
 - look for colonies that could be *H.influenzae*
- Anaerobic blood agar (kanamycin) culture
 - Look for colonies that could be bacteriodes spp.
- In Gram stained smear the organisms appear as pale stained, small Gram-negative rods
- Sabouraud agar culture
 - Look for colonies which could be Candida spp

5. COLLECTION, TRANSPORT, AND EXAMINATION OF EYE SPECIMENS

- The possible pathogens of the eye include:-
 - *S.aureus*
 - *S. agalactiae*
 - *N.gonorrhoeae*
 - *C.trachomatis*
- There are also many commensals in the eye discharges

1 Collection and Transport of **Eye specimens**

➔ **In a hospital with a microbiology Laboratory**

- Specimens from the eye must be cultured as soon as possible after collection, because the natural secretions of the eye contain **anti-bacterial enzyme**
- Using a dry sterile cotton wool swab, collect a specimen of the discharge
- Inoculate the discharge on the following media-
 - Blood agar
 - Chocolate agar
 - MNYC

- Make a smear for a **gram stain**
- If ***C.trachomatis*** suspected make another smear for **Giemsa stain**
- Label both the culture plate and the slides
- As soon as possible deliver to microbiology Laboratory

➔ In a health center for dispatch to a microbiology Laboratory

- Using a sterile cotton wool swab, collect a specimen of the discharge and insert in **Amies transport media**
- Make also a smear of the discharge on a slide
- Send the specimens with a request form to reach the microbiology Laboratory within 6 hrs.

2. Laboratory examination of eye specimens

Day - 1

I. Culture the specimen

- Blood agar and chocolate agar
 - Inoculate the eye discharge on blood agar and chocolate agar
 - Incubate the blood agar plate aerobically at 35-37⁰C overnight
 - Incubate the chocolate agar plate in a CO₂ enriched atmosphere for 48hrs
 - checking the growth after over night incubation

■ **MNYC selective medium** if **gonococcal conjunctivitis** is suspected

- Inoculate the eye discharge on a plate of MNYC medium
- Incubate at 35-37⁰C overnight
- Incubate the chocolate agar plate in a CO₂ enriched atmosphere for 48 hrs.
- checking the growth after over night incubation

II. Examine the specimen microscopically

- Do Gram stain and examine the smear using the 40x and 100x objective for pus cells and bacteria
- Look especially- **G-ve diplococci** intracellular bacteria that could be **N.gonorrhoea**
- **G+ve streptococci or diplococci**, that could be **S.pyogens**
- **Gram -ve rods ----- Haemophilus SPP.**

- Giemsa smear if ***C.trachomatis*** is suspected
 - After staining the fixed smear, examining using 40x and 100x objectives
 - Look for epithelial cells that contain **inclusion bodies**
 - Report the smear as 'chlamydial inclusion bodies present' or '**No chlamydial inclusion bodies seen**'.

III. Examine and report the cultures

On day 2 and on wards

- Blood agar and chocolate agar colonies
- Look especially for colonies that could be:-
 - *H.influenzae*
 - *S.aureus*
 - Beta hemolytic streptococci
 - *S.pneumonia*
 - *P.aeruginosa*
- MNYC medium culture
 - *N.gonorrhoeae* grows rapidly on MNYC selective medium

6. COLLECTION, TRANSPORT AND EXAMINATION OF SKIN SPECIMENS

- There are many pathogens of the skin, for example-
 - *S.aureus*
 - *S.pyogenes*
 - *B.anthraxis*
 - *C.ulcerans*
 - *Y.pestis*
 - *Proteus*
 - *M.leprae*
 - *M.ulceran*
- Fungi Ring worm fungi
 - *Candida spp.*
- There are also commensal organisms that may be found on the skin

1 Collection of skin specimens and ulcer material

→ In a hospital with a microbiology laboratory

- Using a sterile dry cotton wool swab, collect a sample of discharge from infected tissue
- If there is no discharge, use a swab, moistened with sterile physiological saline
- Insert the swab in a sterile tube

- If the tissue is deeply ulcerated and necrotic (Full of dead cells):-
 - aspirate a sample of infected material from the side wall of the ulcer using needle and syringe
- Fluid from pustules and blister :-
 - Aspirate a specimen using a sterile needle and syringe

- Serous fluid from **skin ulcers, papillomas, or papules**, that may contain treponemes:-
 - Collect a drop of the exudates directly on a clean cover glass and invert on a clean slide
 - Deliver immediately the specimen to the Lab. For examination by dark- field microscopy
 - If the specimen has been **aspirated**, transport the needle and syringe in a sealed water proof container
- Skin specimen for ring worm **fungi** should be collected and examined by **KOH technique**

➔ In a health center for dispatch to a microbiology lab.

- Collect the specimen using a **sterile cotton wool swab**
- Inset in a container of **Amies transport medium**
- If the materials is aspirated fluid from a pustule transfer the fluid to a sterile, leak- proof container
- Stopper, and seal in a leak- proof plastic or metal container
- Make a smear of the material on a clean slide of allow air dry
- Label the specimens
- Send the specimens to reach microbiology Lab with in 6 hrs

2. Lab. Examination of skin specimens

Day-1

I-Culture the specimen

- Blood agar and MacConkey agar
 - Inoculate the specimens on these media
 - Incubate at 35-37⁰C over night (aerobically)
for the pus from wounds culture
anaerobically

- **Modified Tinsdale medium (MTM)** if cutaneous diphtheria is suspected:
 - Inoculate the specimen on MTM to isolate *C. ulcerance*
 - Incubate the plate aerobically at 35-37°C / 46hrs
 - Examine for growth after overnight incubation
- **Blood agar and MacConkey agar** at room temperature if **bubonic plague** is suspected
 - Inoculate the specimens on these media
 - Incubate aerobically at room temperature for 48hrs.
 - Examine for growth after overnight incubation

■ **Lowen stein Jensen medium** if **Buruli** ulcer is suspected:

- Make two smear of the specimen
- Then decontaminate the swab by immersing it in 4% NaoH solution for 10 minute
- Inoculate the decontaminated specimen on two slops of LJ media
- Incubate one slop at 35-37⁰C as for *M. tuberculosis*
- And incubate the other slop at 32⁰C for up to 8 weeks

II. Examine the specimen microscopically

- Do gram stain of the smear and examine for:-
 - G+ve cocci → *S.aureus*
 - G+ve streptococci → *S.pyogenes*
 - G-ve Rods → *p.aeruginosa*, *proteus* spp, *E.coli* or other
- If **cutaneous anthrax** is suspected- Look for large gram variable rods lying in chain that could be ***B. anthrax***
- If **bubonic plague** is suspected, look for Gram -ve coccobacilli that could be *Y. pestis*

- Giemsa or **wayson's stained smear** if bubonic plague is suspected
 - Make an evenly smear and Let it air dry
 - Fix it
 - Stain with Giemsa stain or by the rapid wayson technique
 - Examine for **bipolar staining of** *y.pestis*
- **Polychrome Loeffler methylene blue smear** if cutaneous anthrax is suspected
 - Make a smear and Fix it after it air dried
 - And cover it with potassium permanganate (40g/l) soln. For 10 minute
 - Examine the smear under 100x objective

- Ziehl-Neelsen stained smear if Buruli ulcer is suspected
 - Do a **ziehl-Neelson** method I and examine the smear for acid fast bacilli
- Examine by **Dark- field Microscopy** to detect treponemes
 - Examination of a specimen by dark field microscopy for motile treponemes if *T.pertenue* and *T. carateum* is suspected

Examine and report the cultures

On day 2 and on wards

- Blood agar and Macconky agar culture
- Look especially for colonies that could be:-
 - *S.aureus*
 - *P.pyogenes*
 - *P.aeruginosa*
 - *Proteus spp*
 - *E.coli*
- If cutaneous anthrax is suspected
 - Look for **large grey- white irregular colonies** with wary edges on blood agar that could be *B.anthraxis*

- Modified Tinsdale medium culture
 - To identify *C.ulcerans*
- Blood agar and MacConkey agar at room temperature
 - To look for *y.pestis*
- LJ culture
 - To look for *M.ulcerans*

7. COLLECTION, TRANSPORT, AND EXAMINATION OF PUS FROM WOUNDS, ABSCESES, BURNS, AND SINUSES

- Some of the possible bacterial pathogens are:- *S.aureus*, *S.pyogenes*, *C.tetani*, *C. perfringens*, *P.aeruginosa*, *Proteus spp.*, *Bacteriodes spp.*, *E.coli*, *Klebsiella spp.* etc
- Any commensal organisms found in pus are usually those that have **contaminated the specimen from the skin**, clothing, soil, or from the air if an **open wound**

1. Collection of pus from wounds, Abscesses, Burns and sinuses

- ☐ pus from an abscesses is best collected at time the **abscess is incised** and **drained** or after it has ruptured naturally
- **In a hospital with a microbiology Laboratory**
 - ☐ using a sterile technique, aspirate or collect from a drainage tube up to 5 ml pus
 - ☐ Transfer to a leak proof sterile container
 - ☐ If pus is not being discharge, use a sterile cotton wool swab to collect a sample from the infected site
 - ☐ Transport with Amies transport media
 - ☐ Label the specimen and as soon as possible deliver to the lab

- ➔ In a health center for dispatch to a microbiology Lab.
- Collect a sample of the pus on a sterile cotton wool swab
 - Transport it with Amies transport medium
 - If tuberculosis is suspected: aspirate a sample of the pus and transfer it to a sterile leak- proof container
 - Make a smear for Gram stain
 - Label the specimens and send with a request form to reach the lab with in 6 hrs

2 . Lab. Examination

* *Day- 1*

I. Describe the appearance of the specimen

➔ Report the **colour** of the pus and whether it contain **granules**

- **Granules**:- are colonies of organisms white, yellow, brown, red or black granules of varying size, shape , and consistency may be found in pus draining from sinuses in **mycetoma** and in **actinomycosis**
- **Pus from an amoebic abscess**:- This has an acid reaction (PH 5.2-6.7) and is usually **red- brown** or **grey- yellow** in colour

II. Culture the specimen

- **Blood agar, neomycin blood agar, MacConky agar, and cooked meat medium**
 - Inoculate the specimen in a pair on each medium of the above
 - Incubate one blood agar plate and MacConky agar plate aerobically at 35-37°C over night
 - Incubate the neomycin plate and the second blood agar plate anaerobically at 35-37°C for up to 48 hrs
 - Incubate the cooked meat medium at 35-37°C for up to 72 hrs. subculture at 24 hrs, and if indicated at 48hr and 72 hr.

- LJ medium if tuberculosis is suspected
 - Wait until the next morning to see whether there is growth on the blood agar and MacConkey agar plates
 - If sterile, inoculate the specimen directly on a slope of LJ medium
 - If there is growth on the blood agar and MacConkey agar plates decontaminate the specimen by 4% w/v NaOH for 10 minute
 - Then inoculate on **LJ medium**
 - Incubate the LJ medium as described for sputum

III. Examination of the specimen microscopically

■ Gram stain

- Do Gram stain for a smear made from the specimen and examine for the following bacterias:-
- **G+ve cocci** – S.aureus or streptococcus, anaerobic streptococci or enterococci
- **G-ve Rods** - Proteus spp, E.coli, p.aeruginosa or Bacteriodes
- **G+ve large rods** with straight ends **C. perfringens**

■ AFB smear if tuberculosis is suspected

- Do AF stain and Examine the smear for Acid fast bacilli.

- **KOH preparation** if fungal or actinomycete infection is suspected
 - Follow different procedure for granulated pus
 - Examine the preparation under 10x and 40x obj

IV. Examine and report the cultures

- Blood agar, neomycin blood agar, and MacConkey agar cultures
 - Look for colonies especially for those which could be :-
 - *S.aureus*
 - *S.pyogenes*
 - *C. perfringens*
 - *P. aeruginosa*
 - *Proteus spp.*
 - *E.coli*
 - *Enterococci*
 - *Bacteriod spp.*
 - *Anaerobic cocci*

- Cooked meat culture
 - look for the grow of ***C.perfringens*** and ***bacterioides spp.***
 - Then do gram stain of the culture
- LJ- culture
 - Look for growth of ***M.tuberculosis***

8. COLLECTIONS, TRANSPORT, AND EXAMINATION OF UROGENITAL SPECIMENS

- Possible pathogens are: (Inurethral swab):-
 - *S.pyogenes*
 - *C. trachomatis*
 - *T.vaginalis*
 - *N.gonorrhoeae etc*

1.Collection and transport of urogenital specimens

➔ Collection of urethral specimens:-

- **cleans** round the urethral opening using a swab moistened with sterile physiological saline
- **Gently massage** the urethra from above downwards, and
- **collect a sample** of pus on a sterile cotton wool swab
- **Insert the swab** in a container of Amies transport medium

Note:- The patient should not have passed urine preferably for 2hrs before the specimen is collected

- **Make a smear of the discharge** on a slide for staining by the Gram technique

Note:- the smear should be made by rolling the swab on the slide and fixed with methanol

- Label the specimens

➔ Collection of cervical specimens

- **Moisten a vaginal speculum** with sterile warm water and insert in to the vagina
- **Cleans the cervix** using a swab moistened with physiological saline
- Pass a sterile cotton wool swab in to the endocervical cannal and **gently rotate the swab** to obtain a specimen
- Insert the swab in a container of Amies transport medium
- Make a smear of the cervical muco-pus on a slide for Gram staining
- Label the specimens

→ Collection of vaginal specimens

- Collect a sample of vaginal discharge on a sterile cotton wool swab and insert it in amies transport medium
- Make a smear of the discharge on a slide for staining by Gram technique
- Label the specimens
- If ***T. vaginalis*** and ***G. vaginalis*** is suspected
 - do direct wet mount preparation
 - examine under 10x and 40x objective
 - do acridine orange stain of the discharge smeared on clean slide for rapid examination of ***T.vaginalis***

- Appearance of vaginal discharge in infection of :-
 - ***T. vaginalis***:- yellow- green purulent with PH > 4.5
 - ***C.albican***:- white discharge with PH below 4.5
 - ***G.vaginalis***:- Grey, offensive, thin discharge with PH> 4.5
- The normal PH of vaginal discharge is 3.0 --- 3.5 (puberty to menopause).

→ Collection of other urogenital specimens

■ *T.pallidum*:

- wearing rubber gloves, cleans around ulcer (chancre) using a swab moistened in physiological saline
- Collect a sample of serous exudates on a cover glass and invert it on a slide
- Deliver immediately the preparation to the Laboratory for examination

■ *H. ducreyi*:

- After cleaning the ulcerated area as described in the previous text, make a smear from the lesion for staining by the Gram stain method

2 Laboratory examination of the urogenital specimens

Day-1

I. Culture the specimen

■ MNYC medium

- Inoculate the specimen on MNYC selective medium, pre-warmed to room temperature
- With a little delay as possible, incubate the inoculated MNYC plate in a moist CO₂ enriched atmosphere at 35-37⁰C over night

- **Blood agar** (aerobic and anaerobic), **MacConkey agar**, and **cooked meat medium** if sepsis abortion is suspected
 - Inoculate the specimen on two plates of blood agar
 - Incubate one anaerobically and the other aerobically at 35-37⁰C overnight
 - Inoculate the specimen in cooked meat medium and incubate at 35-37⁰C sub culturing as indicated at 24 hr, 48 hr, and 72 hr.
- Serum culture if **chancroid** is suspected

- The following is a simplified technique for diagnosing chancroid in which ***H.ducreyi*** is cultured in patient's serum
 - Collect 10 ml of venous blood from the patient allow to clot
 - Separate the serum from the clot
 - Aseptically transfer the serum to a sterile tube, stopper and heat at 56⁰c for 30 minutes
 - Wait the serum to cool and then inoculate the sample collected from the side wall of the ulcer
 - Incubate at 35-37°C for 48 hrs.

- Sabouraud medium if vaginal candidiasis is suspected
 - If **vaginal candidasis** is suspected and yeast cells are not detected microscopically, inoculate the specimen on **sabouraud agar**
 - Incubate the plate aerobically at 35-37°C for up to 48 hrs. checking for growth after overnight incubation.

II. Examine the specimen Microscopically

Gram smear

- Do gram stain preparation and examine under 40x and 100x obj for pus cells and bacteria

→ smear from a patient with suspected gonorrhoeae

- Look for pus cells containing G-ve diplococci → ***N.gonorrhoeae***

→ Smear from a patient with vaginitis

- Look especially for:- Larg G+ve yeast cells → ***C.albican***
- Epithelial cells with attached Gram variable coccobacilli → ***G.vaginalis*** with few or no pus cells

→ Smear from septically aborted women

■ Look especifically among pus cells For:-

■ Large G+ve rods with straight ends →

C.perfringens

■ G+ve strep.cocci → ***S.pyogenes***

■ G+ve cocci → ***S.aureus***

■ G-ve Rods → ***Bacteriodes spp.***

→ Smear from a patient with **suspected chancroid**

■ Look for G-ve coccobacilli showing bipolar staining

■ Saline preparation ***H.ducreyi*** (often found in chain)

→ ***Gardnerella*** infection

- Look for large Number of ***G.vaginalis*** coccobacilli attached to epithelial cells

→ ***T.vaginalis***- Motile ***T.vaginalis*** among pus cells

- Giemsa stained smear
 - a smear stained with Giemsa will be examined for

C.trachomatis

- Dark-field preparation
 - a preparation for the detection of motile treponemes must be examined as soon as possible after the specimen is collected

III. Examine and report the cultures

* *On day 2 and on wards*

- MNYC medium culture
 - Look for colonies which look like ***gonorrhoeal's colony***
 - Do gram stain from the colonies
 - Look for **Beta- lactamase production**
- Blood agar and MacConkey agar cultures
Look for colonies that could be:-
 - ***S.pyogenes***
 - ***S.aures***
 - ***C. perpringens***
 - ***Proteus spp***
 - ***Enterococci***
 - ***E.coli***

- Sabouraud agar culture
 - Look for the appearance of ***C.albican*** colonies
- Serum culture
 - Examine a Gram stained smear from serum culture to look for ***H.duceyi***

9. COLLECTION, TRANSPORT, AND EXAMINATION OF FAECES

- Some of the possible pathogens found in the stool are:-
 - *C.perfringens* type A and C
 - *B.cereus* (toxin)
 - *S.aureus* (toxin)
 - *Shigella* spp.
 - *Salmonella* spp.
 - *E.coli* (ETEC,EIEC,EPEC)
 - *V.cholerae* ol
 - *Y.enterocolitica*

1. Collection and Transport of faeces

- Faeces for microbiological examination should be collected during the **acute stage of diarrhea**

➔ In hospital with a microbiology laboratory

- Give the patient a clean, dry, disinfectant- **free bed pan** or suitable wide- necked container
- The container need not to be sterile
- ASK the patient to **avoid contaminating with urine**
- Transfer about a **spoon full** of the specimen, especially that which contains mucus, pus, or blood into a clean dry, leak proof container

- Report the **appearance** of the specimen
- If it is not possible to collect specimen (feaces), perform a **rectal swab** by inserting a cotton wool swab into the rectum for about 10 seconds
- Label the specimen and send it with a request form to reach the laboratory with in 2 hrs

- ➔ In a health center for transport to a microbiology Laboratory
- Collect the specimen as described in previous text
 - Transfer **apportion of the specimen to a cotton wool swab**
 - Insert the swab in a container of **sterile carry Blair** transport medium salmonella, shigella, Vibrio, and yersinia spp
 - Survive well in carry-Blair medium for up to 48 hrs and campylobacter for up to 6 hrs

- If cholera is suspected:
- Transfer about 1 ml of specimen into 10 ml of **sterile alkaline peptone water**,
- label and send it to reach a microbiology laboratory with in 8 hrs
- Write the appearance of the sample on the request form
- Label and send the specimen to microbiology laboratory with in 6 hrs.

2. laboratory Examination of faeces

** on day- 1*

I. Describe the appearance of the specimen

- **Normal faeces**: appear brown and formed or semiformal infant faeces are yellow- green and semiformal

II. Examine the specimen microscopically:

- direct wet mount preparation for **ova or parasite**
- **Methylene blue** preparation to detect **fecal leucocytes**
 - Place a drop of methylene blue stain on a slide
 - Mix a small amount of specimen with the stain
 - And cover with a cover glass
 - Examine the preparation for faecal leucocytes using 40x obj

- **Fecal pus cells** are associated with bacteria that cause inflammation of the large intestine (shigella, salmonella & campylobacter)
- Those **mono nuclear cells** are found mainly in typhoid fever and some parasitic infection

- **Basic fuchsin smear** to detect campylobacter
 - Make a thin smear of the specimen on a slide
 - Dry and gently heat fix it
 - Cover it with 10g/l basic fuchsin for 10-20 seconds
 - Examine the smear for ***campylobacters*** using the 100x oil objective

- **Motility and slide immobilization test** if cholera is suspected
 - Examine an alkaline peptone water culture for vibrios with a **rapid** and **darting motility**
 - The preparation is best examined using **dark-field** microscopy but **transmitted light** can also be used
 - If you see a characteristic motility, run a drop of ***V.cholerae*** O-group 1 polyvalent **antiserum** under the cover glass
 - Re-examine for the immobilized bacteria with in 5 minutes

III. culture the specimen

- If the specimen is formed or semi-formed, make a thick **suspension** of it in about 1 ml of sterile peptone water
- Xylose lysine deoxycholate (**XLD**) agar and **selenite broth**
 - Inoculate a loop full of the emulsified faeces or fluid specimen on XLD agar
 - Several loop fulls into selenite F enrichment broth
 - Incubate the XLD agar plate aerobically at 35-37°C over night
 - Incubate the selenite broth at 35-37°C over night

- **Campylobacter medium** if the patient is under two years or *campylobacter enteritis* is suspected
 - Inoculate a large loop full of specimen
 - Place the plate in ajar or tin with a lighted candle and incubate preferably at 42⁰C overnight or if it is not possible at 37⁰C for up to 48hrs
- **Note-** if a selective medium is not available use a **filtration** method to isolate campylobacter (**Butzler medium**)

- **Alkaline peptone water** and **TCBS** if cholera or ***V.parahemolyticus*** food poisoning is suspected
 - Inoculate several loop fulls of specimen in alkaline (PH= 8) peptone water
 - Incubate at 35-37⁰C for 5-8 hrs
 - Sub culture several loopfuls of the peptone water culture on TCBS agar
 - Incubate anaerobically at 35-37⁰C overnight
- **Note:-** ***V.cholerae*** will also grow on TCBS at room temperature
 - The medium is as selective when incubated at room temperature as at (35-37⁰C)

- **MacConkey agar or SS agar if *y. enterocolitica* is suspected**
 - Inoculate a loopful of specimen on MacConkey agar or on **SS agar** to which has been added an additional amount of **sodium deoxycholate** (0.2g/10ml of medium)
 - Incubate **aerobically** at room temperature (20-28⁰C) for up to 48 hrs
 - Examine for growth after over night incubation

- Investigation of **enteritis** caused by pathogenic E.coli
 - Toxin tests have been developed to detect enterotoxigenic E.coli (ETEC)
 - If ETEC is suspected isolate should be sub-cultured on slopes of **nutrient agar** and sent to microbiology lab
 - If EPEC also suspected it should be sent to micro. Laboratory
 - EIEC investigation also require referral micro. Laboratory

IV. Examination and report the cultures

- **XLD agar** and **selenite broth** cultures
 - Look for the appearance of **shigella and salmonella colonies**
 - If no colonies resembling salmonella spp. are seen subculture the selenite broth culture on a plate of XLD agar, and incubate at 35-37⁰C over night

- **Identification** of suspect salmonella and shigella isolates
 - Using a sterile straight wire, touch the surface of an individual smooth colony, and stab into a tube of **MIU medium** (Motility Indole Urea)
 - Incubate at 35-37⁰C for up to 4 hrs, examine at half hourly intervals for **a pink color** through out the medium
 - A **positive urease test** indicates that the organism is probably **proteus** no further tests are required

- If the **urease test is negative** at 4 hours, perform a rapid **APIZ salmonella shigella screening test** as follows:-
 - Place an **indole** strip in the neck of the MIU inoculated tube
 - Then re incubate at 35-37⁰C overnight
 - Inoculate **KIA** with colony from XLD plate, **stabbing first the butt** and then streaking the slope.
 - Close the tube with a loose- fitting cap, and incubate at 35-37⁰C over night
 - After overnight incubation, examine the MIU and KIA cultures for reactions

- After overnight incubation, examine the MIU and KIA cultures for reactions suggestive of salmonella or shigella
 - **All strains** of salmonella and shigella are **urease negative** (no pink colour through out the medium)
 - Test serologically any isolate giving reactions suggestive of salmonella or shigella spp. using a **slide technique**

- Slide agglutination technique:
 - **Emulsify** a small amount of growth from the **KIA** culture in a loop full of physiological saline on a slide
 - **Mix** by tilting the slide back wards and forwards for about 30 second
 - Examine for agglutination against a dark back ground

Note:- if there is agglutination the strain is unsuitable for serological testing

- If there is **no agglutination**, add one loop full of test antiserum and mix
- Examine for agglutination
- **A positive test** will show strong clear agglutination within 1 minute

Note:- To do **O Ag testing** heat a saline suspension of the organism in water bath for 20 minutes

■ **Rapid APIZ salmonella shigella screening test**

- Add 1 drop (not less than 50µl) of sterile distilled water to **couple A & B**
- Using the applicator stick provided, carefully remove a smooth colony from the XLD agar plate and inoculate couple A and then couple B
- Replace the tray lid and incubate at 35-37⁰C for up to 2 hrs
- Examine couple A for a change in color
 - No color change..... **Possibly salmonella or shigella**
 - Yellow, brown, green.... **Not salmonella or shigella**

- If a **red color** develops (and the **oxidase test** is-
ve), suspect salmonella and test the colonies
serologically
- If no red color develops, perform a **motility test**
using the peptone water culture in **couple B**
- If the organism is **motile-** suspect salmonella
and if **non motile, suspect shigella**

■ **Campylobacter medium culture**

- *C.jejuni* and *C.coli* produce **non haemolytic** usually moist spreading colonies on campylobacter selective medium if growth is present, identify the colonies as follow:-
- Perform oxidase and catalase test (campylobacter is +ve for both)
- Examine a basic fuchsin smear
- Examine a physiological saline preparation for **motility**

- **TCBS agar culture**

- ***V.cholerae*** is sucrose fermenting and therefore produces yellow 2-3mm in diameter **shiny colonies** on TCBS agar with **yellow color** in the medium
- It should be differentiated from other vibrio spp.

- Identification of a suspect *V.cholerae* isolate
 - Examine a gram stain of the culture
 - Subculture the organism on a slope of nutrient agar (use a heavy inoculum), and incubate for 4-6 hrs
 - Perform an **oxidase test** on the nutrient agar culture
 - If positive for oxidase test, test the colony with ***v.cholerae* O group 1 polyvalent antiserum** using a slide technique

- Identification of a suspect *V.parahaemolyticus*
 - Perform an oxidase test
 - Examine Gram stain smear of the colonies
 - Sub culture the organism into:-
 - sodium chloride peptone water
 - Peptone water containing 8% w/v Nacl
 - Peptone water containing 10% w/v Nacl
 - Incubate the subcultures at 35-37⁰C for 6-8 hrs.

- MacConkey agar or SS agar room temperature culture
- Identification of a suspect *y. enterocolitica* isolate
- Sub inoculate two tubes of MIU medium, and incubate one at room temperature and the other at 35-37⁰C overnight
- Sub inoculate a tube of KIA, and incubate at room temperature over night
- Perform **a phenylalanine deaminase test** (incubate at room temp)
- Perform oxidase and catalase test
- Examine a Giemsa or **wayson stained smear** for coccobacilli

10. COLLECTION, TRANSPORTATION, AND EXAMINATION OF URINE

- Possible pathogens are :-

- *enterococci*
- *S.saprophyticus*
- *Hemolytic streptococci*
- *E.coli*
- *Proteus spp.*
- *P.aeruginosa*
- *K. lebsiella strains*

1. Collection and Transport of urine

- **Midstream urine** (MSU) collected for Microbiological examination

➔ In a hospital with a microbiology laboratory

- Give the patient a sterile, dry, wide- necked, leak proof container , and explain to collect a clean catch specimen
- Female patients should be instructed to **clean** the area around the urethra openings with clean water, **dry the area** and **collect** the urine with the labia held apart

- About 20 ml of urine should be collected
- Label and deliver the specimen to microbiology Lab. As soon as possible
- If delay in delivery is expected **refrigerate** the sample at 4⁰C, if the delay is more than a hour is **anticipated boric acid** should be added

➔ In a health center for dispatch to a microbiology laboratory

- Collect MSU as explained in the above text
- Add 0.1g/10ml of urine

Note:- Urine for culture should be preserved with bactericidal chemical

- Label and send it with the request paper to reach microbiology laboratory within 48 hrs

➔ Collection of urine if renal tuberculosis is suspected

- Collect the **first urine passed** (entire specimen) on three successive morning
- The collection container should be clean, dry , leak- proof and sufficiently large to contain all the **first passed morning urine**
- The specimens should be stored at 4⁰C **until all three urines have been collected**

2. Laboratory examination of urine

Day- 1

- Describe the appearance of the specimen
- **Report color**, whether it is **clear or cloudy**
 - Normal freshly passed urine is clear and **pale yellow** to yellow depending on concentration
 - When left to stand, a **cloudiness** may develop

- Examine the specimen microscopically

→ **Wet preparation** of fresh uncentrifuged urine

- In the investigation of urinary infection, it is greater value to look for **bacteria in fresh uncentrifuged urine** than **estimating the number of pus cells in centrifuged urine**
- Place three loop full of well- mixed fresh urine on a slide, and cover with a cover glass
- Examine the preparation using 10x and 40x objectives with the condenser iris closed sufficiently to give good contrast

- The following may be found in urine:-
 - Bacteria
 - Pus cells
 - Red cells
 - Yeast cells
 - Epithelial cells
 - Parasites, etc
- The finding of bacteria in a freshly passed uncentrifuged urine indicates **urinary infection**

- Gram smear
 - **Centrifuge** about 10 ml of urine discard the supernatant fluid
 - Make a smear of the sediment and fix and stain by Gram technique
 - Examine it under oil immersion objective
 - Look especially for bacteria associated with urinary infection, especially **gram -ve Rods**
 - Vaginal contamination is indicated by a mixed bacterial flora, including **G+ve rods**

➔ Neisseria gonorrheal in urine

- In male patients with **acute urethritis**, it is often possible to make a presumptive diagnosis of gonorrhea by finding G-ve intra cellular diplococci in pus cells

➔ Dark - field preparation if **leptospirosis** is suspected

- The examination of leptospire can be done using dark field Microscopy

➔ Ziehl-Nelson smear if renal tuberculosis is suspected

- Make a smear of the deposit from three centrifuged early morning urine sediments
- Allow to air dry and fix it with 70%v/v alcohol
- Make AFS stain
- Examine as described in the previous text for AFB

- Culture the specimen
 - **Culture** is required if the urine contains bacteria, cells, casts, protein Nitrite, or has a markedly alkaline or acid reaction
- ➔ Estimating bacterial numbers
 - The approximate number of bacteria **per ml of urine**, can be estimated by using a calibrated loop or a measured piece of filter paper
 - Both methods are based on accepting that **a single colony represents one organism**
 - For example, if an inoculum of 1/500 ml produces 20 colonies, the number of organisms represented in 1/500 ml of urine is 20 or 10,000 in ml (500×20)

→ **Blood agar and MacConkey agar**

- Mix the urine well by inverting the container several times
- Using a sterile calibrated wire loop inoculate a loop full of urine on blood agar and MacConkey agar
- The loop must be held vertical and only the loop must be dipped in the urine
- Incubate the inoculated plates aerobically at 35-37°C over night

➔ **Selenite broth** to detect *S.typhi* carriers

- Centrifuge 7-10 ml of urine in a sterile conical tube at high speed for 5-10 minutes
- Discard the supernatant fluid and resuspend the sediment
- Transfer the entire sediment to a container of **selenite enrichment broth**
- Incubate the broth at 35-37⁰C overnight
- The following morning, subculture on XLD agar
- Incubate the plate aerobically at 35-37⁰C for 24hrs

→ Lowenstein Jensen slope if renal tuberculosis is suspected

- Collect 3 early morning urines
- Allow each urine to sediment overnight (4⁰C)
- Discard the supernatant from each tube and combine the sediment
- Centrifuge the sediment at high speed for 20 minute
- Discard the supernatant fluid and resuspend the sediment

- After making **AFS preparation** , mix the sediment with equal volume of NaOH (40g/l) solution and mix
- Leave the mixture for 10 minutes to decontaminate the urine
- Inoculate 200µl (0.2 ml) of the well-mixed decontaminated urine on the slope of LJ media
- Incubate the inoculum as described previously for AFB

3. Examine and report the culture

- On Blood agar and MacConkey agar cultures look especially for colonies of : -
 - *E.coli*
 - *Proteus spp.*
 - *P.aeruginosa*
 - *Klebsiella strains*
 - *S.saprophyticus*
 - *Enterococci*

Note:- contaminating organisms usually produce a few colonies of mixed growth

- Estimation of bacterial numbers count the approximate number of colonies
- Estimate the number of bacteria per ml of urine
 - Report the bacterial count as less than 10000/ ml
→ contamination
 - 10,000-100,000/ml → infection or contamination
 - More than 100,000/ml → infection

- **XLD agar culture**

- Look for colonies of ***S.typhi***

- **LJ culture**

- Look for colonies of ***M.tuberculosis***

11. COLLECTION, TRANSPORT, AND EXAMINATION OF CEREBROSPINAL FLUID (CSF)

- Possible pathogens :-
 - *S.pneumoniae*
 - *S. aureus*
 - *S. agalactiae*
 - *N. meningitides*
 - *H. influenzae type b*
 - *M.tuberculosis*
 - *B.anthraxis* etc

Note:- CSF has no normal microbial flora

1. Collection of CSF

- It should be collected by an experienced medical officer or Health worker
- It must be collected as aseptically as possible **to prevent organisms being introduced** into the central nervous system
- The fluid usually collected from the **arachnoid space**
- A sterile wide- bore needle is inserted between the fourth and fifth lumbar vertebral
- The C.S.F is allowed to drip into a dry sterile container

→ In a hospital with a microbiology laboratory

- Laboratory staff should be advised before a lumbar puncture is performed so that they can be prepared to receive and examine the specimen immediately

Note:

- A delay in examining CSF reduces the chances of isolating a pathogen.
- It will also lead to a falsely low glucose value due to **glycolysis**

- Take two sterile, dry, screw- cap containers and label as:-
 - First sample collected, to be used for **culture**,
 - Second sample collected, to be used for other **investigations**
- Collect about 1 ml of C.S.F in container No.1 and about 2-3ml in container No.2)
- Deliver immediately the samples with a request form to the laboratory

- In a health center for dispatch to a microbiology laboratory:-
 - Collect the fluid in two sterile containers as described for the hospital collection of C.S.F.
 - Report the appearance

- Culture the CSF, and incubate the inoculated plates in a CO₂ tin or jar
- If unable to perform a cell count and estimate the containing NaF oxalet and mix.
- Send the samples and the inoculated plate to reach to a microbiology lab. With in **2½** hrs.

2. Laboratory examination of cerebrospinal fluid

Day- 1

I. Describe the appearance of the specimen as:

- Whether the C.S.F is clear, slightly cloudy, cloudy, or definitely purulent

Note: If the C.S.F is purulent or markedly cloudy, make immediately a **smear for Gram staining**, and report it as soon as possible

- Express whether CSF sample contains blood or not
- If it contains blood check whether sample No.2 contains as much blood as sample No.1
 - Sample No.1 → contain more blood to traumatic lumbar puncture
 - Sample No.1 and 2 → due to haemorrhage in the CNS
- The C.S.F may appear **xanthochromic** (yellow- Red) due to sub **arachnoid haemorrhage** or **Jaundice** or spinal constriction

- When it contains clots
 - clotted C.S.F indicates an increase in **fibrinogen**
 - It occurs when there is **spinal constriction**
 - It can also occur in **pyogenic meningitis**
 - In tuberculosis meningitis a **skin** may form on the surface of the fluid
- **Normal C.S.F:-** appears clear, bright, and colorless

II. Perform a cell count

- Using a dry Pasteur pipette held vertically, transfer 3 drops of well- mixed C.S.F (sample No.2) to small dry tube
- Using another Pasteur pipette of the same bore size, add 3 drops of **toluidine blue diluting fluid**, and mix well
- This gives a 1 in 2 dilution of the C.S.F
- Count the cells using an improved Neubauer chamber under 10 x objective with condenser, iris closed sufficiently

- Count the cells in 4 of the large squares
- Then multiply the number of cells counted on the 4 squares by 2 if using 1 in 2 dilution; then multiply the figure obtained by 10/4
- Multiply by 10⁶ to give the number of cells per liter of C.S.F
- **Normal C.S.F** contains up to **5x10⁶ cells/ lit**
- In meningitis and other diseases, the C.S.F contains more than 5x10⁶ cells/ liter

III. Test the specimen biochemically

- centrifuge No.2 sample at medium to high speed for about 5 minutes
- Transfer the supernatant fluid to another tube
- Use the clear **supernatant fluid** for **biochemical tests** , and the **sediment** for the **microscopical examination**

IV. culture the specimen (sample No.1)

- Culture of the C.S.F is necessary if the fluid contains cells and ,or, the protein concentration is abnormal

NB: C.S.F should be **cultured** as soon as possible after collection

- If a delay is un avoidable, the fluid should be kept at 35-37⁰C (**never refrigerated**)
- If the C.S.F appears only slightly cloudy, centrifuge it in a sterile tube for 15-20 minutes and use the sediment for inoculating the plates

➔ Chocolate (heated blood)agar

- Inoculate the specimen on chocolate agar
- Incubate it in a CO₂ enriched atmosphere at 35-37°C for up to 48hrs, checking the growth after over night incubation

➔ MacConkey agar and blood agar if the patient is a New born.

- Inoculate the specimen on MacConkey agar and blood agar
- Incubate both plates at 35-37°C over night

- **LJ medium** if tuberculosis meningitis is suspected:
- Inoculate the C.S.F on a slope of LJ medium and Incubate it

Note:-

- It is usual to wait until the following morning to check whether the chocolate agar culture is sterile before inoculation of the LJ medium
- If the culture is not sterile, decontaminate the sediment by adding of 4% w/v NaOH . Then inoculate it.

□ **Sabouraud agar** if cryptococcal meningitis is suspected

- If capsulated yeast cells are seen in the microscopical preparation, inoculate a plate of sabouraud agar
- Incubate at 35-37⁰C for up to 72hrs
- Checking the growth after overnight incubation

V. Examine the specimen Microscopically

- The **microscopic examination** of C.S.F is required if the specimen appears abnormal, contains cells and , or, the total protein is raised with a **positive pandy's test**

→ Gram smear

- Make an evenly spread smear of a drop of purulent C.S.F., or the sediment from a centrifuged sample (No.2) on a slide
- Allow to air dry in a safe place
- Fix with methanol, and stain by the gram technique

- Examine the smear for pus cells and bacteria using 40x and 100 x objectives
- Look for:-
 - G-ve intracellular diplococci> *N .meningitides*
 - G+ve diplococci or short streptococci.....> *S.pneumoniae*
 - G-ve rod, especially if filamentous> *H. influenzae*
 - G+ve cocci ingroup or singly.....> *S. aureus*
 - G+ve strep. Cocci.....> *S.agalactiae*
 - G+ve yeas cells> *C.neoformans*

AFB stain

- If **tuberculosis meningitis** is suspected, transfer several drops of C.S.F sediment to a slide, allowing one drop to dry before adding the next
- Allow the smear to air dry in a safe place
- Fix with methanol and stain by ziehl Neelson Method- I
- Then examine under 40x objective & 100x objective

Note:- AFB are difficult to detect in C.S.F the chances of finding the bacteria are increased if C.S.F is centrifuged for 20-30 minutes and several drops are used

→ **India ink preparation** if cryptococcal meningitis is suspected

- Transfer a small drop of CSF **sediment to the slide**,
- Add a small **drop of India ink** or 20% nigrosine
- **Mix, and cover** with a cover glass
- **Examine** the preparation under 40x objective
- Look for **oval or round stained cells** measuring 5-20 μ m

- If cells resembling *C. neoformans* are seen, culture the C.S.F on **sabouraud agar**
- Notify immediately the medical officer attending the patient
- Wet preparation to detect
 - *Naegleria amoebae*
 - Trypanosomes
- Do Giemsa smear to detect
 - Morula (mott) cells

VI. Examine and report the cultures

On Day- 2 and on wards

Chocolate agar culture

- Look especially for colonies that could be:-
 - *N.meningitides*
 - *S. pneumoniae*
 - *H. influenzae*

MacConkey agar and Blood agar cultures

- Look especially for colonies that could be:-
 - *E.coli* or other coliform
 - *S.agalactiae*
 - *S.aureus*
 - *L.monocytogens*

LJ-culture

- Look for ***M.tuberculosis***

Sabouraud agar culture

- Look for ***C. neoformans***

12. COLLECTION, TRANSPORT, AND EXAMINATION OF BLOOD AND BONE MARROW

■ Possible pathogens are –

- | | | |
|---|-----------------------------|-----------------------|
| ☐ <i>S.aureus</i> | <i>. Klebsiclla strains</i> | <i>. Proteus spp</i> |
| ☐ <i>S.pneumonia</i> | <i>. C.perfringens</i> | <i>. P.aeruginosa</i> |
| ☐ <i>S.pyogenes</i> | <i>. N.meningitides</i> | <i>. Y.pestis</i> |
| ☐ <i>S.typhi</i> | <i>. H.influanzae</i> | |

Note:- Bacteria that can be isolated from **contaminated transfused blood** are usually capable of growing at room temperature or below, such as pseudomonas, coliforms, and A chromobacter spp.

Note:- Neither blood nor bone marrow has a normal microbial flora

1. Collection and culture of blood and bone marrow

- Blood and bone marrow require culturing immediately after collection before clotting occurs

2. Choice of culture media

- The following media are suitable for the routine culture of blood and bone marrow
 - Tryptone soya (tryptic soy) diphasic medium
 - Thioglycolate broth medium

3. Examination of blood and bone marrow

Day- 1

I. Collect and culture the specimen

□ Blood :-

- Blood should be collected before antimicrobial treatment has been started and at the time the patient's temperature is beginning to rise
- To increase the chances of isolating a pathogen, it usually recommended that at least two specimens (collected at different times should be cultured)
- Blood for culture should be collected as aseptically as possible the following technique is recommended:

- Using a pressure cuff, locate a suitable vein in arm
- Cleanse thoroughly the skin over the vein using **tincture of iodine** followed by **ethanol- ether**
- Remove the protective cover from the tops of the culture bottles and cleanse the top of each bottle using an ethanol- ether swab

NB:-

- Do not use a bottle of culture medium if it shows signs of contamination, i.e **forbid broth**
- Do not use a bottle of thioglycolate broth if it appears **oxidized**

- Using a sterile syringe and size 21 gauge needle, with draw 10-12ml of blood
- With care, remove the needle from the syringe, and replace with another sterile needle of similar size
- Insert the needle through the rubber liner of the broth cap
- Dispense 5 ml of blood in to each culture bottle

- Dispense the remaining blood in to a tube or bottle containing EDTA for
 - TLC and
 - parasite examination
- Using an ethanol- ether swab, wipe the top of each culture bottle
- Replace the tape or protective caps.
- Gently mix the blood with the broth

Important:

- The blood must **not be allowed to clot** in the culture media because any bacteria will become trapped in the clot
- Using a lead pencil- label each bottle
 - Name
 - number of the patient,
 - date and time of collection
- Incubate the inoculated media immediately
 - Thioglycolate broth
 - Incubate at 35-37°C for up to 2 weeks
 - Examine and subculture
 - Tryptone soya diphase medium
 - Incubate at 35-37°C for up to 4 weeks
 - Examine for growth

- Culture of blood from new born infants
 - To reduce the risk of contamination, blood from neonates should be collected from a peripheral vein **not from the umbilical vein**

N.B: If **brucellosis** is suspected, loosen the cap of the diphasic culture bottle or insert a sterile needle through the rubber liner, and incubate in a CO₂ atmosphere

■ Bone marrow (BM)

- The aspiration of bone marrow is usually performed by a medical officer
- Using a strict aseptic technique, inoculate the BM in to **tryptone soya diphasic medium**
- Incubate the inoculated diphasic medium as described for blood cultures
- Make a smear of the BM for Giemsa staining

Note:- If only a **small amount of marrow** is obtained, add aseptically 3-5 ml of sterile nutrient broth to the inoculated broth

II. Examine the specimen Microscopically

- Giemsa smear

- **Blood**

- Centrifuge a sample of the EDTA anti-coagulated blood, and make a smear of the **buffy coat layer**
 - When dry, fix with methanol for 2 minutes, and stain by the Giemsa method
 - Examine the smear for malaria parasites and if indicated, look also for *Borrelia*, microfilariae, and trypanosomes

- **Bone Marrow**

- Examine the BM for, malaria, *L.donovani*, Microfilariae, trypanosomes

- Dark field preparation
 - If **leptospirosis** is suspected, centrifuge a sample of fresh EDTA blood and examine a drop of the plasma for motile leptospires using dark field microscope
- Thioglycolate broth culture
 - Examine daily for up to 14 days
 - Look for visible signs of bacterial growth such as

- Turbidity above the red cell layer,
- colonies growing on top of the red cells (cotton balls)
- Haemolysis,
- gas bubbles, and
- clots
- A sterile culture usually remains clear. A slight turbidity may develop after several days incubation

- If there are signs of bacterial growth, subculture the broth and examine a Gram stained smear for bacteria

Important:

- If the patient is seriously ill, subculture the broth (even in the absence of visible bacterial growth) after overnight incubation, after 48hrs, and twice weekly for up to 2 weeks.

Sub culturing a blood culture broth

- **A strict aseptic technique** must be used to avoid contaminating the culture
- Using an ethanol- ether swab, cleanse the top of the bottle
- Using a sterile needle and small syringe, insert the needle through the rubber liner in the cap and with draw about 1 ml of the broth culture
- Inoculate the broth on :-
 - Blood agar
 - Chocolate agar
 - MacConkey agar
- Swab the top of the culture bottle, and re-incubate

■ **Tryptone soya diphasic media:**

- Examine daily for the first of days and twice a week for up to 4 weeks
- Look for colonies on the agar slope (preferably using a hand lense) and signs of bacteria growth in the broth

Note:

- Colonies of staphylococci, S.typhi, brucellae and most coliforms can usually be **seen easily**,
- Where as colonies of pneumococci, S.pyogenes, and y.pestis are **not easily seen**.
- Pseudomonas and proteus spp. produce **a film of growth** on the agar

- If growth is present:-
 - Sub culture on blood agar, chocolate agar and MacConkey agar
 - Examine a Gram stained smear of the colonies
 - Depending on the colonies examine for-coagulase, catalase, oxidase, urease and motility

- If large G+ve rods resembling ***C.perfringes*** are seen, subculture also on **lactose egg yolk milk agar** and incubate the plate anaerobically
- If **motile, urease** and **oxidase -ve**, G-ve rods are isolated, subculture the colonies on **KIA** to screen for salmonella
- If **catalase positive**, G-ve coccobacilli are isolated from a diphasic culture that has been incubated in CO₂ atmosphere, suspect Brucella spp.

Important:- Always **report immediately a positive blood culture**, and send a preliminary report of Gram smear and other useful test results