# PROCESSING OF SAMPLES

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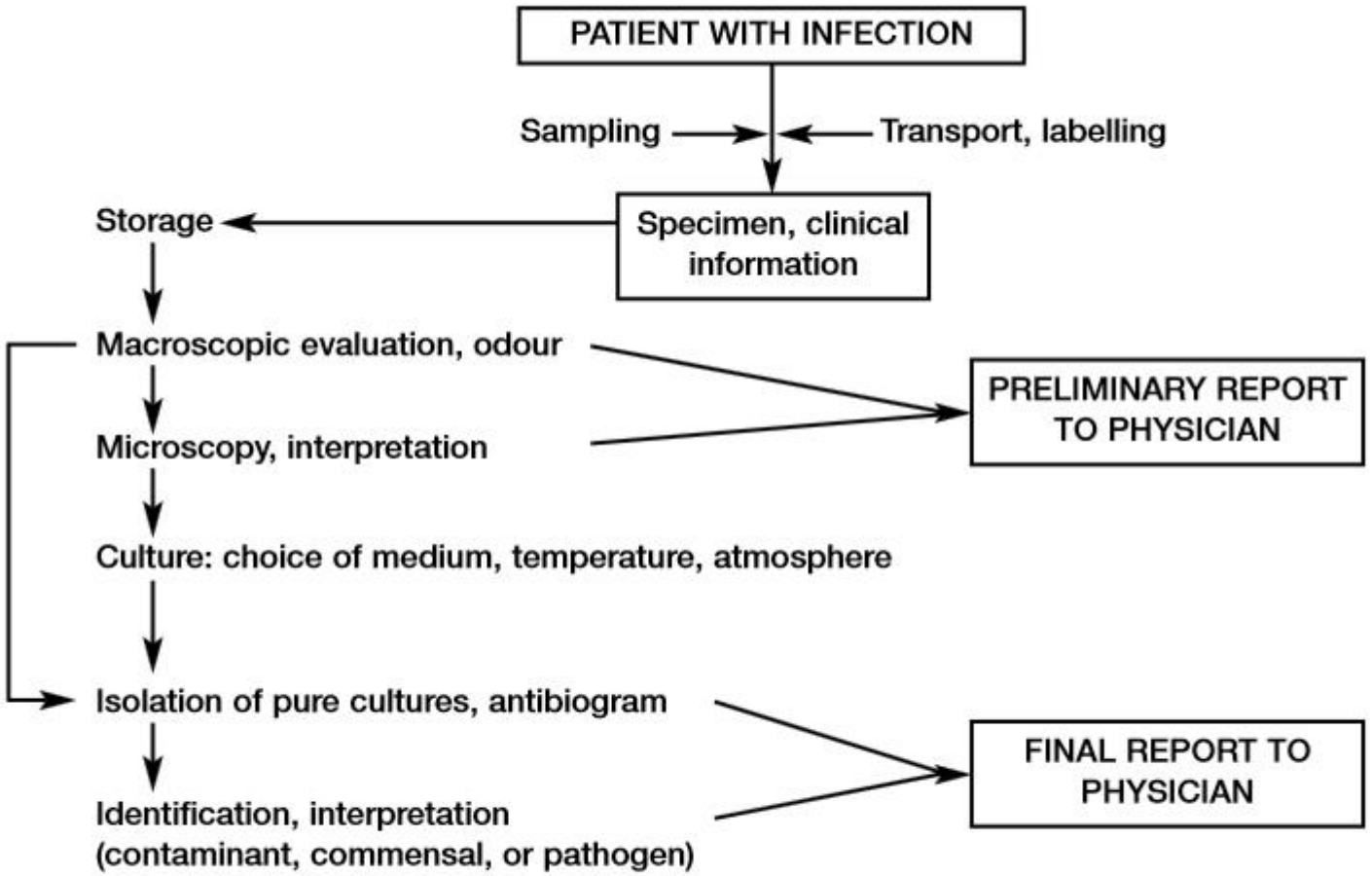
Cobam

UOP




Steps in the laboratory investigation of an infected patient.
Reproduced from *Basic Laboratory procedures in clinical bacteriology*, 2nd edition, 2003, World Health Organization.

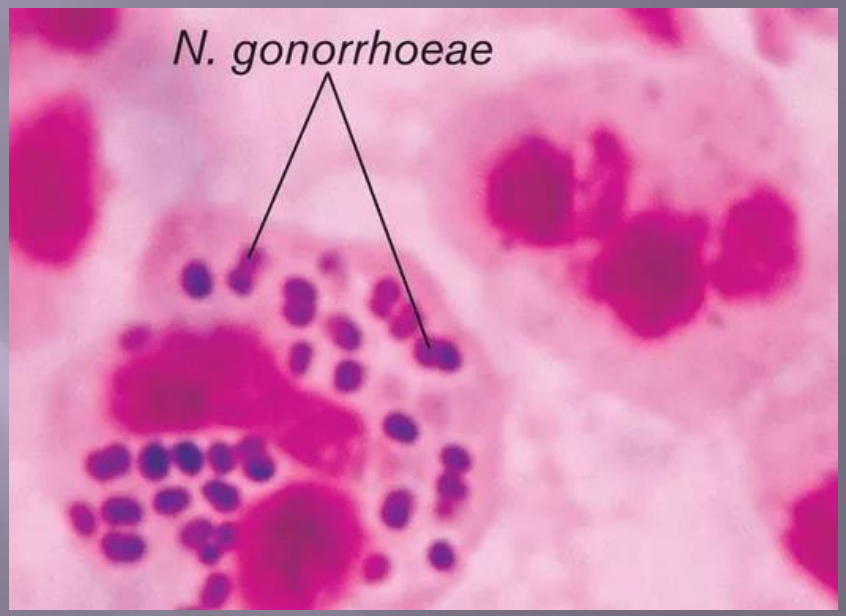
# LABORATORY INVESTIGATION OF MICROBIAL INFECTIONS

- The laboratory investigation of microbial diseases involves:
- Examining specimens to detect, isolate, and identify pathogens or their products using: –
  - Microscopy
  - Culture techniques
  - Biochemical methods
- Immunological (antigen) tests Testing serum for antibodies produced in response to infection, i.e. serological response.

# MICROSCOPY

## microorganisms can be examined microscopically for their motility, morphology, and staining reactions

- Motile Vibrio cholerae in a rice watery faecal specimen from a person with cholera.
- Gram negative reaction and characteristic morphology of Neisseriae gonorrhoeae (intracellular diplococci) in a urethral discharge from a man with gonorrhoea.
- Gram positive reaction and morphology of pneumococci in cerebrospinal fluid from a patient with pneumococcal meningitis.
- Gram positive reaction and morphology of yeast cells in a vaginal discharge from a woman with vaginal candidiasis.



- Acid fast reaction of Mycobacterium tuberculosis in Ziehl-Neelsen stained sputum from a person with pulmonary tuberculosis
- Treponema pallidum in chancre fluid (using dark-field microscopy), establishing a diagnosis of primary syphilis.
- Fungal hyphae and arthrospores in a sodium hydroxide preparation of skin from a person with ringworm.

# Examining pathogens in wet preparations

- examination of wet preparations is mainly used: –
- to examine specimens and cultures for motile bacteria.
- to examine c.s.f. for type of pathogen and immune cells
- – to examine specimens for fungi.

# Technique using transmitted light microscopy

1 .Place a small drop of suspension on a slide and cover with a cover glass. Avoid making the preparation too thick. It is advisable to seal the preparation with nail varnish or molten petroleum jelly to prevent it drying out.

- Hanging drop preparation: Placing a drop of suspension on a cover glass and inverting this over a cavity slide or over a normal slide supported on a ring of plasticine is not recommended. Vibration of the fluid makes the prep- aration difficult to examine.

2. Examine the preparation microscopically for motile organisms, using the 10 and 40 objectives.

# Preparing fixed smears for staining

- Smears should be spread evenly covering an area of about 15–20 mm diameter on a slide.

● **Purulent specimen:** Using a sterile wire loop, make a thin preparation. Do not centrifuge a purulent fluid, e.g. c.s.f. containing pus cells.

● **Non-purulent fluid specimen**: Centrifuge the fluid and make a smear from a drop of the well-mixed sediment.

● **Culture**: Emulsify a colony in sterile distilled water and make a thin preparation on a slide. When a broth culture, transfer a loopful to a slide and make a thin preparation.

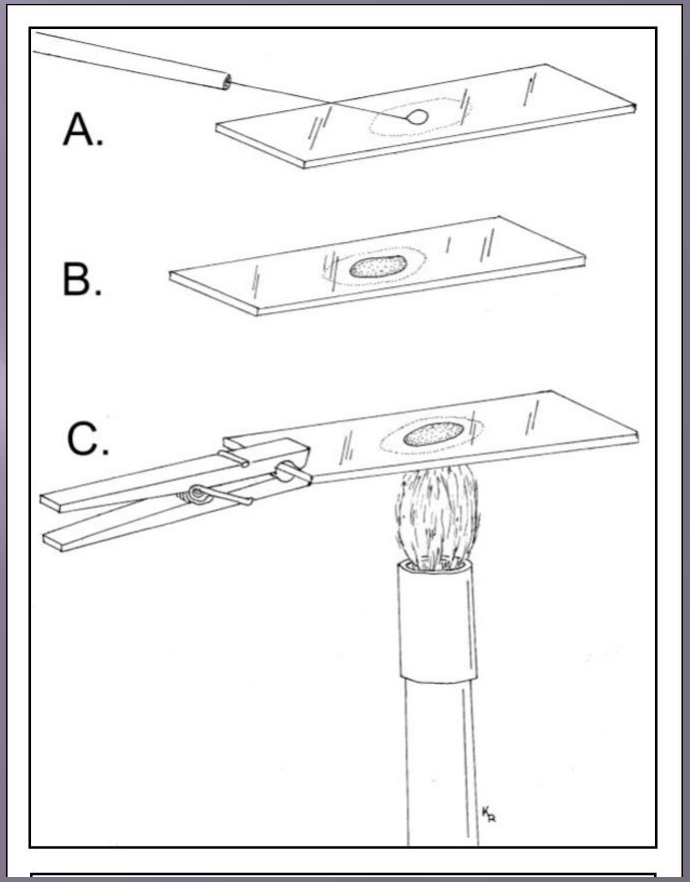
● **Sputum**: Use a piece of clean stick to transfer and spread purulent and caseous material on a slide. Soak the stick in a phenol or hypochlorite disin- fectant before discarding it.

● **Swabs:** Roll the swab on a slide. This is particu- larly important when looking for intracellular bacteria such as N. gonorrhoeae (urethral, cervical, or eye swab). Rolling the swab avoids damaging the pus cells.

● **Faeces: Use** a piece of clean stick to transfer pus and mucus to a slide. Decontaminate the stick before discarding it. Spread to make a thin prep- aration.

# Smear fixation

- The purpose of fixation is to preserve micro- organisms and to prevent smears being washed from slides during staining.
- Smears are fixed by heat, alcohol (70% ethanol or methanol), or occasionally by other chemicals (formaldehyde).



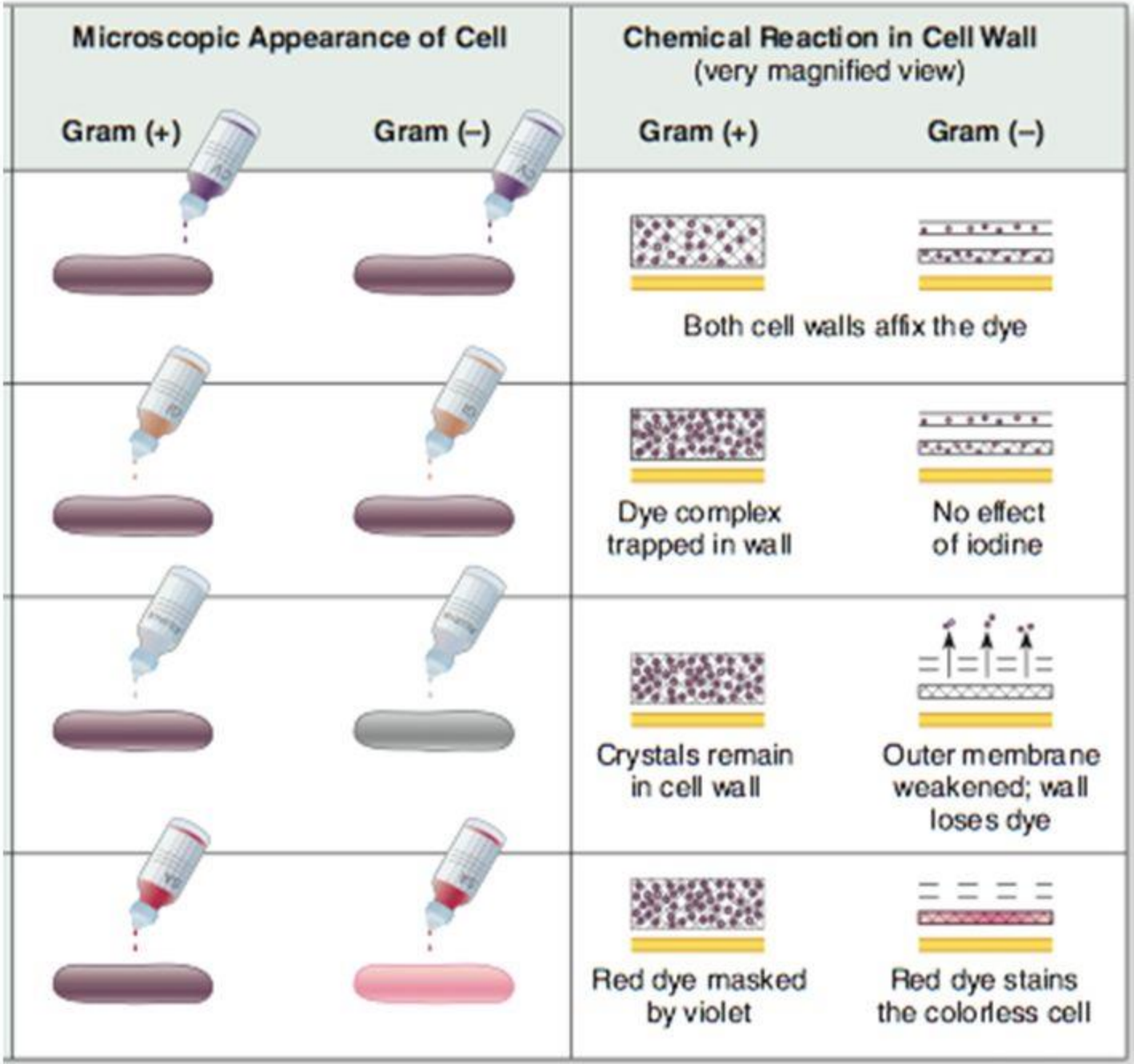
# GRAM STAIN PROCEDURE

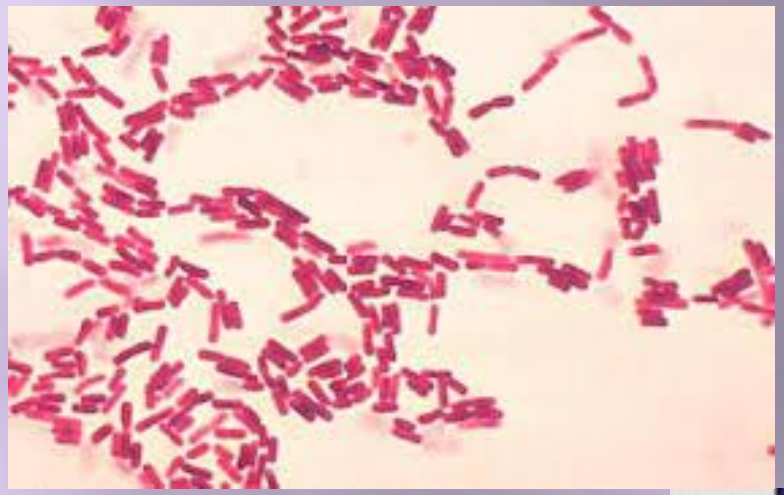
1. Flood with crystal violet for 1 minute. Rinse

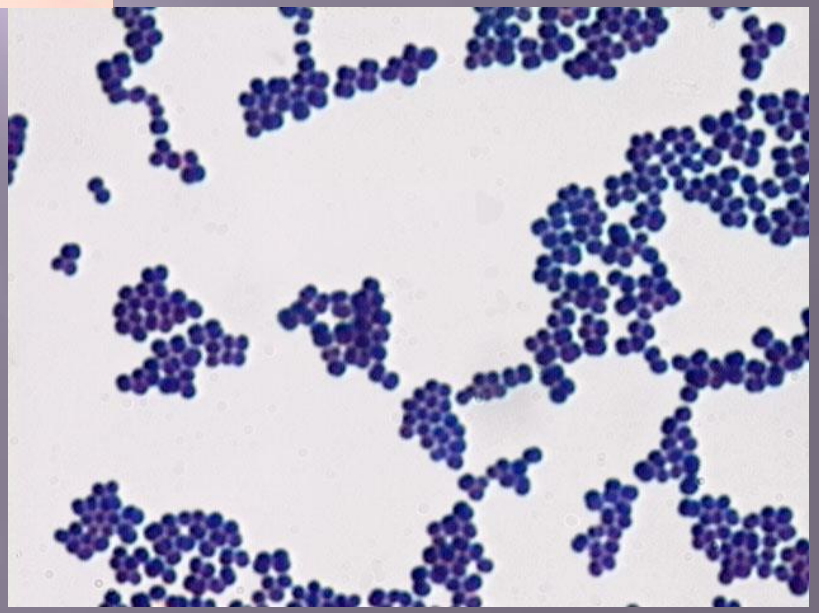
2. Flood with iodine for 1 minute. Rinse

3. Decolorize with alcohol until no more purple comes off the slide. Rinse

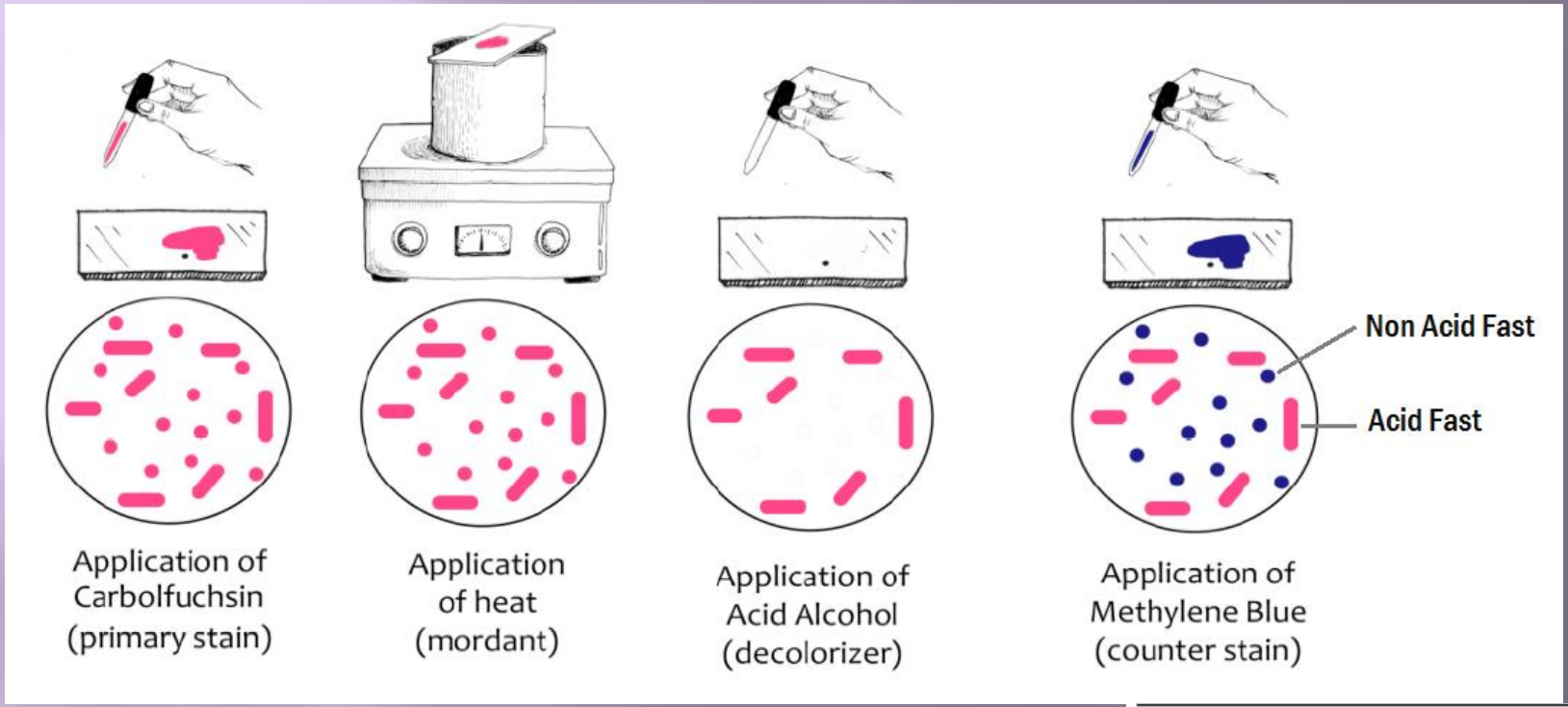
4. Flood with safranin. Rinse and dry.







# Acid fast staining




More than 10 AFB/field . . . . . report +++
1–10 AFB/field . . . . . report ++
10–100 AFB/100 fields . . report +
1–9 AFB/100 fields . . . . . report the exact number

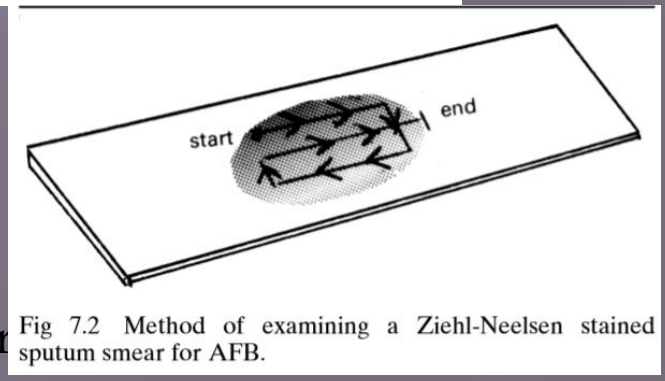
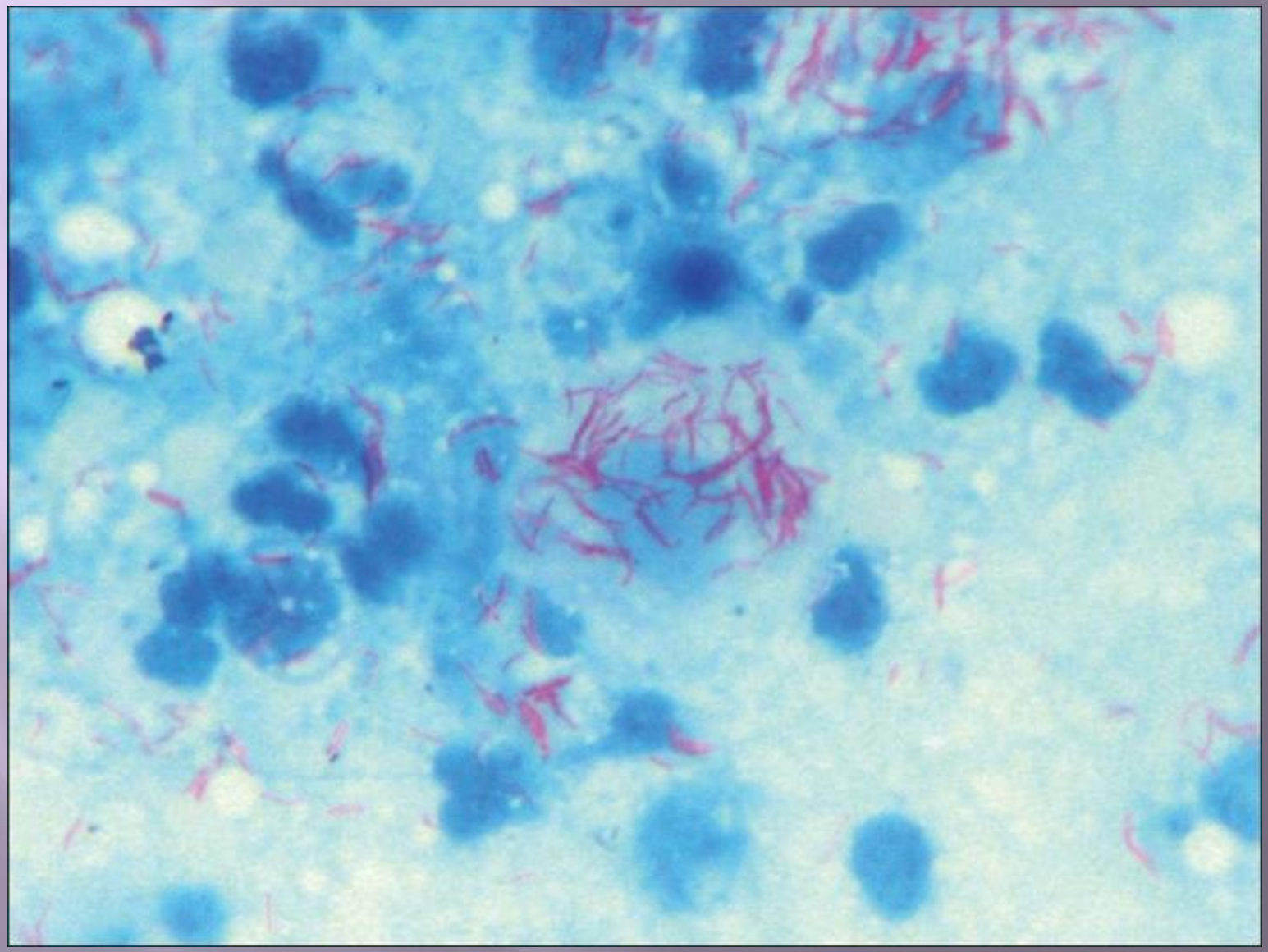
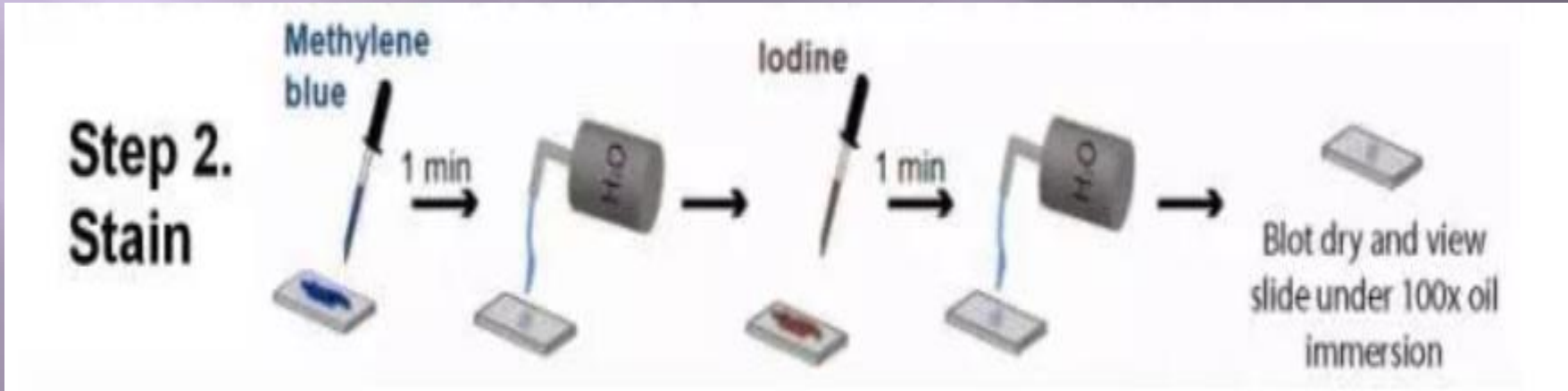



Fig 7.2 Method of examining a Ziehl-Neelsen stained sputum smear for AFB.

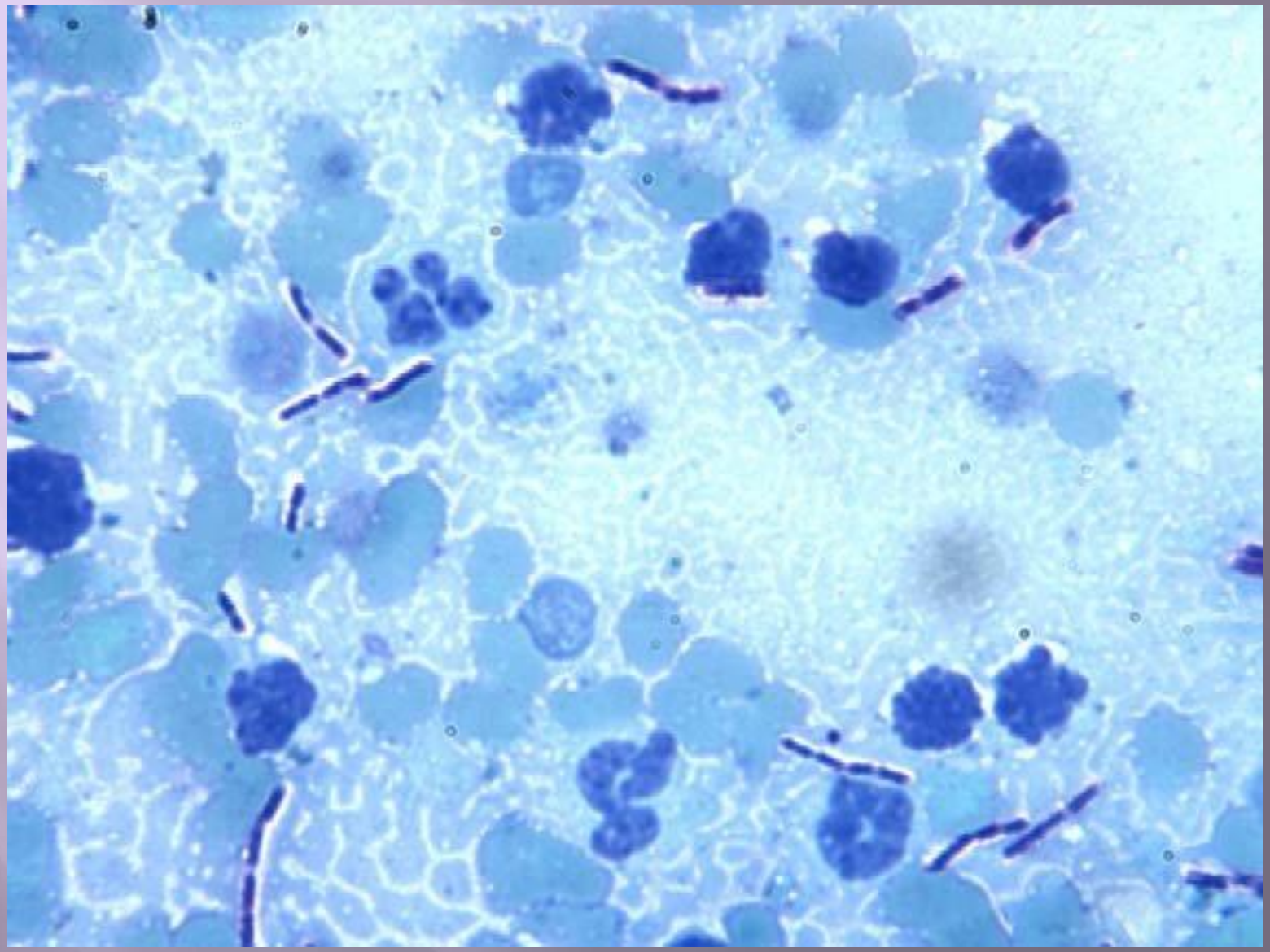


# Methylene blue staining

- Loeffler's methylene blue Reagent No. 51 or Polychrome Loeffler methylene blue (B. anthrax).
- When anthrax is suspected, fix the smear with potassium permanganate for 10 minutes.




Simple Staining Procedure

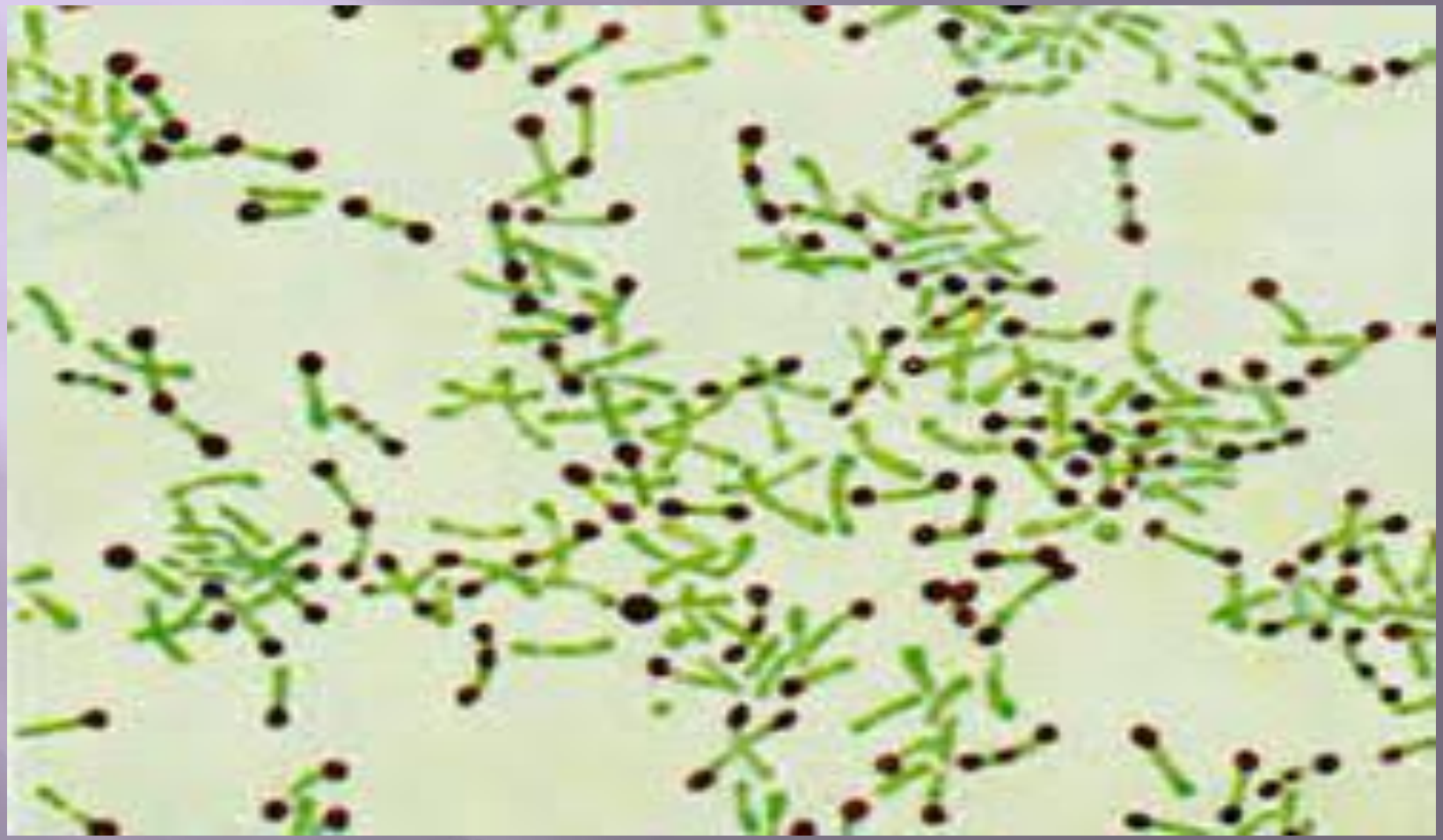


# Albert's staining

1. Fix the dried smear using alcohol
2. Cover the smear with the toluidine blue- malachite green stain for 3–5 minutes.
3. Wash off the stain with clean water.
4. Tip off all the water.
5. Cover the smear with Albert's iodine for 1 minute. Wash off with water.
6. Wipe the back of the slide clean, and place it in a draining rack for the smear to air-dry.
7. Examine the smear microscopically, first with the 40 objective to check the staining and to see the distribution of material and then with the oil immersion lens to look for bacteria containing metachromatic granules.

For Corynebacterium species

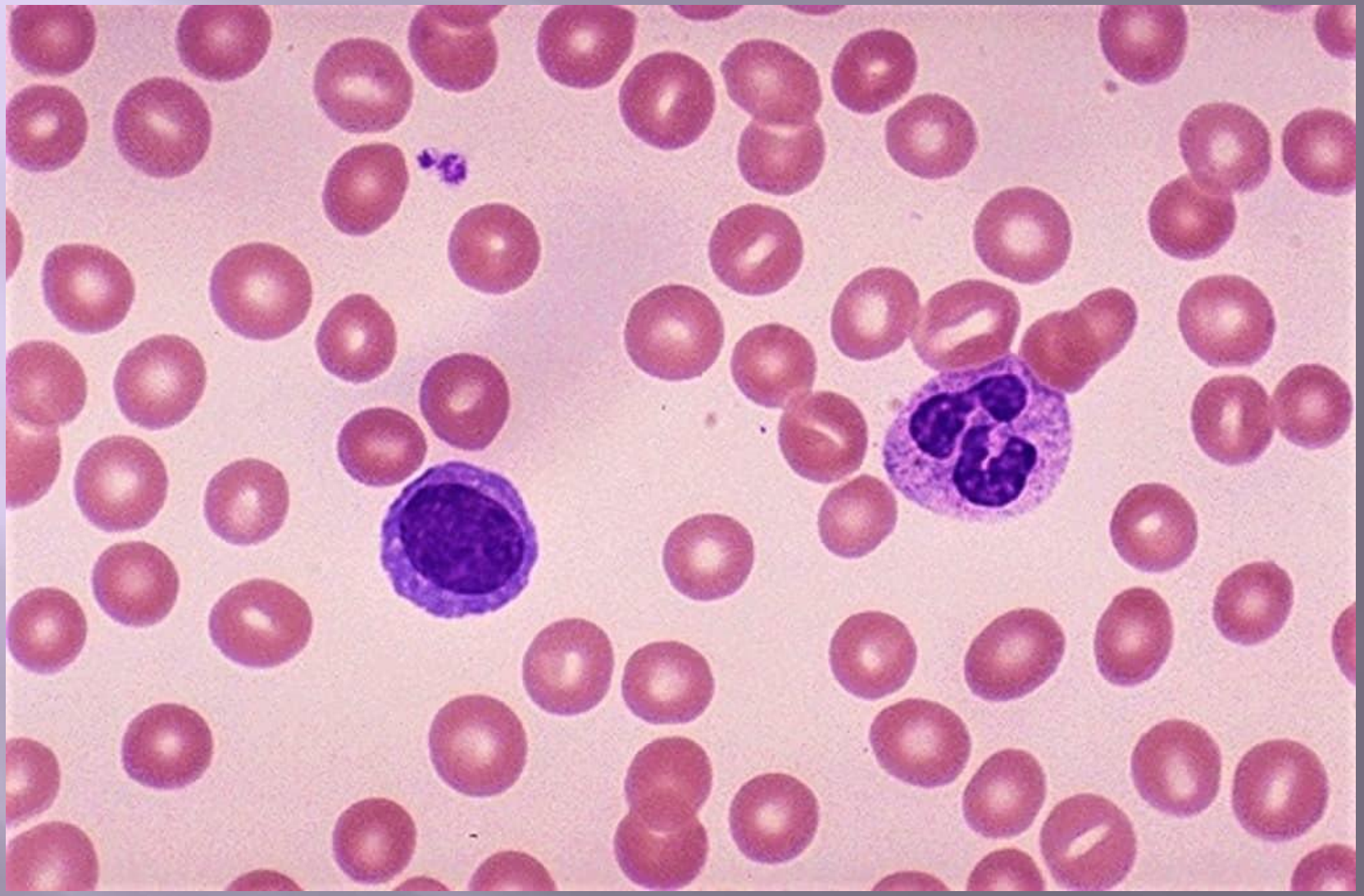
# Corynebacterium diphtheria



# Geimsa staining

- a type of Romanowsky stain, named after Gustav Giemsa, a German chemist who created a dye solution.
- was primarily designed for the demonstration of malarial parasites (1:40 dilution) in blood smears, but it is also employed in histology for routine examination of blood smear.

  - Fix air-dried film in absolute methanol by dipping the film briefly (two dips) in a Coplin jar containing absolute methanol.
  - Remove and let air dry.
  - Stain with diluted Giemsa stain (1:20, vol/vol) for 20 min *(For a 1:20 dilution, add 2 ml of stock Giemsa to 40 ml of buffered water in a Coplin jar).*
  - Wash by briefly dipping the slide in and out of a Coplin jar of buffered water (one or two dips).
  - Let air dry in a vertical position. Observe under the microscope first at 40X and then using oil immersion lens



# CULTURING

# DIFFERENT TYPES OF CULTURE MEDIA

- For a culture medium to be successful in growing the pathogen sought it must provide all essential nutrients, ions, and moisture, maintain the correct pH and osmotic pressure, and neutralize any toxic materials produced.
- It is also essential to incubate the inoculated medium in the correct atmosphere, at the optimum temperature and for an adequate period.
- The main types of culture media are:
  - ● Basic
  - ● Enriched
  - ● Selective
  - ● Indicator
  - ● Transport
  - ● Identification

# Types of media

- **Basic media**: simple media such as nutrient agar and nutrient broth supporting the growth of microorganisms that do not have special nutritional requirements.
  - often used in the preparation of enriched media + to maintain stock cultures of control strains of bacteria + for sub-culturing pathogens from differential or selective media
- **Enriched media**: required for the growth of organisms with exacting growth requirements such as H. influenzae, Neisseria species, and some Streptococcus species.
  - **Basic media** may be enriched with whole or lyzed blood, serum, peptones, yeast extract, vitamins and other growth factors.
- **Selective media**: These are solid media which contain substances (e.g. bile salts or other chemicals, dyes, antibiotics) which inhibit the growth of one organism to allow the growth of another to be more clearly demonstrated.
  - P. aeruginosa is inhibited by anaerobic conditions.
  - V. cholerae can be isolated on an alkaline medium such as TCBS agar.
  - Listeria monocytogenes can grow at 4 C
- **Indicator (differential) media**: These are media to which dyes or other substances are added to differentiate microorganisms. Many differential media distinguish between bacteria by incorporating an indicator which changes colour when acid is produced following fermentation of a specific carbohydrate e.g. MacConkey agar.

Preparation of culture media→ dissolve powder media (contents) into water → agar for semi solid media

Sterilization of media + all other glass wares required

Pouring of media (solid= sterile petri-plates, broth= sterilize test tube)

24 hours sterility test

Inoculation of sample onto media

Incubation of cultured plates at 37oC for 18-24hrs

Observation of appearance of colonies


Fig 7.6 Inoculation of a plate of culture medium to give single colonies

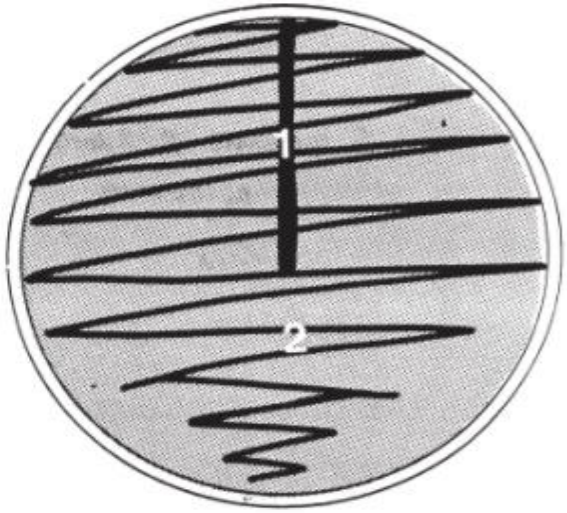



Fig 7.7 Simplified technique of inoculating a plate of culture medium, suitable for use in clinics

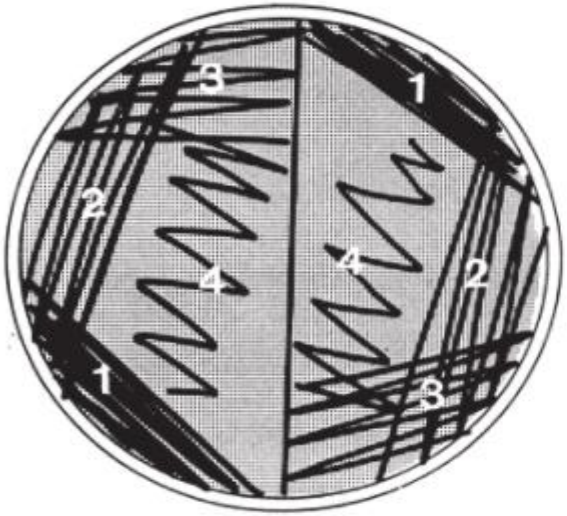



Fig 7.8 Inoculation of half a plate of culture medium

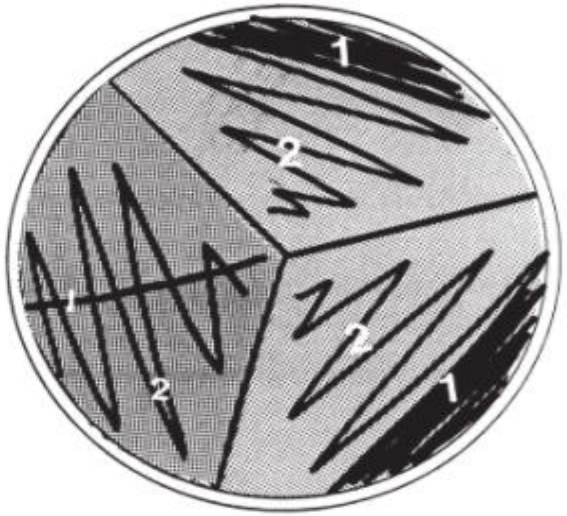



Fig 7.9 Different ways of inoculating a third of a plate of culture medium

Fig 7.10 Inoculation of an agar slope

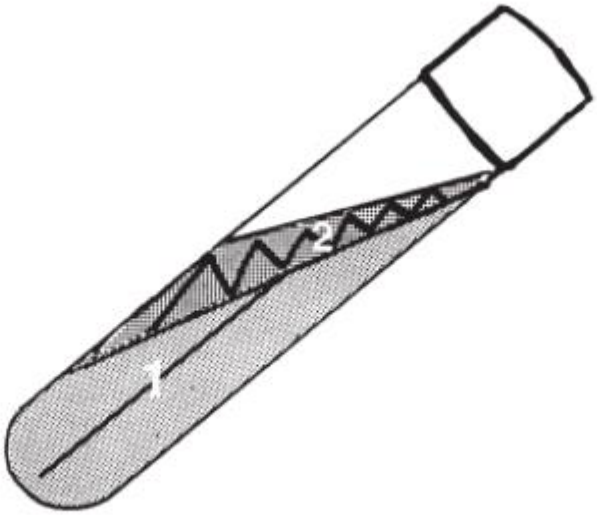



Fig 7.11 Inoculation of a butt and slope. Use a straight line to inoculate the butt first


Fig 7.12 Inoculation of a deep (stab)

# EXAMINATION OF SPUTUM

# Possible pathogens

- BACTERIA

| Gram positive | Gram negative |
| --- | --- |
| Streptococcus pneumoniae<br>Haemophilus influenzae<br>Staphylococcus aureus<br>Streptococcus pyogenes | Klebsiella pneumoniae<br>Pseudomonas aeruginosa<br>Proteus species Yersina pestis Moraxella catarrhalis |

- Also Mycobacterium tuberculosis, Mycoplasma pneumoniae, and Legionella pneumophila.
- FUNGI AND ACTINOMYCETES
- Pneumocystis jiroveci, Blastomyces dermatitidis, Histoplasma capsulatum, Aspergillus species, Candida albicans, Cryptococcus neoformans, and Nocardia species.
- PARASITES
- Paragonimus species

# Describe the appearance of the specimen

- Describe whether the sputum is:
- Purulent: Green-looking, mostly pus
- Mucopurulent: Green-looking with pus and mucus
- Mucoid: Mostly mucus
- Mucosalivary: Mucus with a small amount of saliva
- When the sputum contains blood, this must also be reported.

## Summary of Microbiological Examination of Sputum

**Day 1**

| | | | ADDITIONAL INVESTIGATIONS |
|---|---|---|---|
| 1 | **Describe Specimen** | ■ ***Report whether specimen:***<br>– purulent, mucopurulent, mucoid, salivary<br>– contains blood | |
| 2 | **Examine Microscopically** | ■ ***Gram smear:*** For pus cells and bacteria<br>■ ***Zn smear:*** For AFB | ■ ***Giemsa smear:*** When pneumonic plague or histoplasmosis is suspected<br>■ ***KOH preparation:*** When *Aspergillus* infection is suspected<br>■ ***Toluidine blue-O and Giemsa smears:*** When *Pneumocystis* pneumonia is suspected<br>■ ***Eosin preparation:*** When an allergic condition requires investigation<br>■ ***Saline preparation:*** When paragonimiasis is suspected |
| 3 | **Culture Specimen** | ■ ***Blood agar***<br>– Add an optochin disc<br>– Incubate aerobically<br>■ ***Chocolate agar***<br>– Incubate in $CO_2$ | ■ ***Culture for M. tuberculosis*** (In Reference Laboratory) See text |

## Day 2 and Onwards

**4 Examine and Report Cultures**

- ***Blood and chocolate agar cultures***
  Report *significant* growth of:

  *S. pneumoniae*
  *H. influenzae*
  *S. aureus*

  Less commonly found pathogens:

  *K. pneumoniae, P. aeruginosa, M. catarrhalis, S. pyogenes, Proteus, C. albicans*

- Test *H. influenzae* for *beta*-lactamase production
- Antimicrobial susceptibility tests as required

# EXAMINATION OF PUS/ULCERS

# Possible pathogens

- It is impossible to list all the pathogens that may be found in pus. Those listed are the more commonly isolated pathogenes from wounds, abscesses, burns, and draining sinuses.
- **BACTERIA**
- Gram positive — Gram negative
  - Staphylococcus aureus — Pseudonomas aeruginosa
  - Streptococcus pyogenes — Proteus species
  - Enterococcus species — Escherichia coli
  - Anaerobic streptococci
  - Bacteriodes species Other streptococci — Klebsiella species
  - Clostridium perfringens — Pasteurella species and other clostridia
- Actinomycetes : Actinomyces israeli
- Mycobacterium tuberculosis
- **FUNGI** : Histoplasma c. duboisii, Candida albicans,
- **PARASITES :** Entamoeba histolytica (in pus aspirated from an amoebic liver abscess),
- **COMMENSAL**s Any commensal organisms found in pus are usually those that have contaminated the specimen from skin, clothing, soil, or from the air if an open wound

## Summary of Microbiological Examination of Pus, Ulcer Material and Skin Specimens

**Day 1**

| | | | ADDITIONAL INVESTIGATIONS |
|---|---|---|---|
| 1 | **Describe Specimen** | | ■ ***Look for granules:*** When mycetoma or actinomycosis is suspected |
| 2 | **Culture Specimen** | ■ ***Blood agar*** Incubate aerobically<br>■ ***MacConkey agar*** Incubate aerobically<br>■ ***Cooked meat medium*** Subculture at 24 h, 48 h, and 72 h as indicated<br>■ ***Neomycin blood agar when anaerobic infection is suspected*** Incubate anaerobically up to 48 h | ■ ***Culture for M. tuberculosis or M. ulcerans*** Requires facilities of a Tuberculosis Reference Laboratory |
| 3 | **Examine Microscopically** | ■ ***Gram smear*** For pus cells and bacteria | ■ ***Ziehl-Neelsen smear:*** When tuberculosis or *M. ulcerans* disease is suspected<br>■ ***KOH preparation:*** When a fungal or actinomycete infection is suspected<br>■ ***Giemsa or Wayson's smear:*** When bubonic plague is suspected<br>■ ***Polychrome methylene blue:*** When cutaneous anthrax is suspected<br>■ ***Dark-field microscopy:*** To detect treponemes when yaws or pinta is suspected |

## Day 2 and Onwards

**4 Examine and Report Cultures**

- ***Blood agar and MacConkey agar cultures***
  Look particularly for:
  - *S. aureus*
  - *S. pyogenes*
  - *P. aeruginosa*
  - *Proteus species*
  - *E. coli*
  - *Enterococcus* species
  - *Klebsiella* species
  - Anaerobes:
    - *C. perfringens*
    - *Bacteroides fragilis* group
    - *Peptostreptococcus* species
- ***Antimicrobial susceptibility tests***
  As indicated

# EXAMINATION OF URINE SAMPLE

# Possible pathogens

- **BACTERIA**
- Gram positive

Staphylococcus saprophyticus
Haemolytic streptococci

Gram negative
Escherichia coli s
Proteus species
Pseudomonas aeruginosa
Klebsiella strains
Salmonella Typhi
Salmonella Paratyphi
Neisseria gonorrhoeae

These species are not primarily pathogens of the urinary tract, but may be found in urine.

- **Mycobacterium tuberculosis, Leptospira interrogans, Chlamydia, Mycoplasma and Candida species.**
- **PARASITES** Schistosoma haematobium, Trichomonas vagi- nalis, and occasionally Enterobius vermicularis, Wuchereria bancrofti and Onchocerca volvulus.

# Summary of the Microbiological Examination of Urine

**Day 1**

| | | | |
|---|---|---|---|
| **1** | **Describe Appearance** | ■ *Describe*<br>– Colour<br>– Whether clear or cloudy | ADDITIONAL INVESTIGATIONS |
| **2** | **Examine Microscopically** | ■ *Wet preparation*<br>Report:<br>– WBCs (pus cells)<br>– Red cells<br>– Casts<br>– Yeast cells<br>– *T. vaginalis* flagellates<br>– *S. haematobium* eggs<br>– Bacteria (fresh urine only)<br>– Crystals of importance | ■ ***Gram smear***: When bacteria or WBCs (pus cells) are seen in wet preparation. |
| **3** | **Test Biochemically** | ■ ***Tests to help diagnose UTI***<br>– Protein<br>– Nitrite (Greiss test)<br>– Leukocyte esterase (when microscopy for WBCs not possible) | ■ ***Glucose, ketones, bilirubin, urobilinogen***:<br>As indicated |
| **4** | **Culture Specimen** | ■ *CLED agar*<br>When bacteria and, or pus cells are present:<br>– Inoculate CLED medium<br>– Incubate aerobically | |

## Day 2 and Onwards

**5 Examine and Report Cultures**

- ***CLED culture***

  Look particularly for:

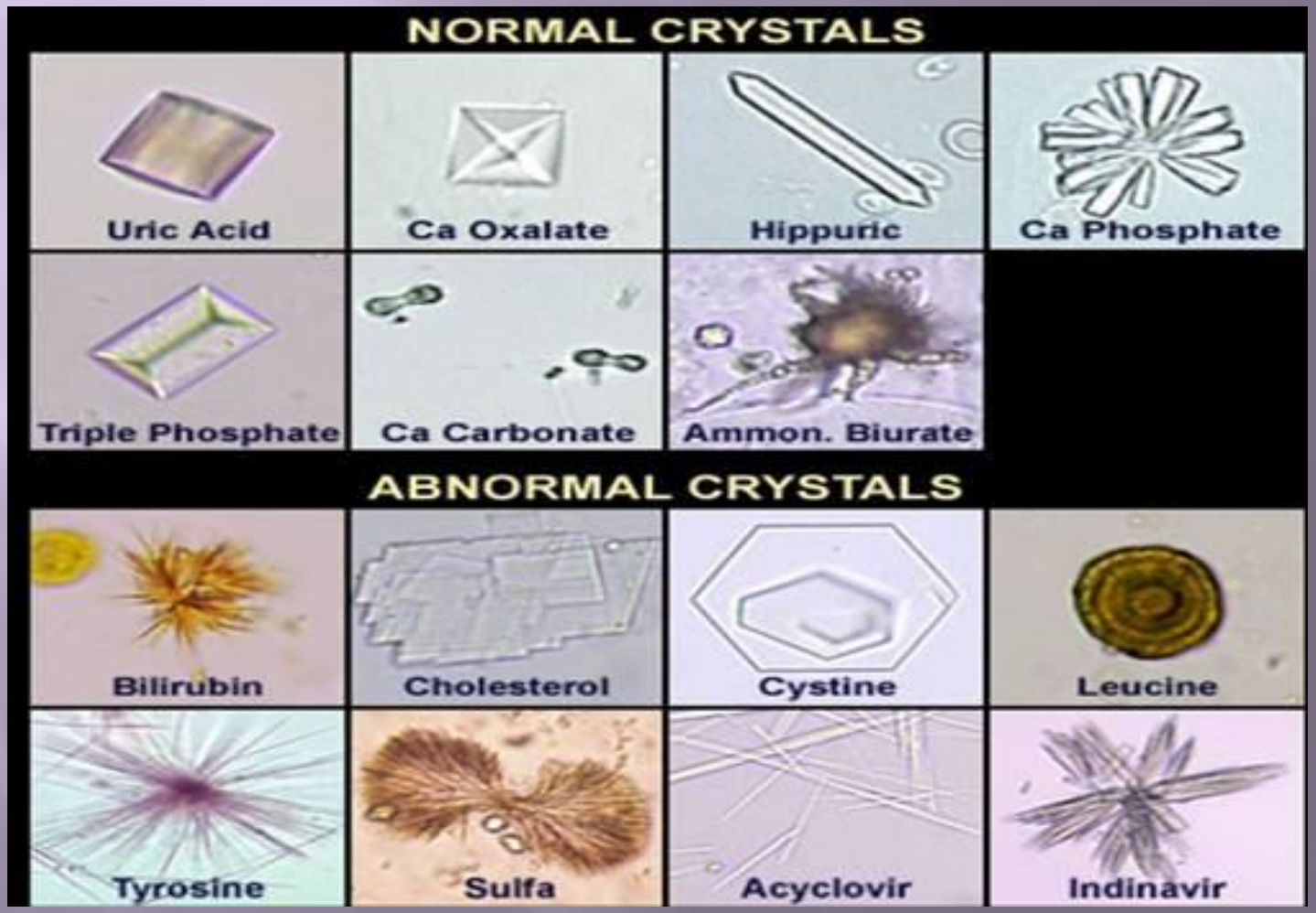
  *E. coli* (common cause UTI)
  *Proteus* species
  *P. aeruginosa*
  *Klebsiella*
  *E. faecalis*
  *S. aureus*
  *S. saprophyticus*

  Report bacterial numbers:
  - Less than $10^4$/ml, not significant
  - $10^4$–$10^5$/ml, doubtful significance
  - More than $10^5$/ml, significant bacteriuria.

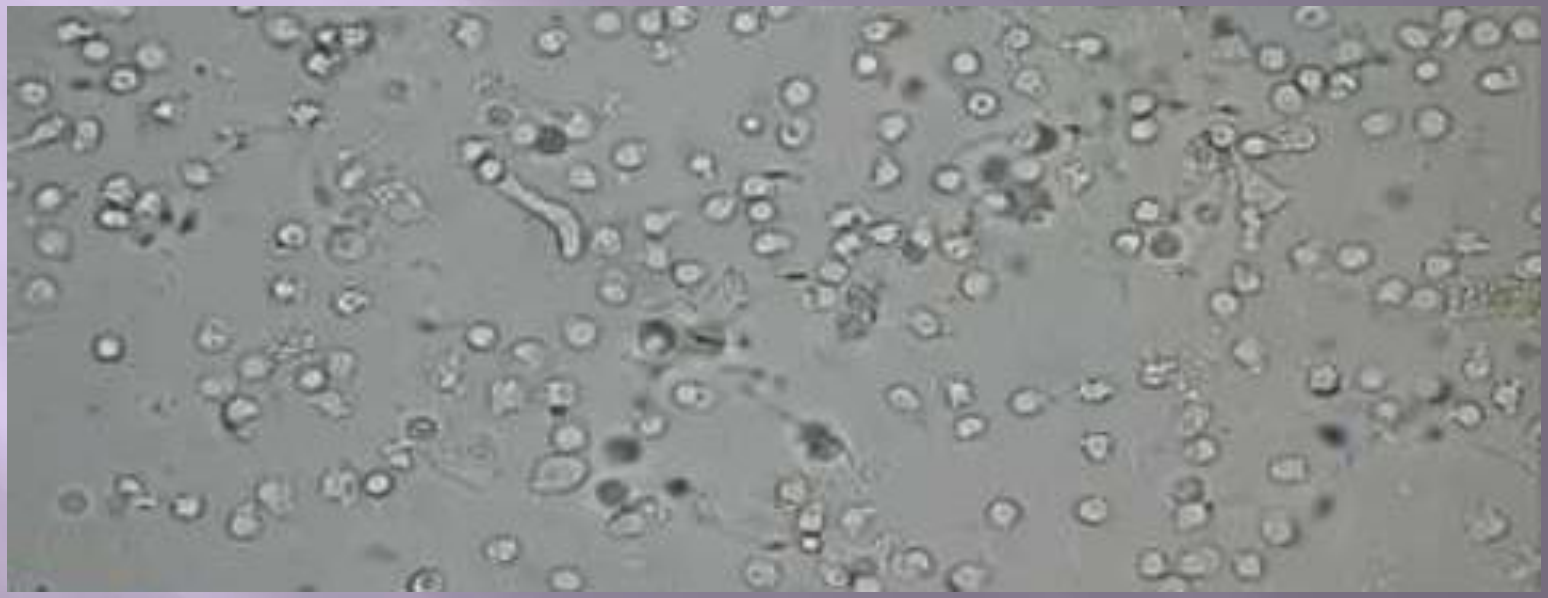
- ***Antimicrobial susceptibility testing*:**

  As indicated

# Crystals in urine



# Pus cells in urine



# EXAMINATION OF URO-GENETICAL SPECIMEN

## Summary of Microbiological Examination of Urogenital Specimens

**Day 1**

| | | ADDITIONAL INVESTIGATIONS |
|---|---|---|
| **1 Culture Specimen** | ■ ***MNYC medium or Thayer-Martin medium***<br>– Incubate in $CO_2$ (moist environment) | When puerperal *or septic abortion is suspected*:<br>■ ***Blood agar (2 plates)***<br>– Incubate aerobically<br>– Incubate anaerobically<br>■ ***MacConkey agar***<br>– Incubate aerobically<br>■ ***Cooked meat medium***<br>– Incubate overnight. Subculture as indicated at 24 h, 48 h, 72 h. |
| **2 Examine Microscopically** | ■ ***Gram smear***<br>– *Urethral:* Intracellular Gram negative diplococci<br>– *Vaginal*: Yeast cells (candidiasis) Clue cells (bacterial vaginosis)<br>– *Vaginal/cervical:* Pus cells and bacteria associated with puerperal sepsis and septic abortion<br>■ ***Wet preparation***<br>Motile *T. vaginalis* | ■ ***Dark-field:*** When syphilis is suspected<br>■ ***Giemsa smear:*** When *K. granulomatis* infection (donovanosis) is suspected<br>■ ***Cervical smear(s) sent to histology/cytology laboratory:*** When malignancy is suspected |

## Day 2 and Onwards

**3 Examine and Report Cultures**

- ***MNYC plate or Thayer-Martin plate***
  - Examine for *N. gonorrhoeae*

***Colonies resembling N. gonorrhoeae:***

- Oxidase test
- Gram stain colonies
- *Beta*-lactamase test

- ***Blood agar, MacConkey agar plates***
  - Look especially for:

    *S. pyogenes*
    *S. aureus*
    *C. perfringens*
    *Proteus*
    *Enterococcus*
    *E. coli*
    *Bacteroides*
  - Antimicrobial susceptibility tests as required