Main Steps in Bacteriological Diagnosis

The diagnostic cycle

Laboratory testing – series of events:

I. Pre-analytical

II. Analytical = observation & isolation & identification & enumeration

III. Post-analytical

Performance must be monitored throughout the entire cycle for quality assurance i.e. accurate, reliable results

Clinical & bacteriological diagnosis of infectious diseases

PRE-ANALYTICAL:

Patient consulted by physician ▼

Physician → tentative clinical diagnosis ▼

Specimens: Collection & labels on containers ▼

Request form + specimens ▼

Lab receives samples; data recorded

ANALYTICAL ▶ ▶

Direct examination: smears, stains ▶

POST-ANALYTICAL: Final report elaborated & sent to physician

Subcultures + results of identification systems ▲

Cultures examined; identification systems ▲

Culture media selected, inoculated, incubated ▲

Presumptive reports, preliminary results ▲

Pre-analytic phase: Specimen collection

- Crucial for confirming a certain microorganism as cause of the clinically suspected infectious disease
- Improper specimen collection may cause:
  - Failure to recover the microorganism (no growth on culture medium)
  - Incorrect / harmful therapy e.g directed against a comensal / contaminant microorganism

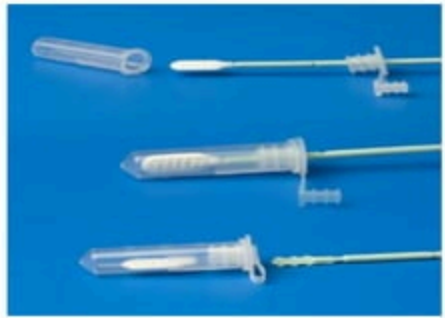
  E.g. *Klebsiella pneumoniae*:

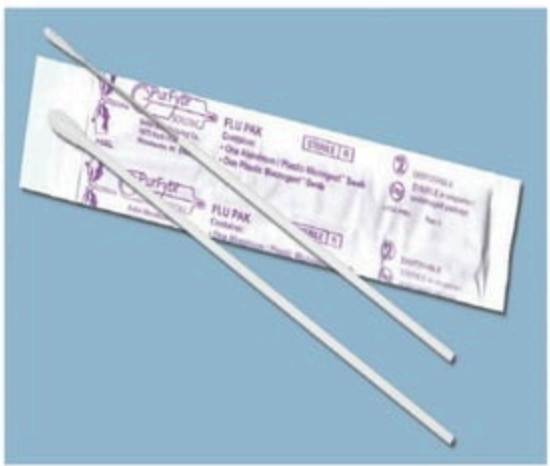
  - recovered from sputum of pneumonia patient;
  - recognised causative agent of pneumonia BUT also may colonize the naso-pharynx
  - If sputum sample consisted mostly of saliva then isolating *K.pneumoniae* might not reflect the true cause of the patient's pneumonia but saliva contamination of the sputum sample

Pre-analytic phase: Specimen collection (continued)

Rules for correct specimen collection:

1. **Source**: actual infection site; minimal contamination from adjacent tissues, organs, secretions e.g. throat swabs from peritonsillar fossae and posterior pharyngeal wall, avoiding contact with other oral areas
2. **Optimal moment**: depending on the natural history and pathophysiology of the infectious process e.g. Typhoid fever: blood – 1st week; feces and urine – 2nd-3rd week
3. **Sufficient quantity**

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FLU PAK

Pre-analytic phase: Specimen collection (continued)

Rules for correct specimen collection (continued):

4. **Appropriate collection devices, containers**

   + transport systems (container ± transport medium): main objective to decrease time between collection and inoculation to prevent lack of recovery of certain bacteria

5. **Sample collection before antibiotics** (if possible)

6. **Smears performed to supplement culture** (if possible)

   - Assessment of inflammatory nature of specimen → aid the clinical integration (meaningfulness) of the culture result
   - Gram smears e.g. Gram negative bacilli + no growth on aerobic culture (wrong atmosphere or wrong media i.e. fastidious microbes e.g. *Legionella*)

Pre-analytic phase: Specimen collection (continued)

Rules for correct specimen collection (continued):

7. Labeling of specimen containers & **Request form**:

- Legible
- Minimun information:
  - Patient name; identification number (hospital file, practice log book, etc)
  - Source of specimen; clinician + contact data (phone no)
  - Date and hour of collection
  - Clinical diagnosis (suspected infection)
  - Treatments (antibiotics?...)

Pre-analytic phase: Transport

Main transport related objectives:

- Sample related: Transport media
  - Maintain the sample as similar to its original state as possible
- Human & environment related: Packaging and transport systems and regulations
  - Prevent contamination/infection of healthcare staff & environment & general population (biosafety)

Pre-analytic phase: Transport (continued)

Transport media

Pre-analytic phase: Transport (continued)

"Triple" Packaging of biological samples:

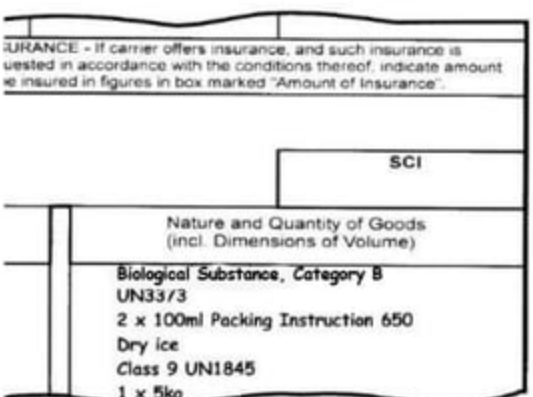
- Outer box (usually cardboard, rigid, secure closure system, adequately labeled to state content)
- Inner container (waterproof, resistant to pressure, usually plastic, securely closed by lid, contains additional materials to absorb shocks e.g. bubbled plastic bags and leackages e.g. absorbent material
- Sample containers (tubes, plates) inserted in the inner plastic container, wrapped in above mentioned shock- and fluid absorbent materials
- + request form and other documents inserted in sealed plastic bags, inserted in outer box or sticked to its exterior

Triple packaging

Pre-analytic phase: Transport (continued)

Shipment of biological specimeens:

Packaging and transport systems and regulations: IATA (ICAO), ADR...



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Specimen receipt & preliminary observations (continued)

Examples of acceptance/rejection criteria (checklist):

1. Request form & labels contain all info required (check for consistency !!!)
2. Improperly packaged, leacking / broken containers
3. Time from collection to receipt – too long to allow recovery
4. Improper / lack of transport media
5. Insufficient quantity e.g. single swab for multiple requests
6. Overgrown / dried out culture plates
7. .....etc.....

Each lab must have such a list and share it with collaborators!

Rejecting samples must be avoided as much as possible!

Collection & transport requirements must be shared with clinicians!!!

Specimen receipt & preliminary observations

- Specially designed area / room for receiving and recording samples
- Rules for manipulating samples and accompanying documents (UNIVERSAL PRECAUTIONS):
  - Samples: biological safety cabinet (BSC), personal protective equipment (PPE): lab coat, gloves, eye&respiratory protection
  - Documents – handled by different person / at different stage e.g. either before or after preliminary examination/processing of sample (after removal of gloves & hand washing) – purpose: avoid cross contamination of objects (log record book, computer, pens, etc)

Specimen receipt & preliminary observations (continued)

Preliminary actions upon receipt of specimens

1. Data entry into lab log book/computer database
2. (Unpacking and) visual examination – check for acceptance criteria (see next slide)

   +

3. Microscopic examination of direct mounts/stained smears → presumptive diagnosis
4. Sample(s) taken to area/room where the analytical phase begins

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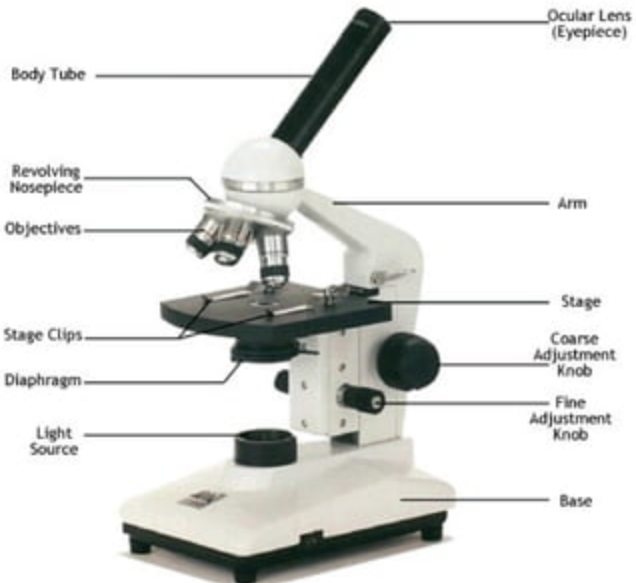
Bacterial infections:
direct identification & characterization methods

- Microscopy
- Cultivation
- Antimicrobial sensitivity

Microscopy

- Types of microscopes
  - Optical - Magnification objectives
    - 10x; 40x; **100x for bacteria**
  - Phase contrast
  - Dark field (dark ground)
  - Fluorescence – UV light
  - Electron

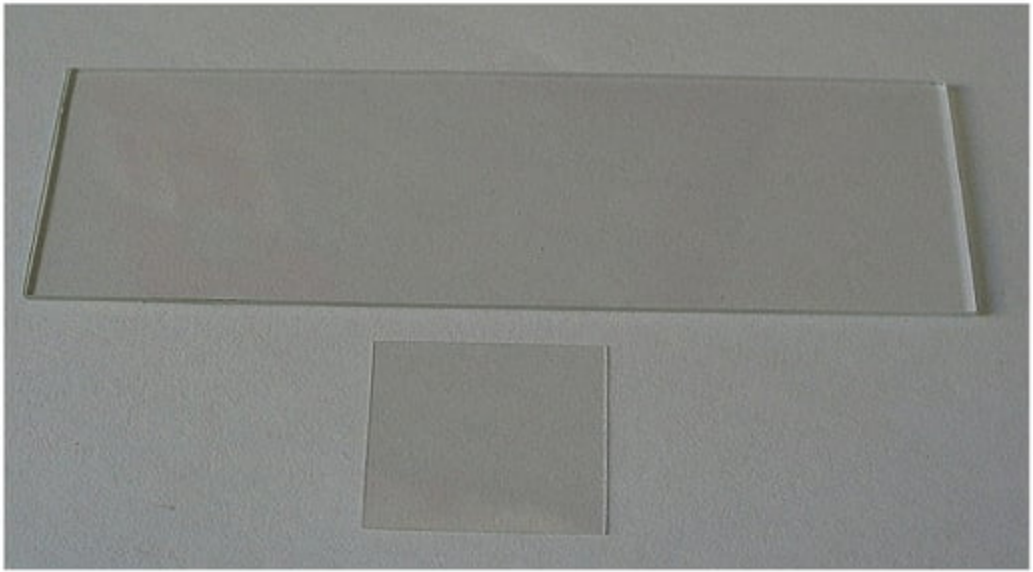
Optical microscope



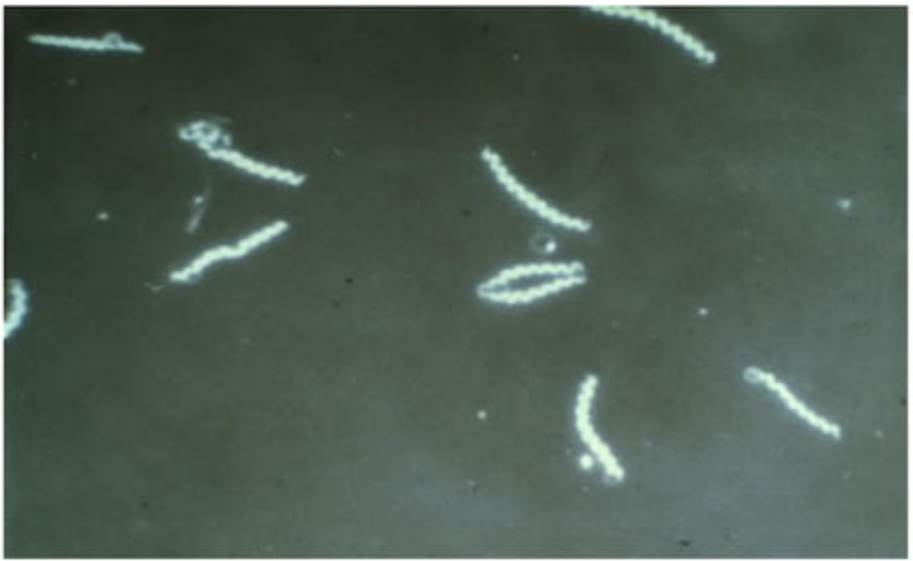
Microscopic examination

- Wet mounts (unstained materials)
  - Direct light
  - Observation of cells (PMN, macrophages), mobile germs in liquid samples (urine, CSF), shape and disposition of germs (cocci/bacilli/spirilli/vibrios)
- Stained smears

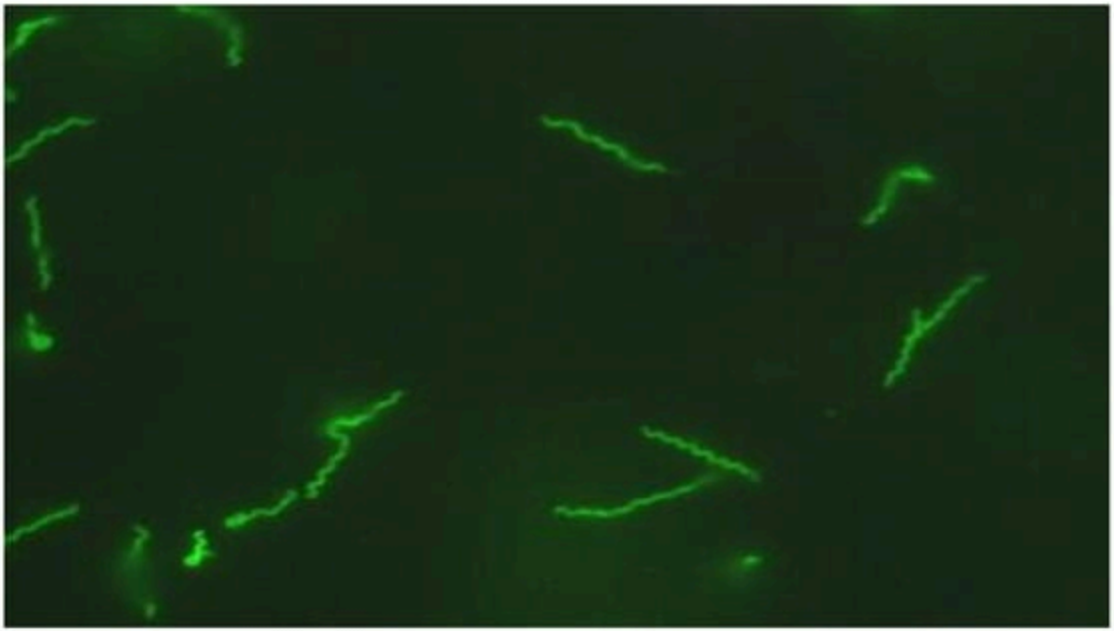
Microscope glass slide and cover slip



Spirochetes – wet mount by dark field microscopy



Treponema denticola – dark field microscopy + fluorescent dye staining



Stained smears

Main steps:

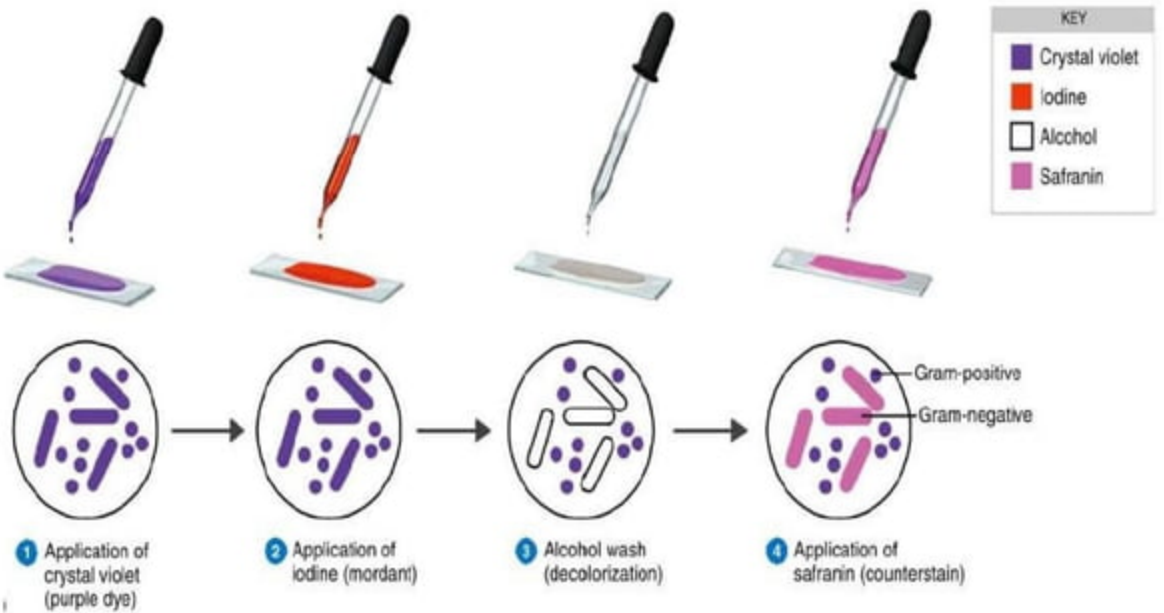
- Smear specimen on microscope glass slide
- Air Drying
- Heat Fixation (flame): help adhesion of specimen to slide, kill bacteria, favour absorbtion of stain on bacterial surface
- Staining:
  - Monostaining e.g. Methyl blue
  - Combined e.g. Gram, Ziehl Nielsen

Gram staining

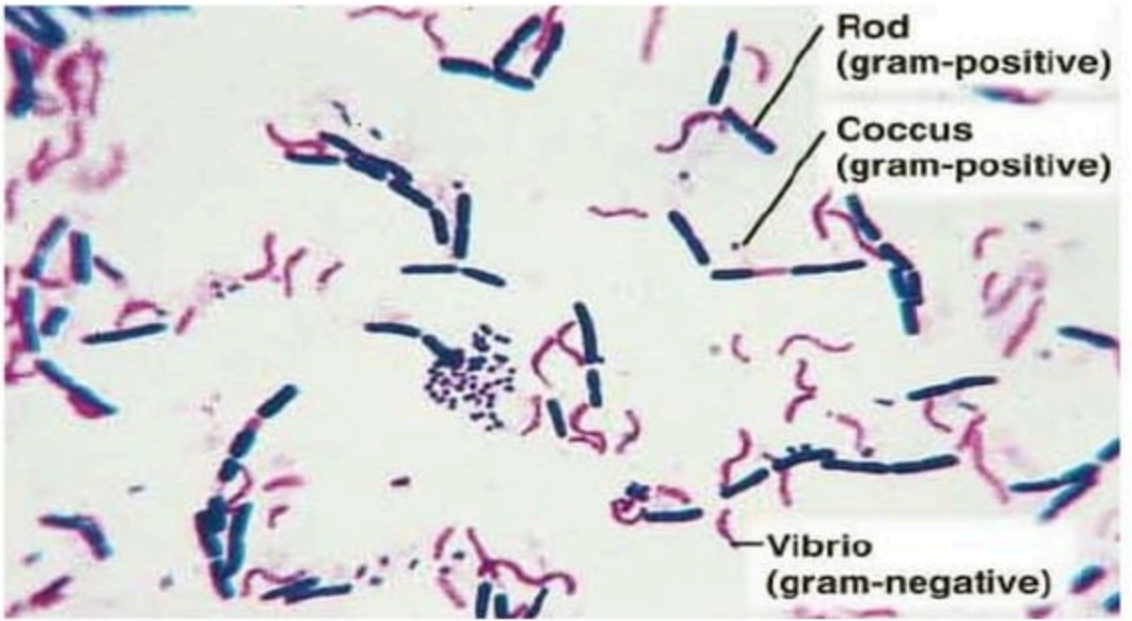
1. heat-fixed smear flooded with crystal violet (**primary stain)**
2. crystal violet is drained off and washed with distilled water
3. smear covered with "Gram's iodine" (Lugol) (a**mordant or helper)**
4. iodine washed off: all bacteria appear dark violet or purple
5. slide washed with alcohol (95% ethanol) or an alcohol-acetone solution (**decolorizing agent)**
6. alcohol rinsed off with distilled water
7. slide stained with safranin, a basic red dye (**counter stain)** 2-3 minutes
8. smear washed again, heat dried and examined microscopically

Exact protocol – depending on the kit

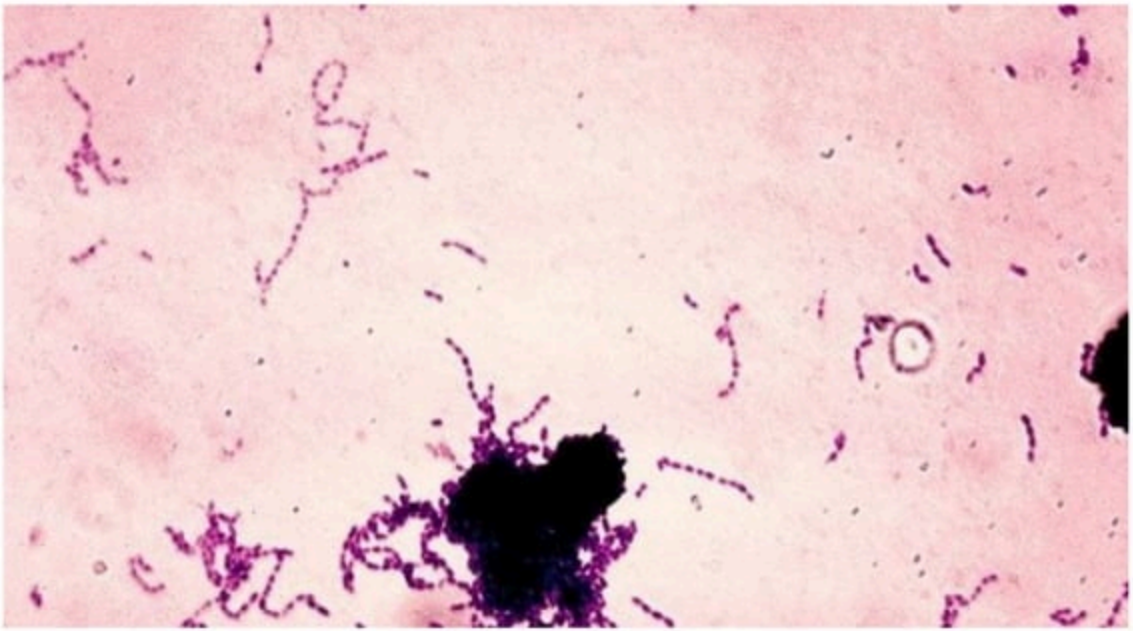
Gram staining



Gram stained smear



Streptococcus mutans – Gram stained smear



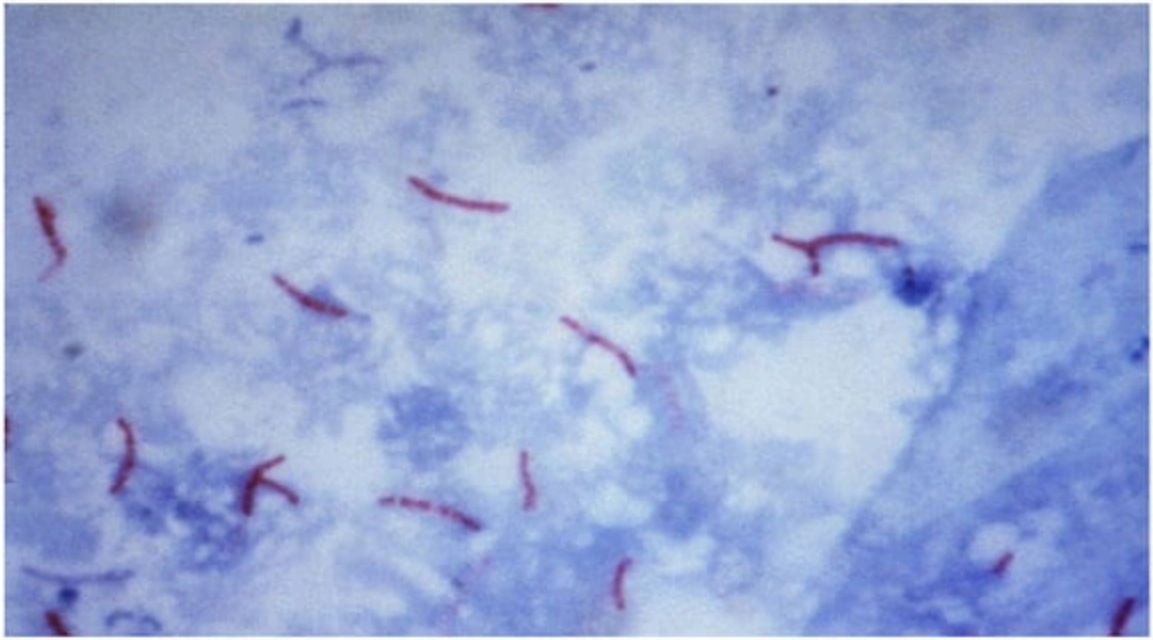
Ziehl-Neelsen Staining

- used to stain *Mycobacterium tuberculosis* and *Mycobacterium leprae* = acid fast bacilli: stain with carbol fuschin (red dye) and retain the dye when treated with acid (due to lipids i.e. mycolic acid in cell wall)

Reagents

- **Carbol fuchsin** (basic dye) - red
- Mordant (heat)
- 20% sulphuric acid (decolorizer) – acid fast bacilli retain the basic (red) dye
- **Methylene blue** (counter stain) – the other elements of the smear, including the background will be blue

Mycobacterium tuberculosis - Ziehl-Neelsen Staining



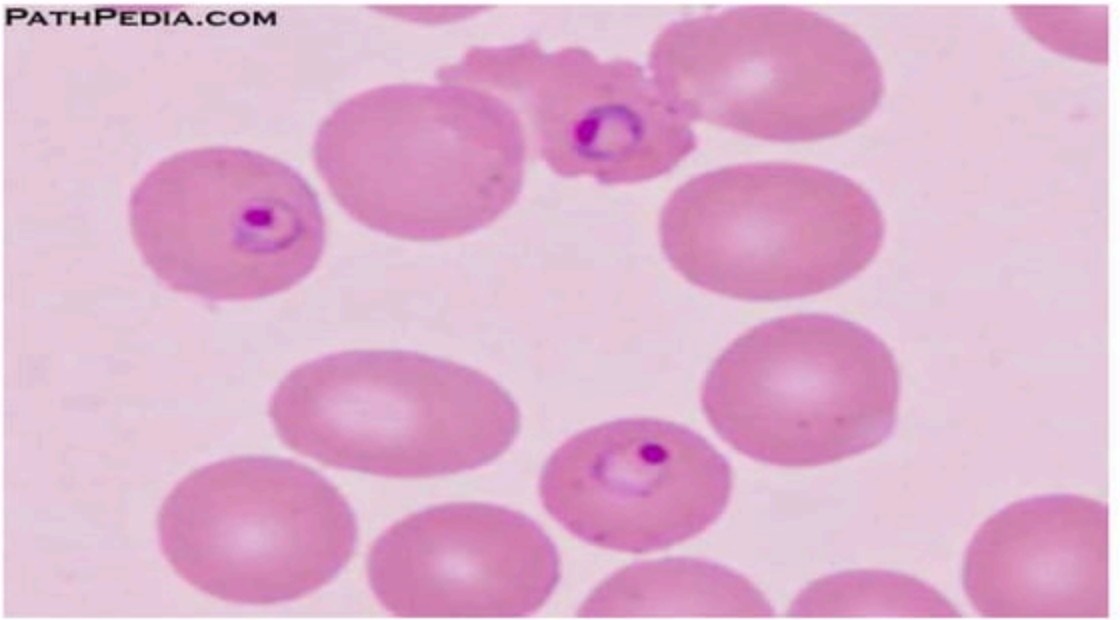
Giemsa staining

- Smears from blood, vaginal / urethral secretion, bone marrow aspirate

Steps:

- Fixation with methanol (2-3 min)
- Coloration with Giemsa solution
- Washing – buffered water
- Drying
- Microscopic examination

Malaria parasites in blood smear (Wright/Giemsa staining)



Microscopy for various biological specimens

- CSF:
  - wet mounts – assess type & no of cells (white/red blood cells)
  - Stained smears from centrifugation sediment: Gram, Ziehl-Neelsen + aditional smear
  - Presumptive causative agents:
    - High no of PMN on wet mount→ bacterial meningitis *Neisseria meningitidis, Haempohilus influenzae*
    - Ziehl-Neelsen stained smear – very important in case M.tuberculosis is suspected (cultures take 2-3 weeks)

Microscopy for various biological specimens

- Pus
  - Gram stained smears: PMN + staphylococci, streptococci
- Urine
  - Gram and Ziehl-Neelsen stained smears prepared from sediment (after centrifugation of specimen)
  - Urinary infection: smear with germs + high no of PMN
- Sputum
  - Prewashing of specimen in several, successive Petri dishes (to remove germs from the pharynx attached to sputum)
  - Gram (staphylococci, streptococci), Ziehl-Neelsen (*M.tuberculosis*)

Bacterial infections: direct identification & characterization methods

- Microscopy
- **Cultivation** (see presentation on culture media)
- Antimicrobial sensitivity

Cultivation of bacteria on culture media

- Purpose: isolated colonies (single microbial species)

- Identification:
  - Gram stained smears (from colonies)
  - Morphology of colonies (shape, dimensions, margins, colour, ...)
  - Changes in the culture medium (e.g. Hemolysis)
  - Biochemical characters:
    - Enzyme secretion (coagulase, catalase, oxydase)
    - Fermentation of sugars
    - Production of H_2S
  - Immune assays: agglutination, immune fluorescence, ELISA

Bacterial infections:
direct identification & characterization methods

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- **Antimicrobial sensitivity**

Antimicrobial sensitivity

- ***In vitro*** testing for the sensitivity of microbes to various antibiotics; expressed as:
  - MIC (minimal inhibitory concentration) – the lowest quantity of amntibiotic completely inhibiting the multiplication of a bacterial strain
  - MBC (minimal bactericidal concentration) – the lowest quantity of antibiotic able to kill 99.9-100% of the germs of a tested bacterial strain

- ***In vivo*** – concentration of antibiotic at the infection site