Microbiology

Principles of Diagnosis

Parameters of Diagnostic معالم Bacteriology

Background Information

Prokaryotes

- Prokaryotes represent two domains, bacteria and archaea.
- Archaea live in Earth's extreme environments.
- Bacteria are the most abundant and diversified organisms on Earth.

Differences between prokaryotic & eukaryotic cells

Character		Prokaryotes	Eukaryotes
Nucleus	Nuclear membrane	Absent	Present
	Nucleolus	Absent	Present
	Chromosome	One circular	One or more paired and linear
Cell division		Binary fission	Mitosis
Cytoplasmic membrane	Structure and Composition	fluid phospholipid bilayer, lacks sterols	fluid phospholipid bilayer containing sterols
	Function	Incapable of endocytosis (phagocytosis and pinocytosis) and exocytosis	Capable of endocytosis and exocytosis

Differences between prokaryotic & eukaryotic cells

Character		Prokaryotes	Eukaryotes
Cytoplasm	Mitochondria	Absent	Present
	Lysosomes	Absent	Present
	Golgi apparatus	Absent	Present
	Endoplasmic reticulum	Absent	Present
	Vacuoles	Absent	Present
	Ribosomes	70 S	80 S

eukaryotic cells

Character		Prokaryotes	Eukaryotes
Cell Wall		Present	Animals & Protozoans – Absent Plants, Fungi & Algae - Present
	Composition	Peptidoglycan – complex carbohydrate	Cellulose or chitin
Locomotor organelles		Flagella	Flagella/ Cilia

Prokaryotic Cells

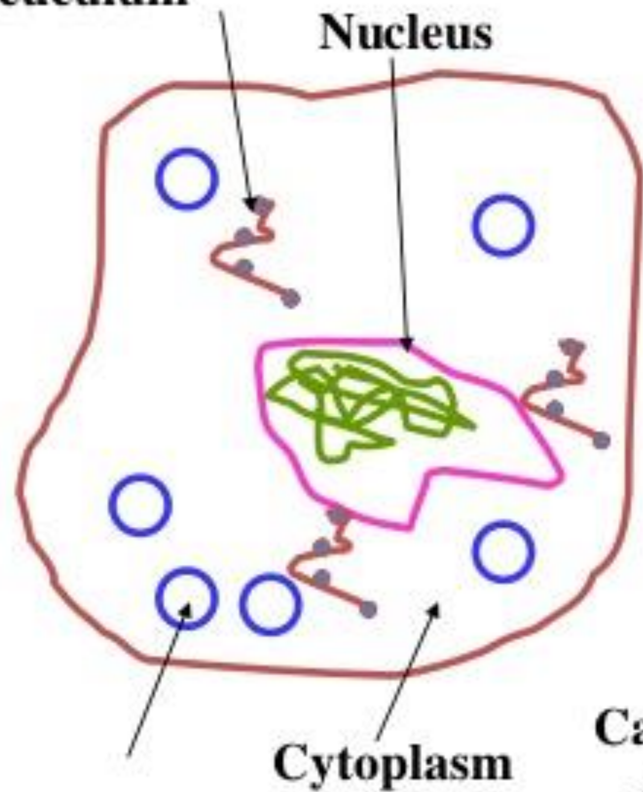
- Much smaller (microns) and more simple than eukaryotes
- Prokaryotes are molecules surrounded by a membrane and cell wall.
- They lack a true nucleus and don't have membrane bound organelles like mitochondria, etc.
- Large surface-to-volume ratio : nutrients can easily and rapidly reach any part of the cells interior

Size of Bacteria

- Unit of measurement in bacteriology is the micron (micrometre, µm)
- Bacteria of medical importance
  - 0.2 – 1.5 µm in diameter
  - 3 – 5 µm in length

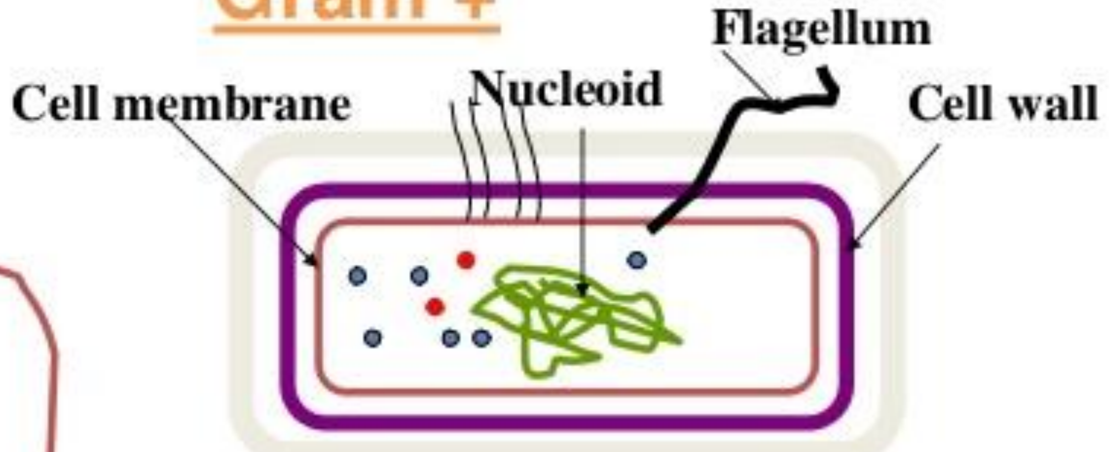
Eukaryotic cell

(e.g. animal)

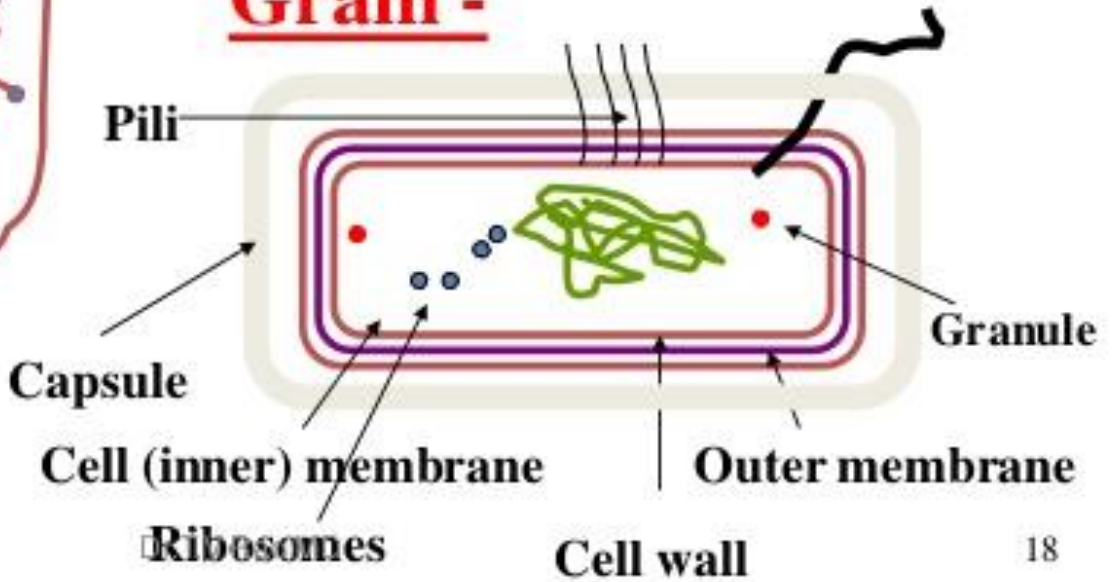


Prokaryotic cell

Gram +



Gram -

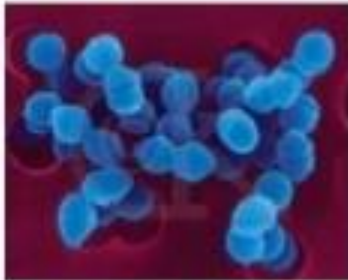


Shapes of Bacteria

- Cocci – spherical/ oval shaped | major groups
- Bacilli – rod shaped
- Vibrios – comma shaped
- Spirilla – rigid spiral forms
- Spirochetes – flexible spiral forms
- Actinomycetes – branching filamentous bacteria
- Mycoplasmas – lack cell wall

Bacteria Have One of Three Cellular Shapes

- Rods (bacilli)



- Coccoid-Shaped

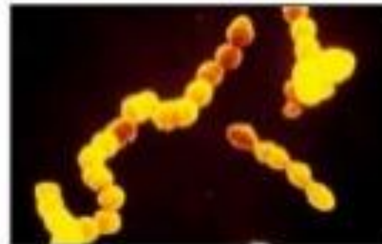
- Spirilla

Arrangement of bacteria: Cocci

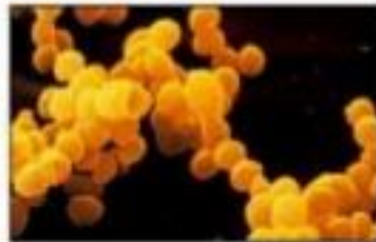
Cocci in pair – Diplococcus

Coccus

Tetrad – groups of four



Cocci in chain - Streptococci



Cocci in cluster - Staphylococci

Sarcina – groups of eight

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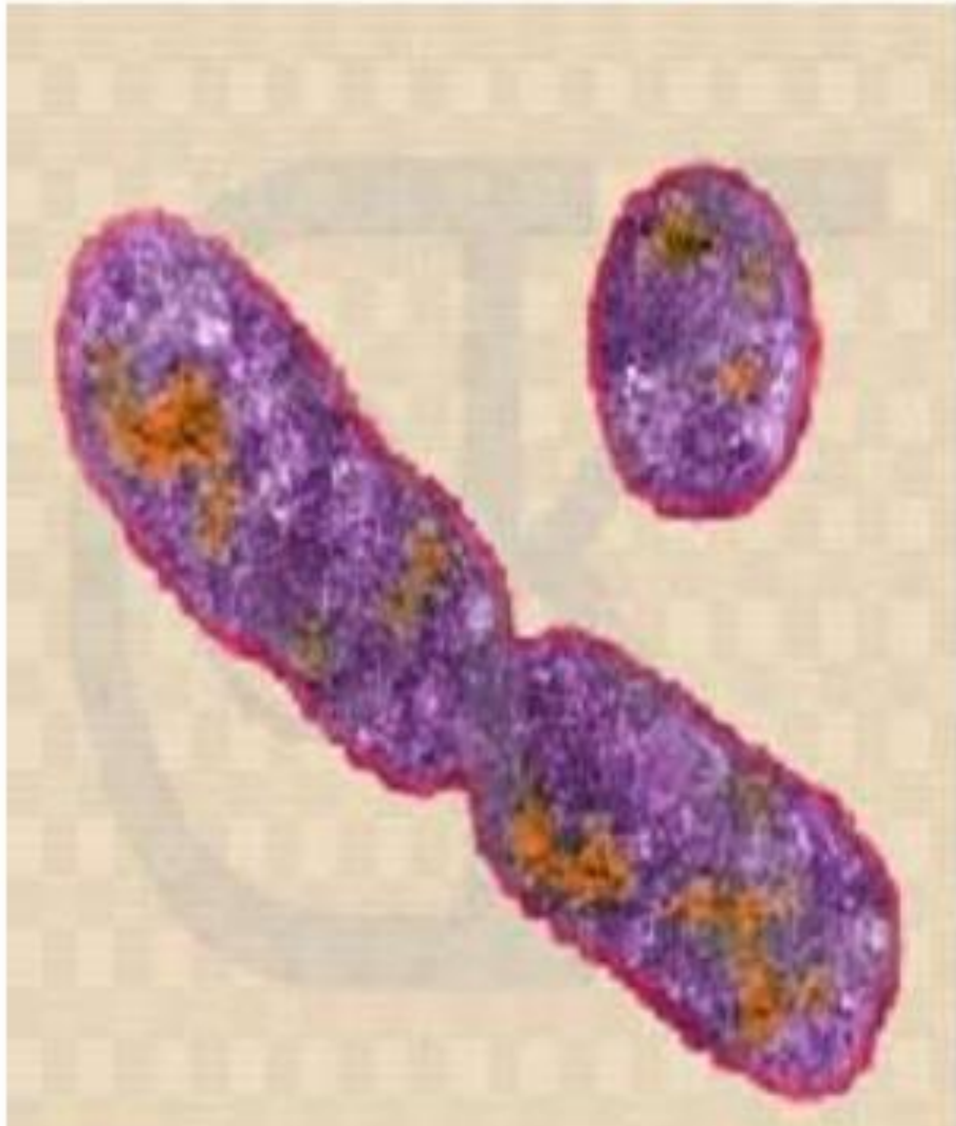
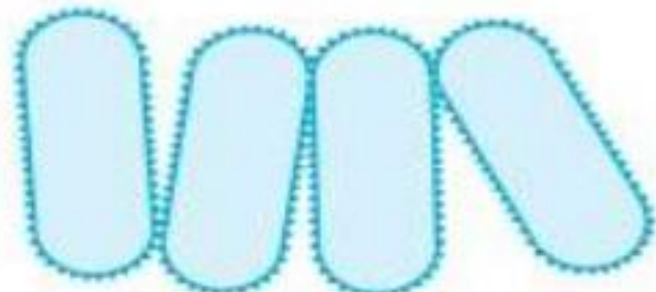


- Prokaryotic cell division is binary fission.
  - Single DNA molecule that first replicates.
  - Attaches each copy to a different part of the cell membrane.
  - Cell begins to pull apart.
  - Following cytokinesis, there are then two cells of identical genetic composition.

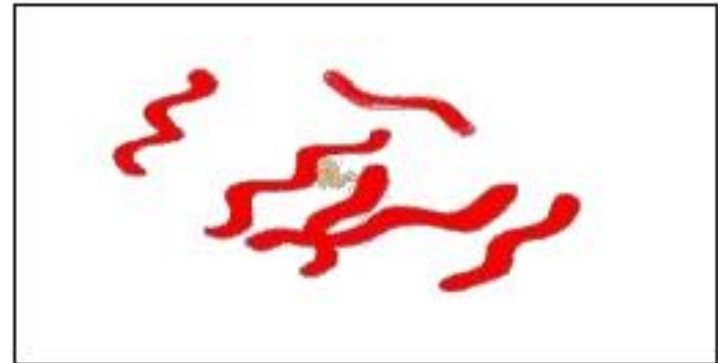
Arrangement of bacteria: Bacilli

Arrangements of Bacilli



Other shapes of bacteria

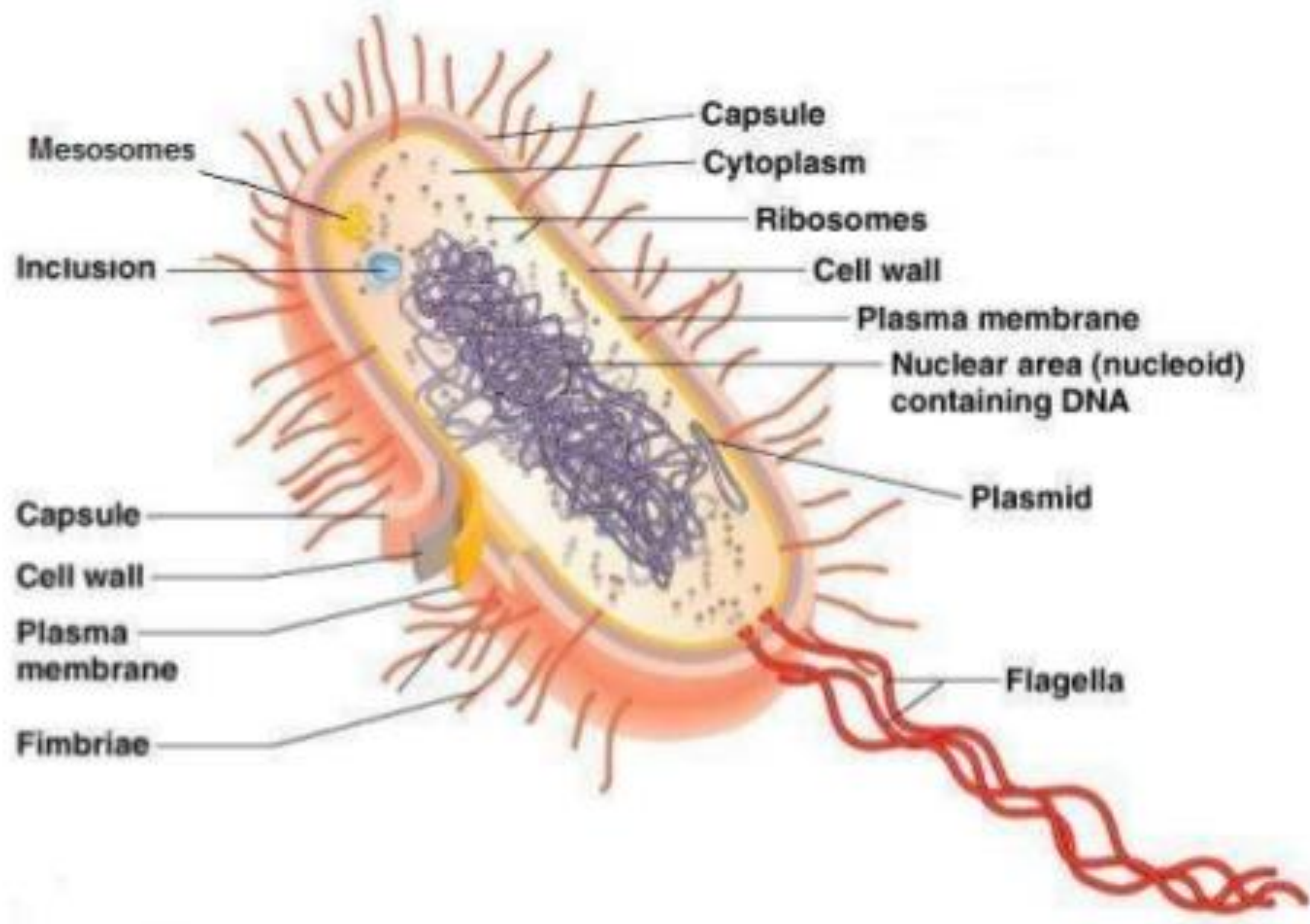
Comma shaped



Spirilla

Spirochetes

Anatomy of a Bacterial Cell



Anatomy of A Bacterial Cell

- Outer layer – two components:
  1. Rigid cell wall
  2. Cytoplasmic (Cell/ Plasma) membrane – present beneath cell wall
- Cytoplasm – cytoplasmic inclusions, ribosomes, mesosomes and nucleus
- Additional structures – plasmid, slime layer, capsule, flagella, fimbriae (pili), spores

Structure of Bacteria

- All cells have 3 main components:
  - DNA ('nucleoid")
    - genetic instructions
  - surrounding membrane ("cytoplasmic membrane")
    - limits access to the cell's interior
  - cytoplasm, between the DNA and the membrane
    - where all metabolic reactions occur
    - especially protein synthesis, which occurs on the ribosomes
- Bacteria also often have these features:
  - cell wall
    - resists osmotic pressure
  - flagella
    - movement
  - pili
    - attachment
  - capsule
    - protection and biofilms

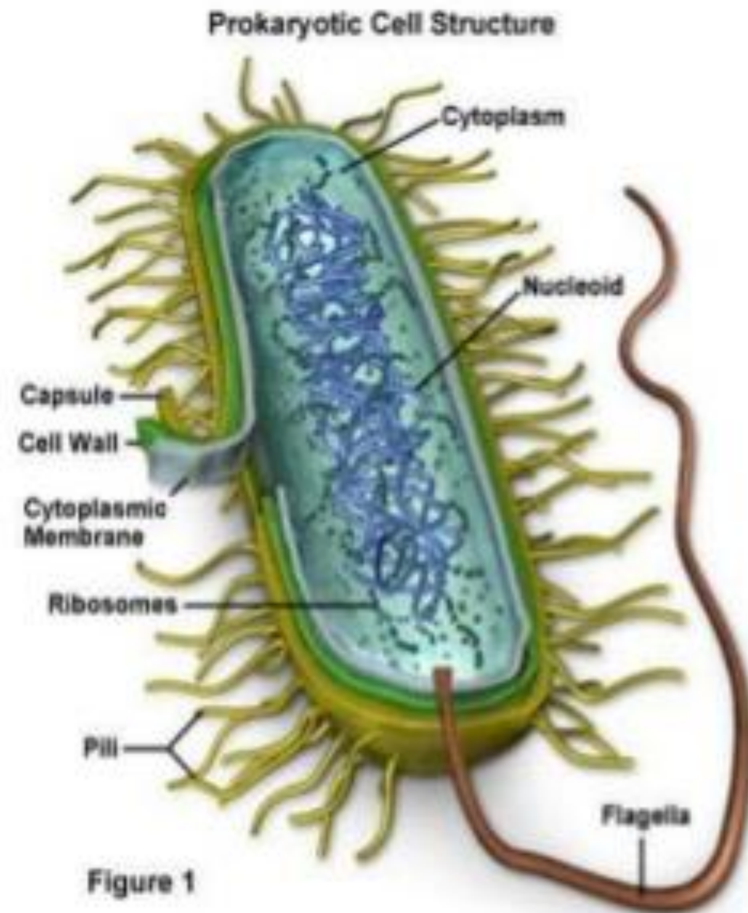


Figure 1

Typical shapes of bacteria

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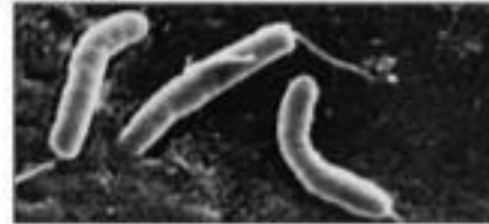
(a) Coccus

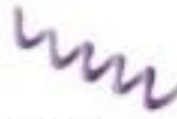
(d) Vibrio



(b) Rod (bacillus)

(e) Spirillum

(c) Coccobacillus



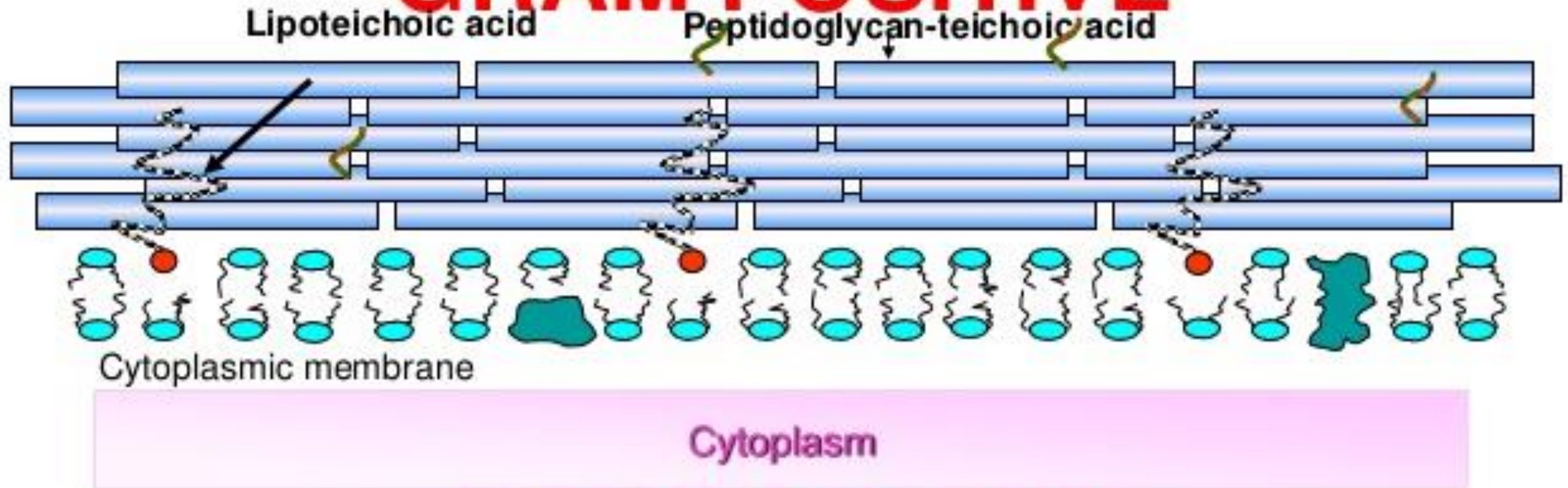
(f) Spirochete

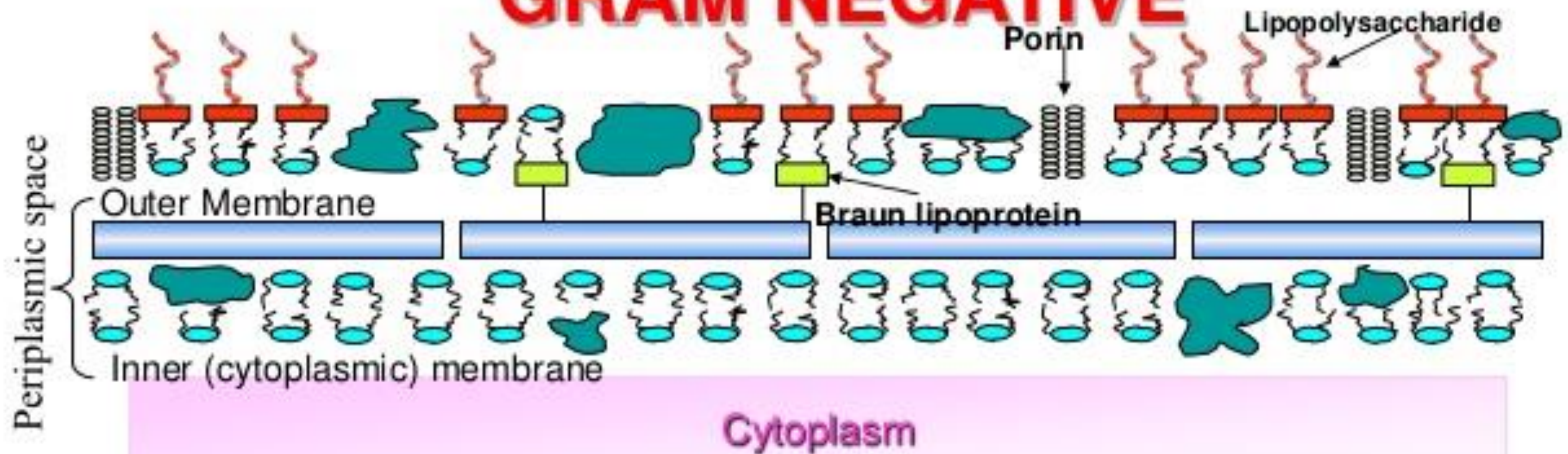
Most bacteria retain a particular shape; a few are pleiomorphic

The Cell Envelope

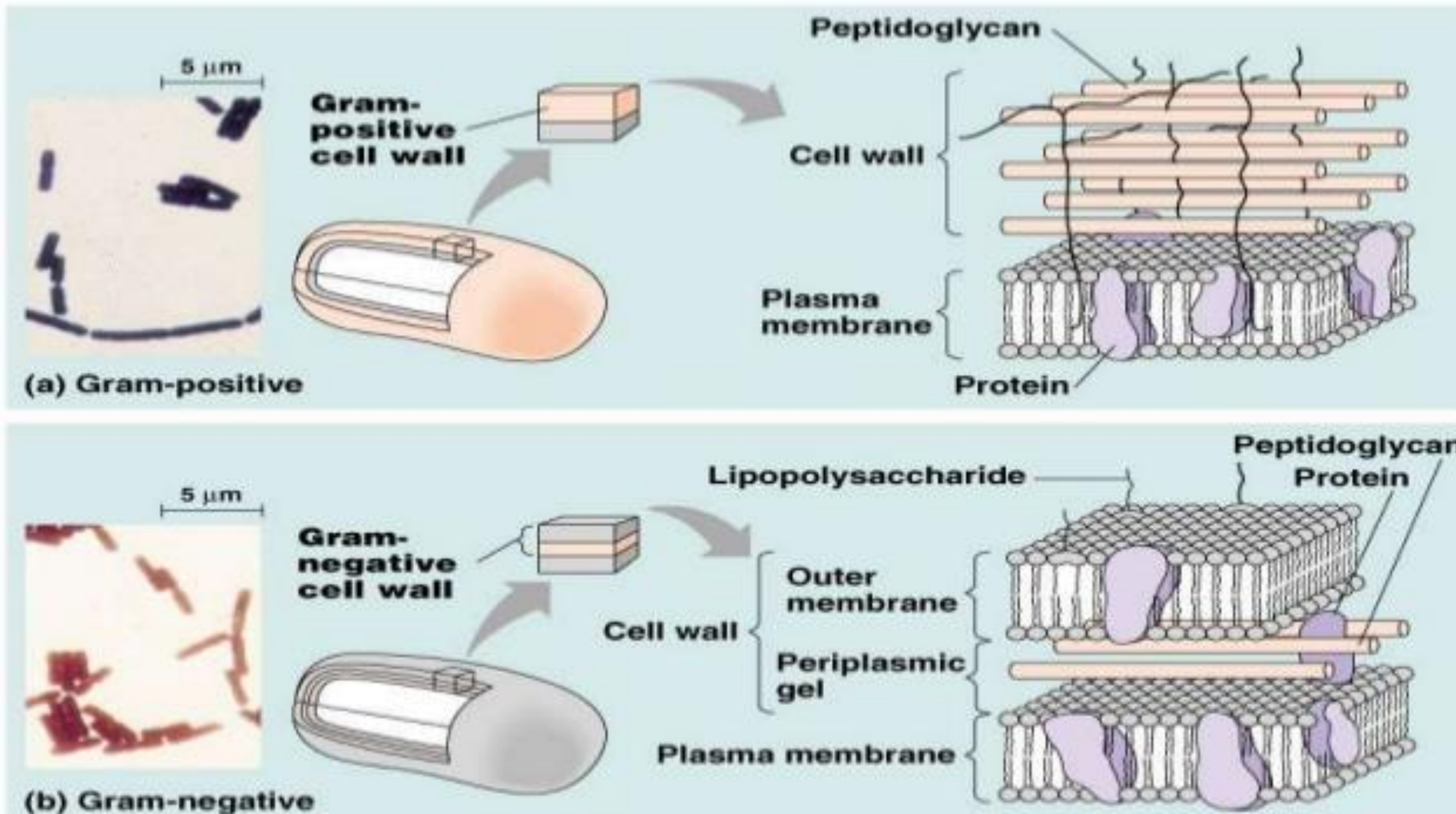
Gram Stain

Gram Positive **Gram Negative**


GRAM POSITIVE
Lipoteichoic acid
Peptidoglycan-teichoic acid
Cytoplasmic membrane
Cytoplasm


GRAM NEGATIVE
Lipopolysaccharide
Porin
Outer Membrane
Braun lipoprotein
Periplasmic space
Inner (cytoplasmic) membrane
Cytoplasm

Gram-positive and gram-negative bacteria

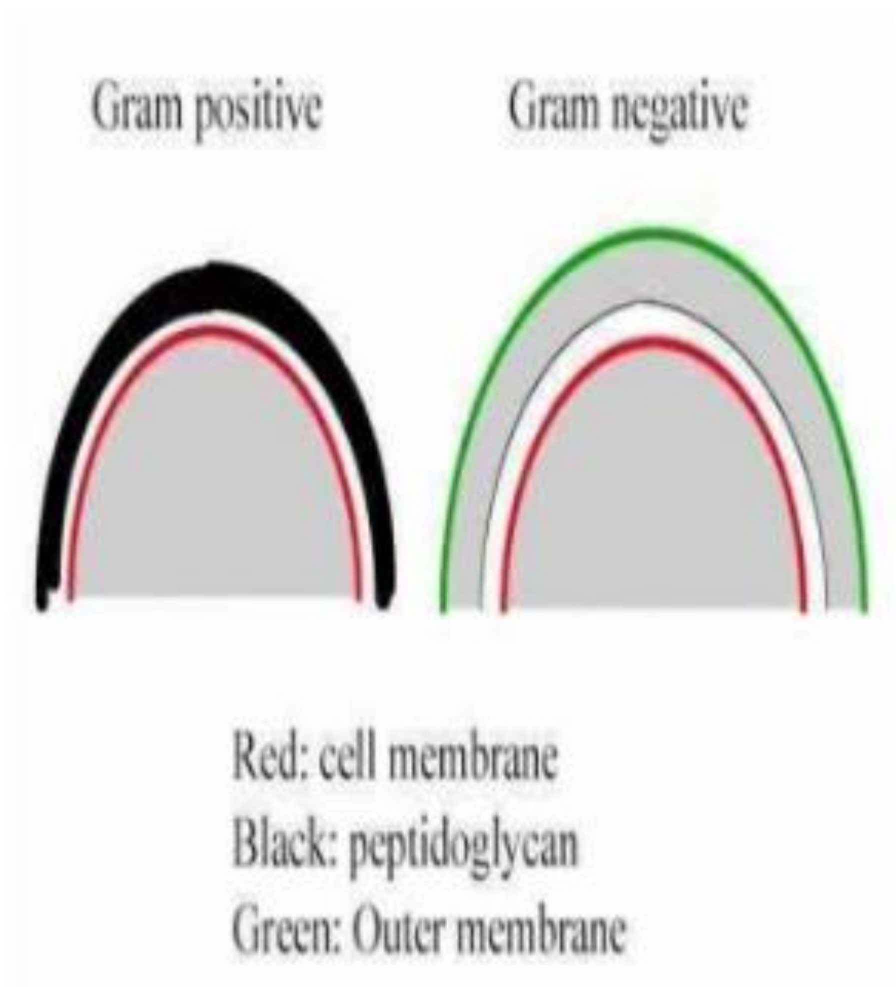


Difference Between Gram-Negative and Gram-Positive Bacteria

Gram-Negative Bacteria	Gram-Positive Bacteria
More complex cell wall.	Simple cell wall.
Thin peptidoglycan celll wall layer.	Thick peptidoglycan celll wall layer.
Outer lipopolysaccharide wall layer.	No outer lipopolysaccharide wall layer.
Retain safranin.	Retain crystal violet/iodine.
Appear pink/red.	Appear blue/purple.

Cell Envelope

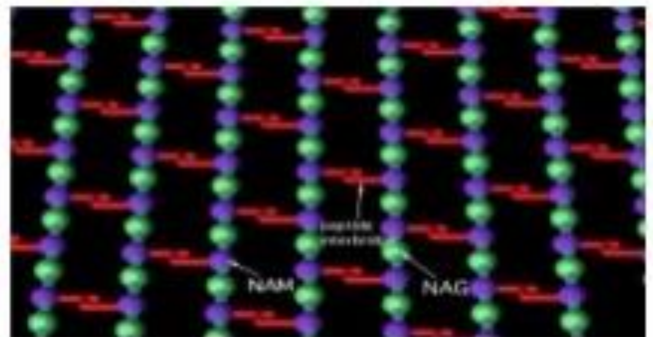
- The cell envelope is all the layers from the cell membrane outward, including the cell wall, the periplasmic space, the outer membrane, and the capsule.
  - All free-living bacteria have a cell wall
  - periplasmic space and outer membrane are found in Gram-negatives
  - the capsule is only found in some strains



Structure & Function of Cell Components

CELL WALL

- Outermost layer, encloses cytoplasm
  1. Confers shape and rigidity
  2. 10 - 25 nm thick



1. **Composed of complex polysaccharides (peptidoglycan/ mucopeptide) - formed by N acetyl glucosamine (NAG) & N acetyl muramic acid (NAM) alternating in chains, held by peptide chains.**

Cell Wall

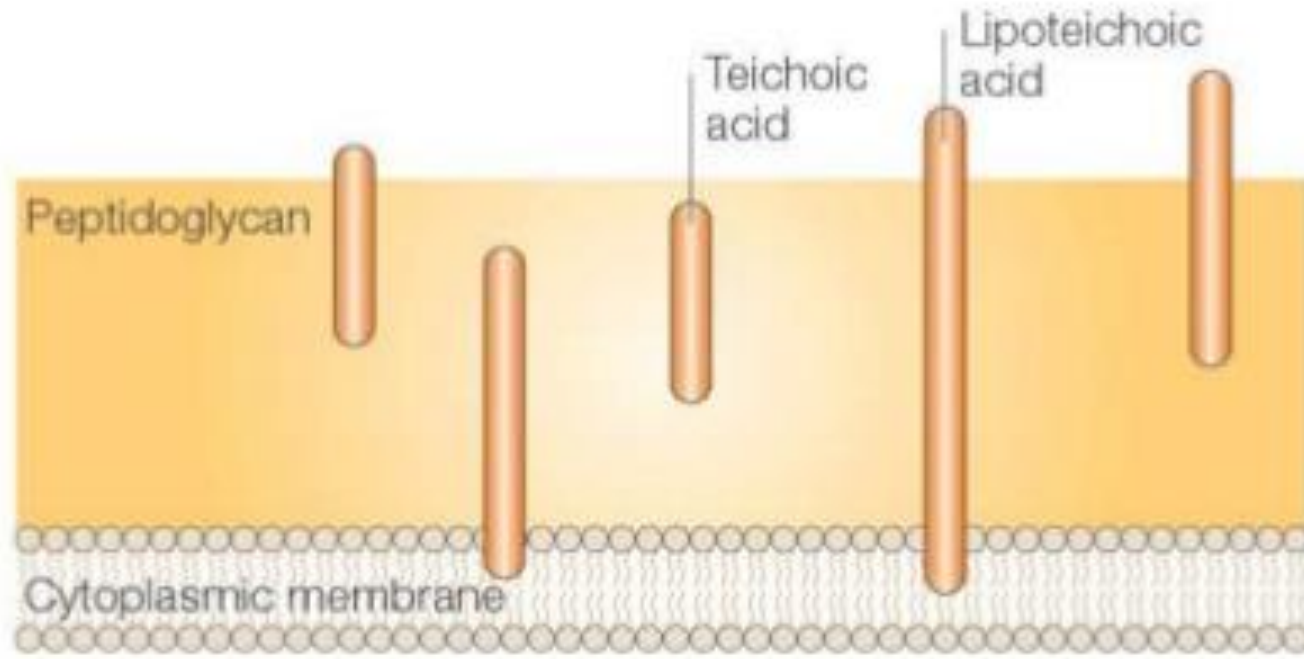
- Cell wall –
  4. Carries bacterial antigens – important in virulence & immunity
  5. Chemical nature of the cell wall helps to divide bacteria into two broad groups – Gram positive & Gram negative
  6. Gram +ve bacteria have simpler chemical nature than Gram –ve bacteria.
  7. Several antibiotics may interfere with cell wall synthesis e.g. Penicillin, Cephalosporins

Outer Membrane

Gram negative bacteria

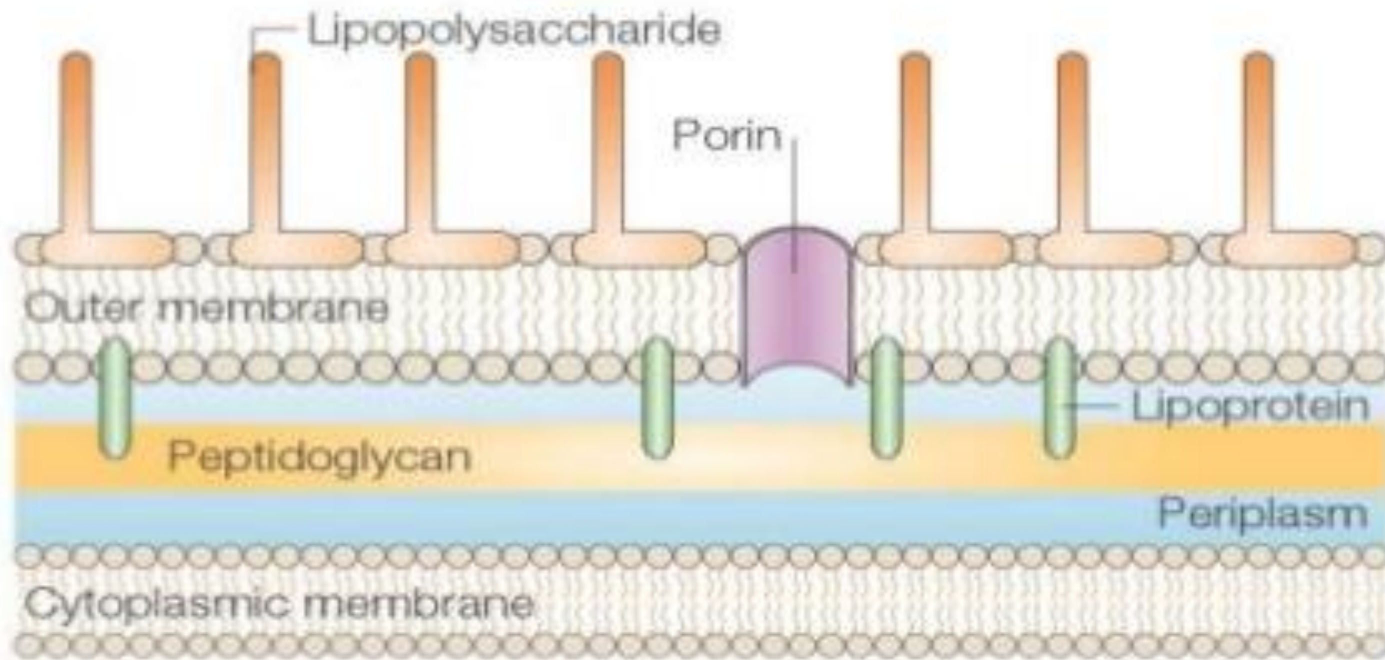
- major permeability barrier
- space between inner and outer membrane
  - Periplasmic space
    - store degradative enzymes
- <u>Gram positive bacteria</u>
- no Periplasmic space

Gram positive cell wall



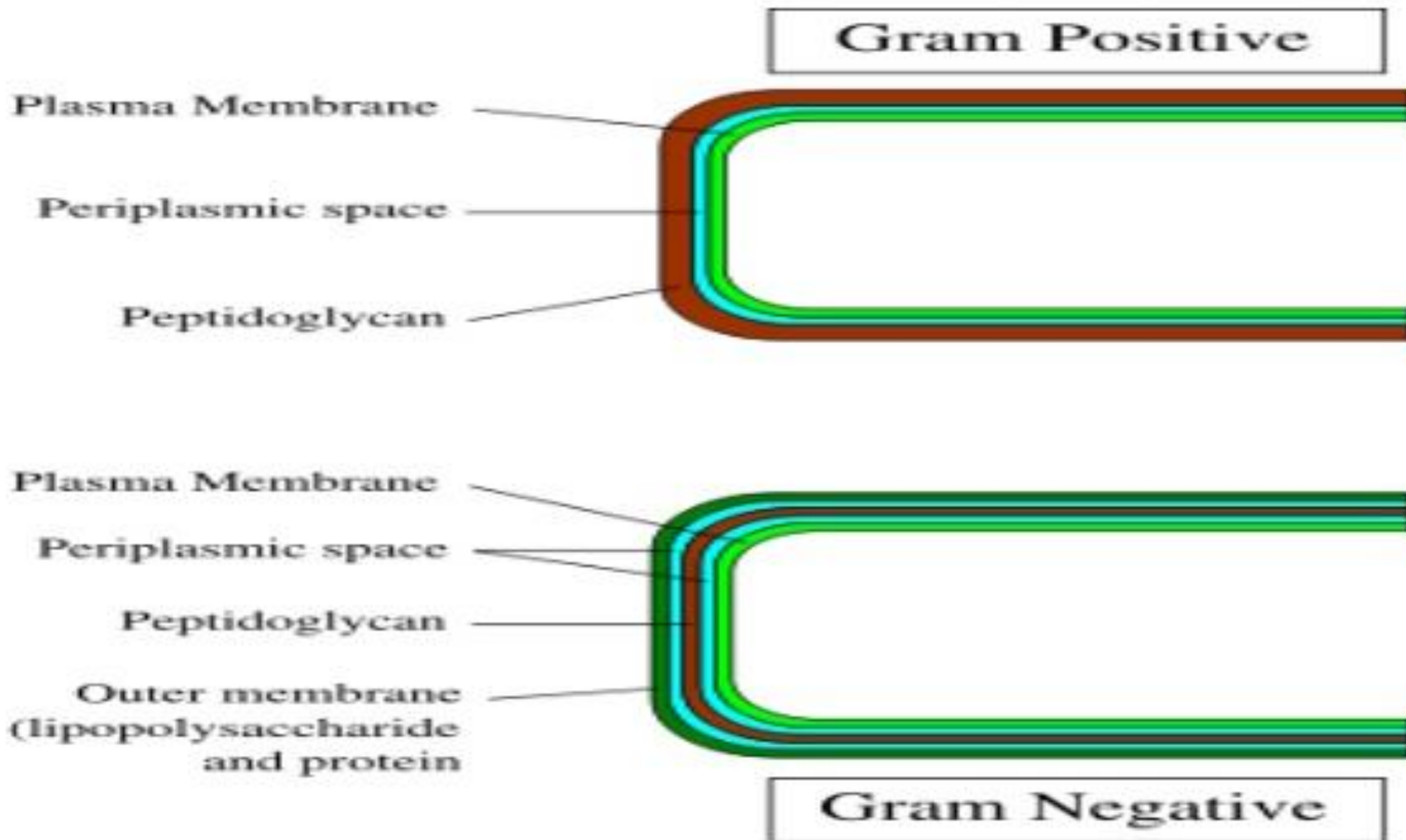
The Gram-positive cell wall is composed of a thick, multilayered peptidoglycan sheath outside of the cytoplasmic membrane. Teichoic acids are linked to and embedded in the peptidoglycan, and lipoteichoic acids extend into the cytoplasmic membrane

Gram negative cell wall



The Gram-negative cell wall is composed of an outer membrane linked to thin, mainly single-layered peptidoglycan by lipoproteins The peptidoglycan is located within the periplasmic space that i created between the outer and inner membranes. The oute membrane includes porins, which allow the passage of smal hydrophilic molecules across the membrane, and lipopolysaccharide molecules that extend into extracellular space.

Cell Wall



Summary of the differences between Gram positive & Gram negative bacteria

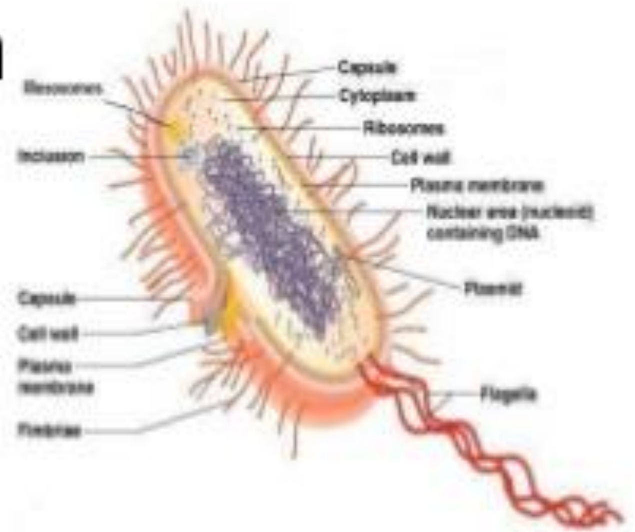
Property of bacteria	Gram Positive	Gram Negative
Thickness of wall	20-80 nm	10 nm
Number of layers in wall	1	2
Peptidoglycan content	>50%	10-20%
Teichoic acid in wall	+	-
Lipid & lipoprotein content	0-3%	58%
Protein content	0%	9%
Lipopolysaccharide	0	13%
Sensitive to penicillin	Yes	Less sensitive
Digested by lysozyme	Yes	Weakly

Cytoplasmic (Plasma) membrane

- Thin layer 5-10 nm, separates cell wall from cytoplasm
- Acts as a semipermeable membrane: controls the inflow and outflow of metabolites
- Composed of lipoproteins with small amounts of carbohydrates

Nucleus

- No nucleolus
- No nuclear membrane
- Genome –
  - single, circular double stra
  - Haploid
  - Divides by binary fission



Additional Organelles

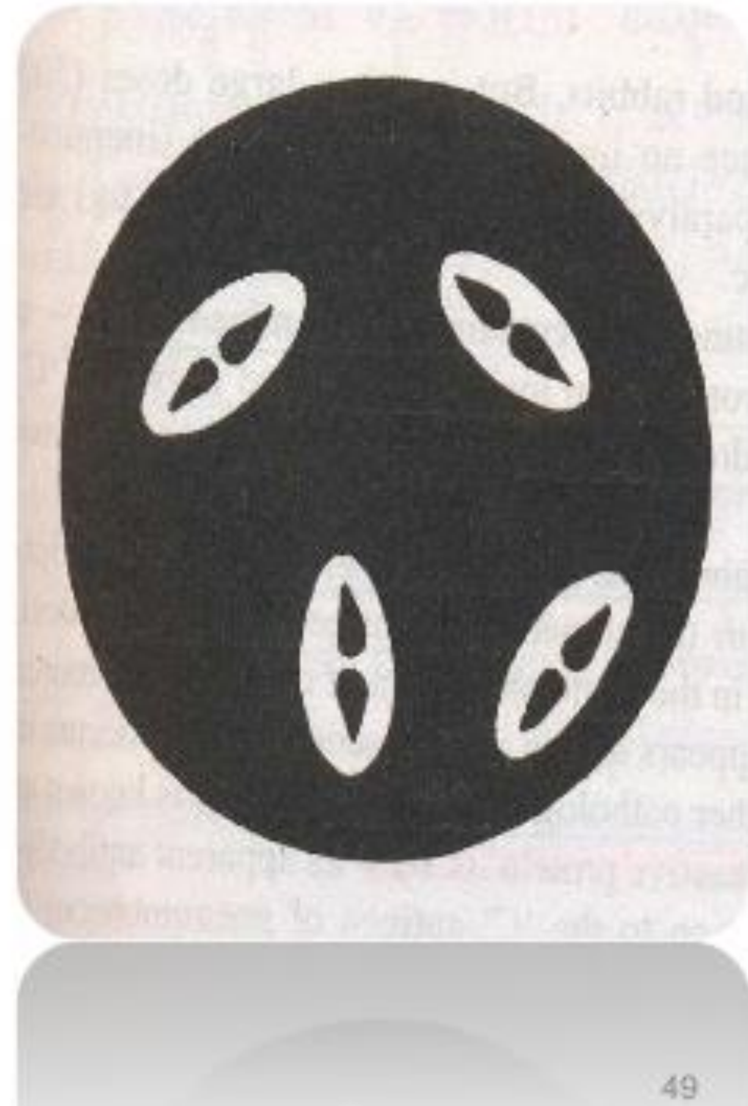
1. Plasmid –
   - Extra nuclear genetic elements consisting of DNA
   - Transmitted to daughter cells during binary fission
   - May be transferred from one bacterium to another
   - Not essential for life of the cell
   - Confer certain properties e.g. drug resistance, toxicity

Additional Organelles

2. Capsule & Slime layer –
   - Viscous layer secreted around the cell wall.
   - Polysaccharide / polypeptide in nature

a) **Capsule** – sharply defined structure, antigenic in nature

- Protects bacteria from lytic enzymes
- Inhibits phagocytosis
- Stained by negative staining using **India Ink**
- Can be demonstrated by Quellung reaction (capsule swelling reaction)

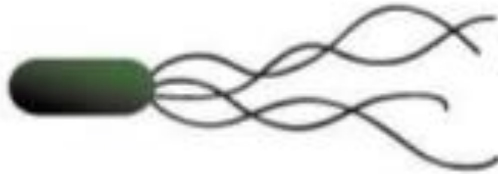


Additional Organelles

3. Flagella –
   - Long (3 to 12 µm), filamentous surface appendages
   - Organs of locomotion
   - Chemically, composed of proteins called flagellins
   - The number and distribution of flagella on the bacterial surface are characteristic for a given species - hence are useful in identifying and classifying bacteria
   - Flagella may serve as antigenic determinants (e.g. the H antigens of Gram-negative enteric bacteria)
   - Presence shown by motility e.g. hanging drop preparation

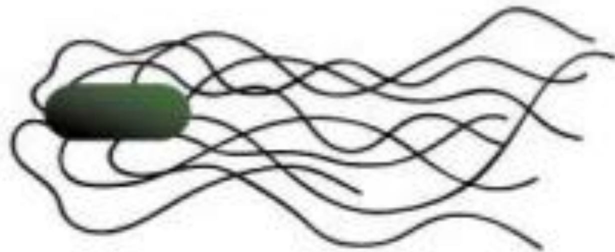
Types of flagellar arrangement

Polar/ Monotrichous – single flagellum at one pole



Lophotrichous – tuft of flagella at one pole

Amphitrichous – flagella at both poles



Peritrichous – flagella all over

Amphilophotrichous – tuft of flagella at both ends

Cocci do not have flagella

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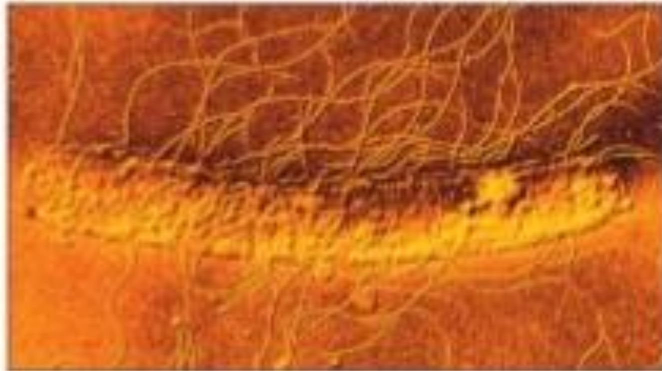


Peritrichous monotrichous
(or amphi, or lophotrichous

FLAGELLA

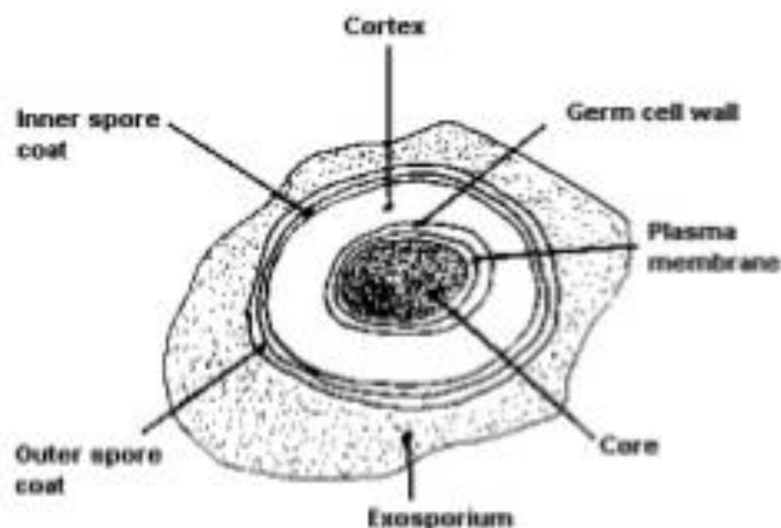
- Some bacteria are motile
- Locomotory organelles- flagella
- Taste environment
- Respond to food/poison
  – chemo taxis

Additional Organelles

4. Fimbriae/ Pili –
   - Thin, hairlike appendages on the surface of many Gram-negative bacteria
   - 10-20µ long, acts as organs of adhesion (attachment) - allowing bacteria to **colonize environmental surfaces** or **cells** and **resist flushing**
   - Made up of proteins called pilins.
   - Pili can be of two types –
     - **Common pili** – short & abundant
     - **Sex pili** - small number (one to six), very long pili, helps in conjugation (process of transfer of DNA)

Additional Organelles

5. Spores –
   - Highly resistant resting stages formed during adverse environment (depletion of nutrients)
   - Formed inside the parent cell, hence called Endospores
   - Very resistant to heat, radiation and drying and can remain dormant for hundreds of years.
   - Formed by bacteria like Clostridia, bacillus



Shape & position of bacterial spore

Oval central

Spherical central

Oval sub terminal

Non bulging

Oval sub terminal

Oval terminal

Spherical terminal

Bulging

Free spore

Spores

- Spores can also survive very high or low temperatures and high UV radiation for extended periods. This makes them difficult to kill during sterilization.
- Anthrax
- Spores are produced only by a few genera in the Firmicutes:
- Bacillus species including anthracis (anthrax) and cereus (endotoxin causes ~5% of food poisoning)
- Clostridium species including tetani (tetanus), perfringens (gangrene), and botulinum (botulism: food poisoning from improperly canned food)

Bacterial Taxonomy

- Includes three components:

1. Classification : orderly arrangement
2. Identification of an unknown unit
3. Nomenclature : naming the units

Bacterial Taxonomy: Classification

- Orderly arrangement : Kingdom – Division – Class – Order – Family – Tribe – Genus – Species
  - Phylogenetic classification – represents a branching tree like arrangement. One characteristic being used for division at each branch or level
  - Molecular or Genetic classification – based on the degree of genetic relatedness of different organisms
  - Intraspecies classification – based on biochemical properties (biotypes), antigenic features (serotypes), bacteriophage susceptibility (phage types)

Bacterial Taxonomy: Nomenclature

- Two kinds of name are given to bacteria
  - Casual / common name – for local use, varies from country to country
    e.g. “typhoid bacillus”
  - Scientific / International Name – same all over world, consists of two words (in Italics)
    e.g. *Salmonella typhi*, *Staphylococcus*

Why we should be Stain Bacteria

Bacteria have nearly the same refractive index as water, therefore, when they are observed under a microscope they are opaque or nearly invisible to the naked eye.

Different types of staining methods are used to make the cells and their internal structures more visible under the light microscope.

Practical Bacteriology

What is a Stain

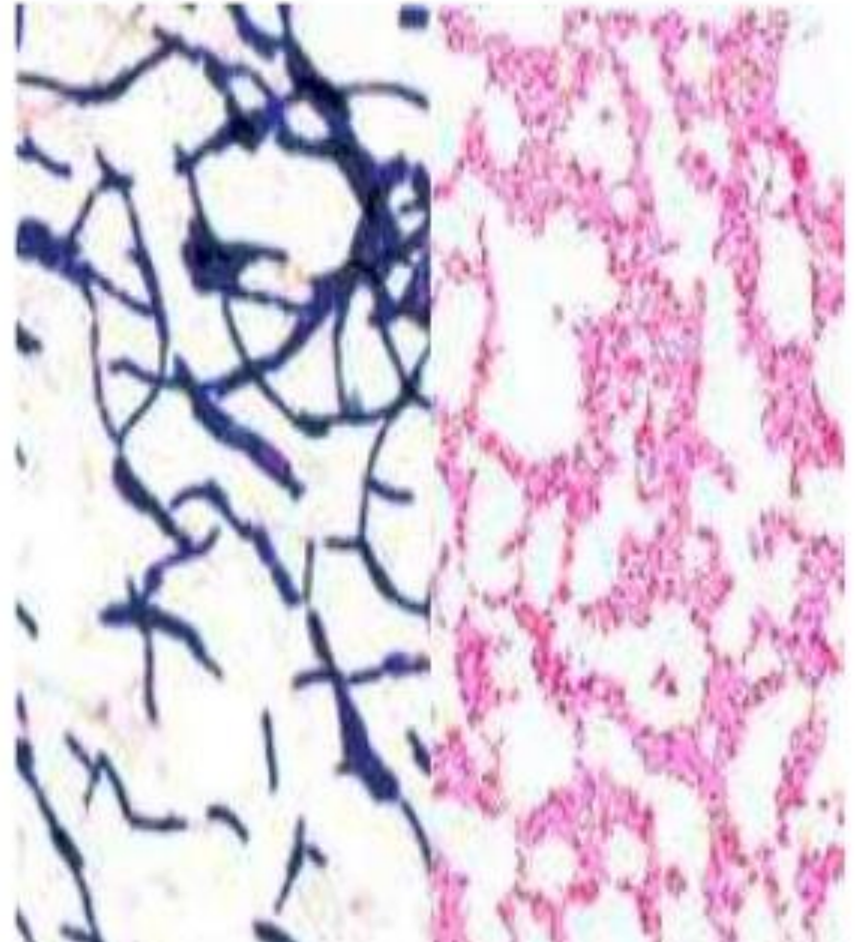
- A stain is a substance that adheres to a cell, giving the cell color.
- The presence of color gives the cells significant contrast so are much more visible.
- Different stains have different affinities for different organisms, or different parts of organisms
- They are used to differentiate different types of organisms or to view specific parts of organisms

Simple staining

- Methylene blue, Basic fuchsin
- Provide the color contrast but impart the same color to all the organisms in a smear
- Loffler's ethylene blue: Sat. solution of M. blue in alcohol - 30mlKoH, 0.01% in water - 100mlDissolve the dye in water, filter. For smear: stain for 3’. For section: stain

Simple Staining Easier to Perform But has Limitations

- Simple easy to use; single staining agent used; using basic and acid dyes.
- Features of dyes: give coloring of microorganisms; bind specifically to various cell structures



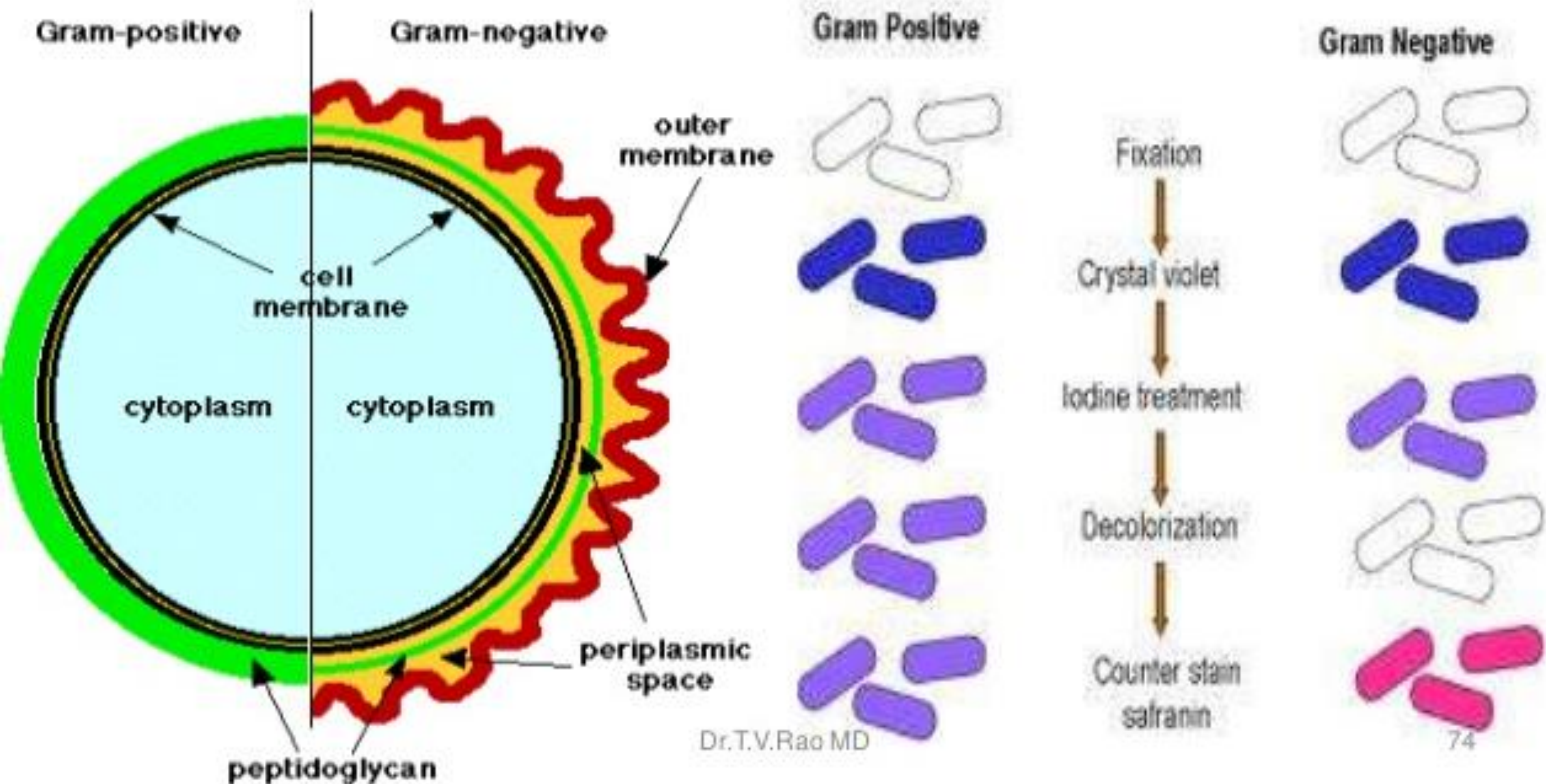
Differential Stains

- Differential Stains use two or more stains and allow the cells to be categorized into various groups or types.
- Both techniques allow the observation of cell morphology, or shape, but differential staining usually provides more information about the characteristics of the cell wall (Thickness).

Gram Staining Steps

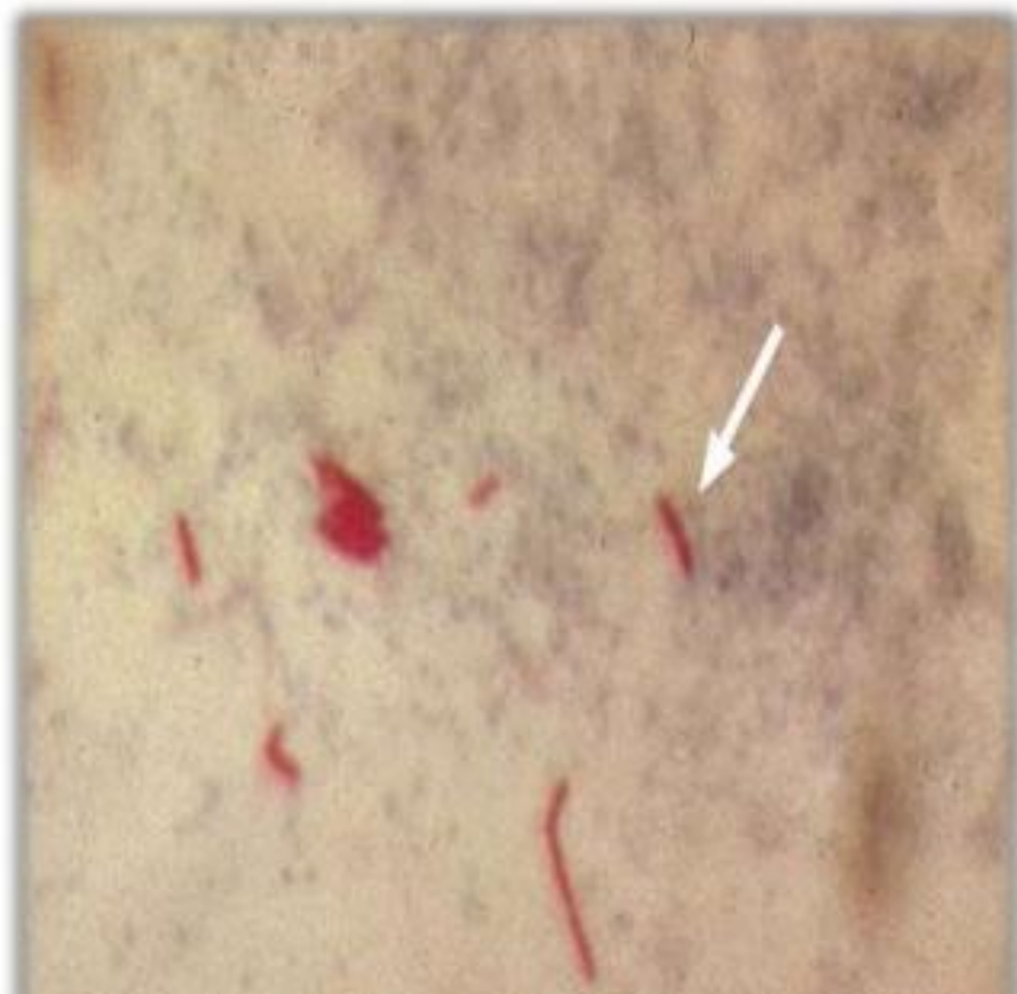
1. **Crystal violet** acts as the primary stain. Crystal violet may also be used as a simple stain because it dyes the cell wall of any bacteria.
2. **Gram's iodine** acts as a mordant (Helps to fix the primary dye to the cell wall).
3. **Decolorizer** is used next to remove the primary stain (crystal violet) from Gram Negative bacteria (those with LPS imbedded in their cell walls). Decolorizer is composed of an organic solvent, such as, acetone or ethanol or a combination of both.)
4. Finally, a counter stain (Safranin), is applied to stain those cells (Gram Negative) that have lost the primary stain as a result of decolorization

Structure and Reactivity to Gram Staining.



Acid-Fast Stain

- Acid-fast cells contain a large amount of lipids and waxes in their cell walls
  - **primarily <u>mycolic acid</u>**
- Acid fast bacteria are usually members of the genus Mycobacterium or Nocardia
  - **Therefore, this stain is important to identify Mycobacterium or Nocardia**



Ziehl-Neelsen stain

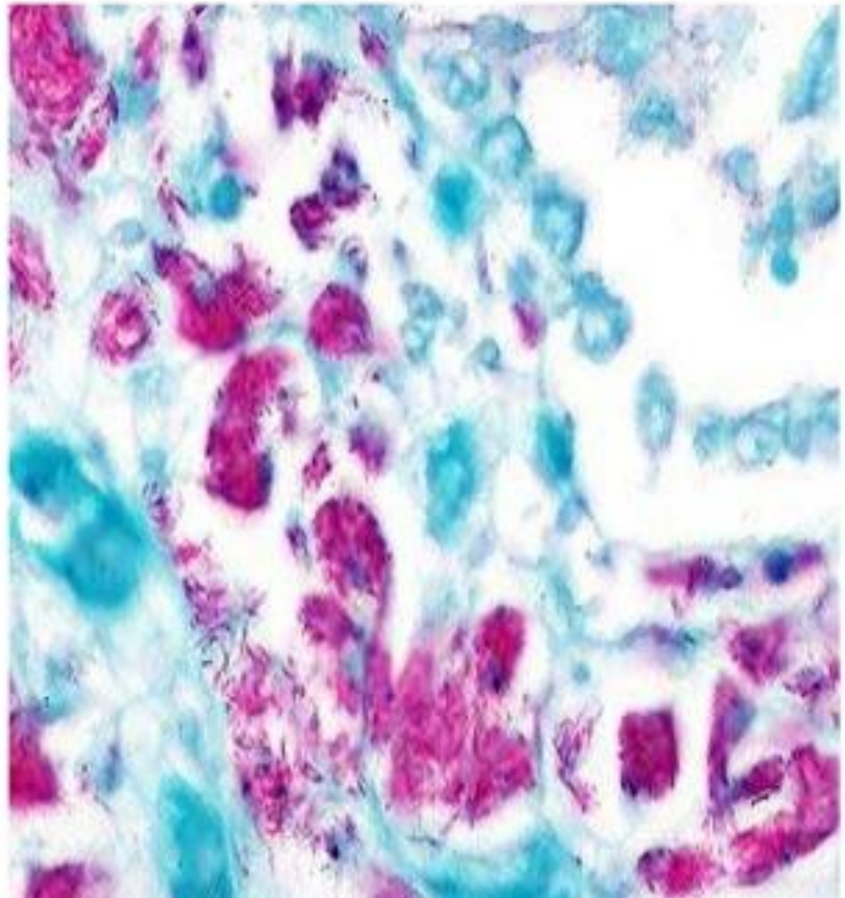
- Ziehl-Neelsen staining is used to stain species of *Mycobacterium tuberculosis* that do not stain with the standard laboratory staining procedures like Gram staining.
- The stains used are the red colored Carbol fuchsin that stains the bacteria and a counter stain like Methylene blue or Malachite green

Acid-Fast Organisms

- Primary stain binds cell wall mycolic acids
- Intense decolorization does not release primary stain from the cell wall of AFB
- Color of AFB-based on primary stain
- Counterstain provides contrasting background

AFB Staining Methods

- Zeihl Neelsen's-hot stain
- Kinyoun's-cold stain
- Modifications

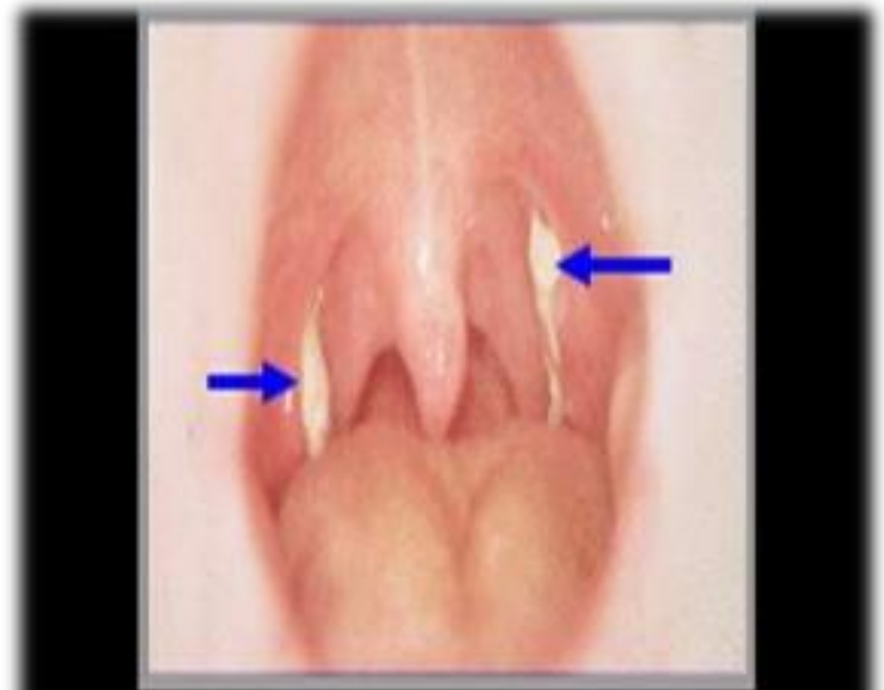


ALBERT'S STAINING FOR C.diptheria

Diphtheria is Serious Disease

When you suspect Diphtheria

- In all cases of suspected cases of Diphtheria, stain one of the smears with Gram stain
- If Gram stained smear shows morphology suggestive of C.diptheria, proceed to do Albert staining which demonstrates the presence or absence of metachromatic granules.



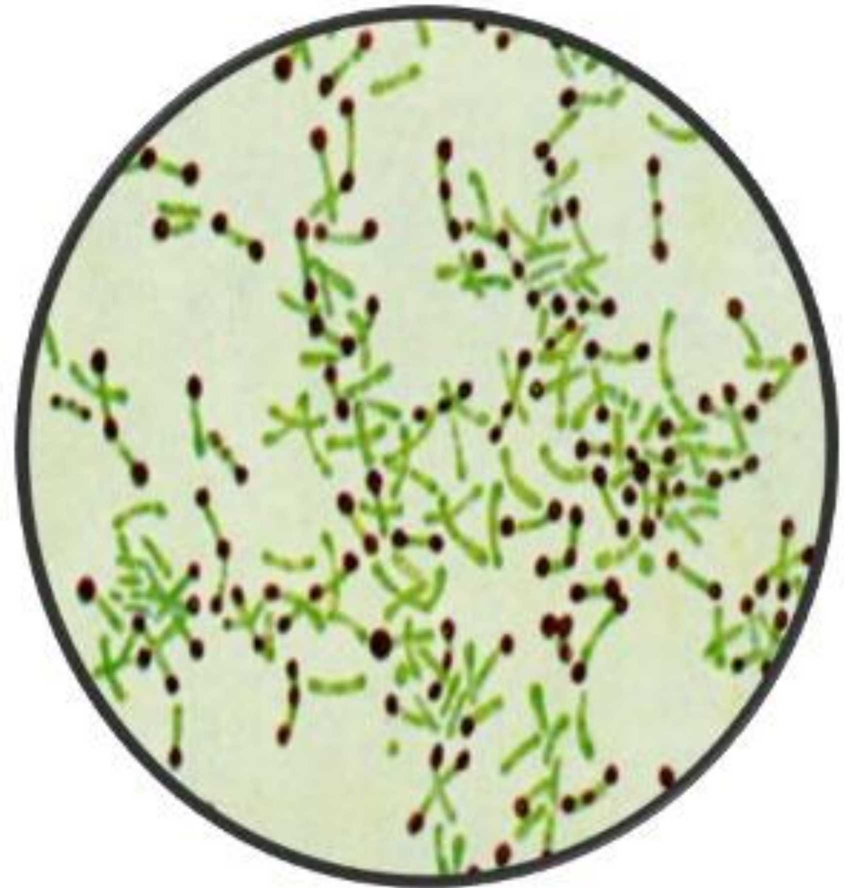
Albert staining

- ***Albert stain I***
- Toluidine blue 0.15 gm
  Malachite green 0.20 gm
  Glacial acetic acid 1.0 ml
  Alcohol(95%) 2.0 ml
  Distilled water 100 ml

- ***Albert stain II***
- Iodine 2.0 gm
  Potassium iodide 3.0 gm
  Distilled water 300 ml

How the C.diptheria appear

- To demonstrate metachromatic granules in *C.diptheria*. These granules appear bluish black whereas the body of bacilli appear green or bluish green.



Phenotypic Methods

- ‘Old fashioned’ methods via biochemical, serological and morphological are still used to identify many microorganisms.
- **Phenotypic Methods**
- Microscopic Morphology include a combination of cell shape, size, Gram stain, acid fast rxn, special structures e.g. endospores, granule and capsule can be used to give an **initial putative identification**.

putative وهمي

Phenotypic Methods

- **Macroscopic morphology** are traits that can be accessed with the naked eye e.g. appearance of colony including texture, shape, pigment, speed of growth and growth pattern in broth.
- **Physiology/Biochemical** characteristic are traditional mainstay of bacterial identification.
- These include enzymes (catalase, oxidase, decarboxylase), fermentation of sugars, capacity to digest or metabolize complex polymers and sensitivity to drugs can be used in identification.

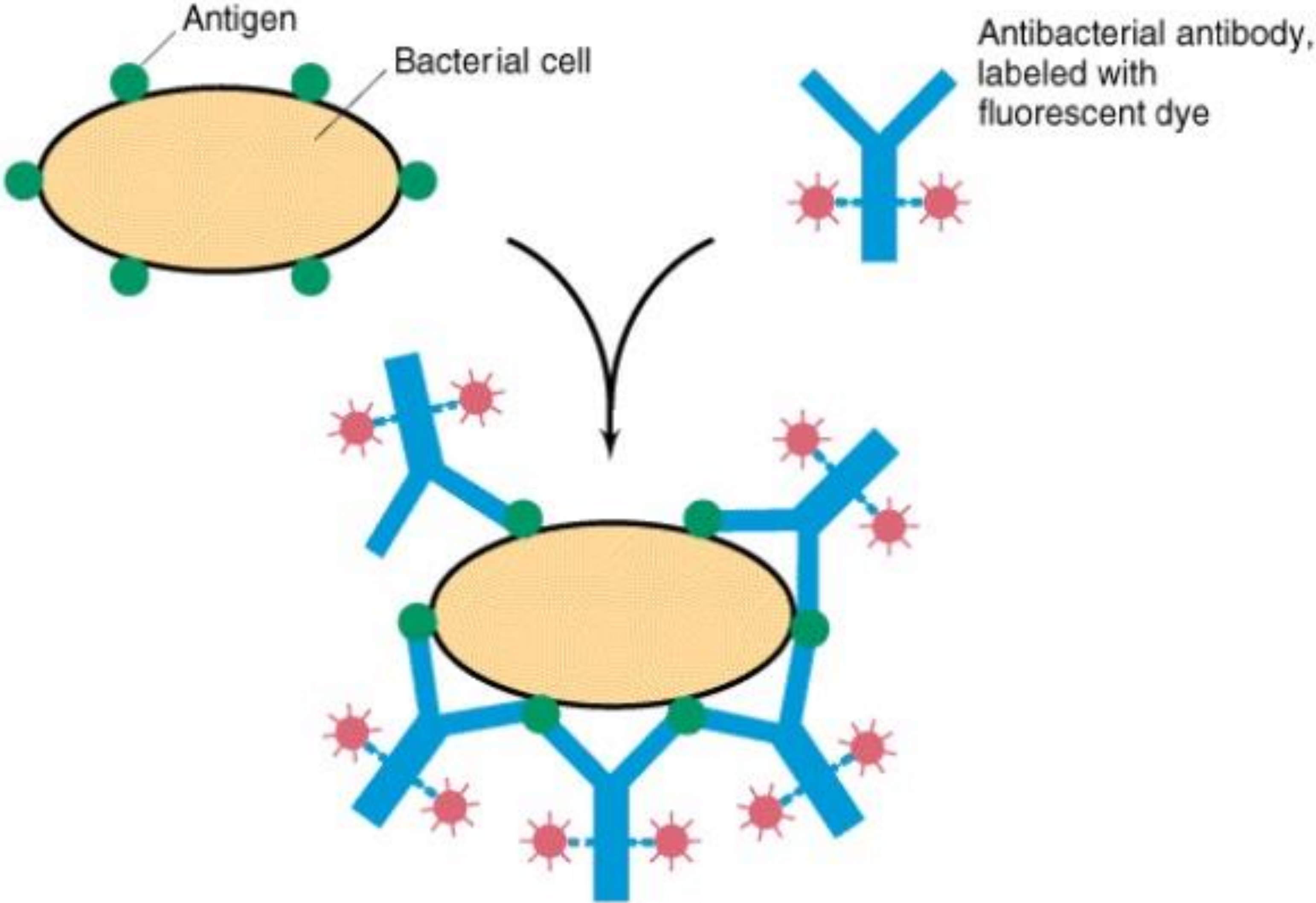
Microscopy

- **Magnification**
  - enhancement of size using ocular and objective lenses.
    - Ocular: eyepiece (10X)
    - Objective: 4X – 100X
  - allows for visualization of bacteria, fungi, and parasites, not viruses
- **Resolution**
  - ability to distinguish two objects as distinct
  - resolving power is closest distance between two objects
  - immersion oil is added when using 100X objective to prevent light scatter
- **Contrast**
  - use stains to enhance visualization; allow organism to stand out from background

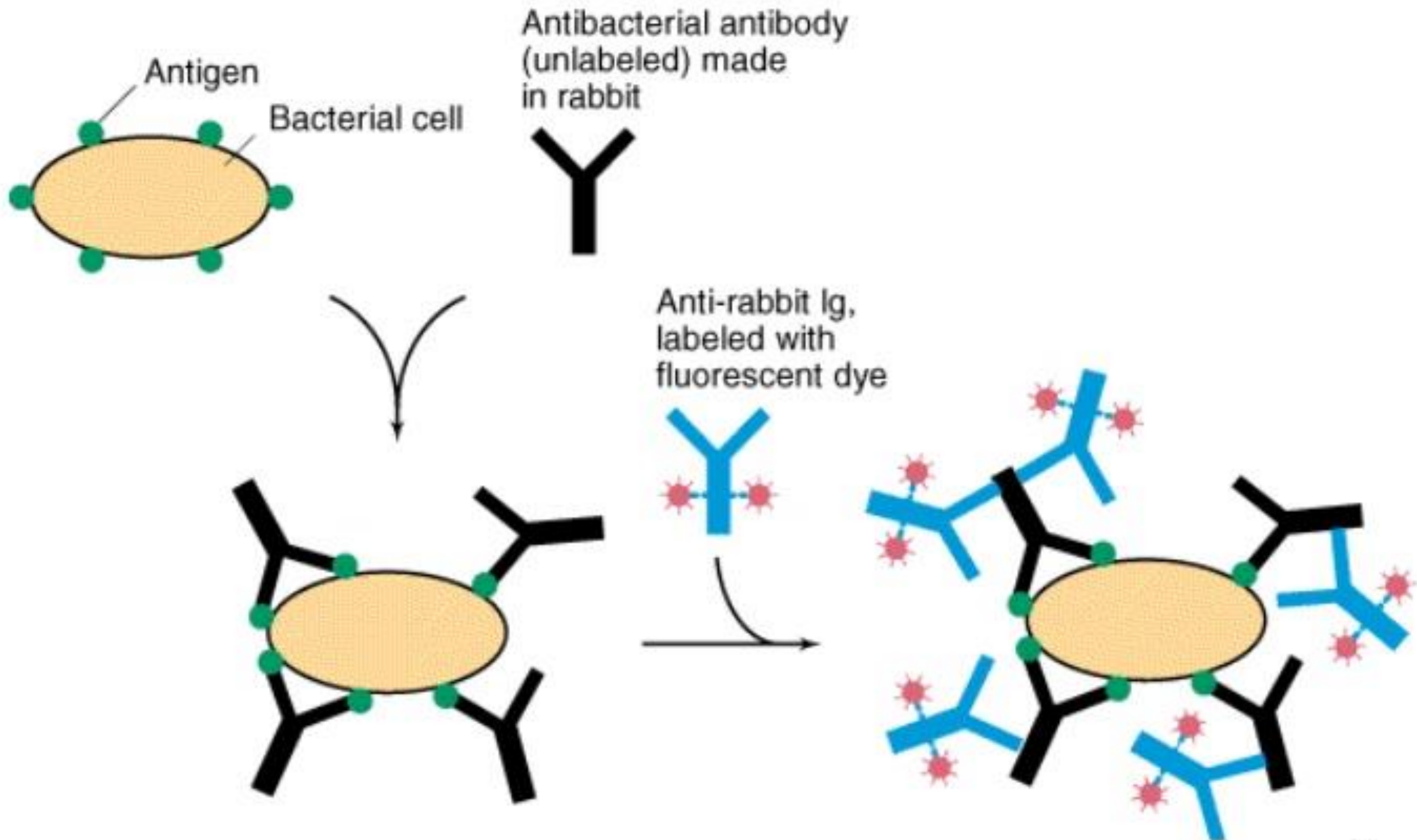
Staining techniques by Grams Method

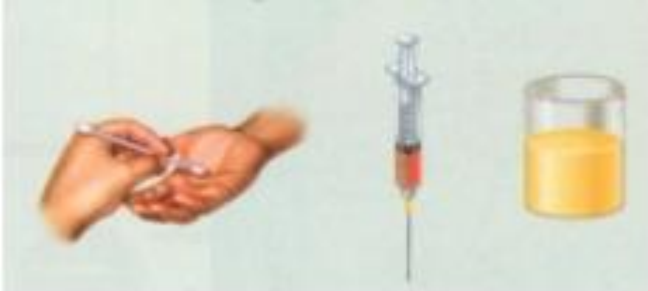
- make slide by smear, drop, or cytocentrifuge
- dry, then fix by heat (flame, 10 min at 60ºC) or fix by methanol (95% 1min)
- Gram stain
  - Crystal violet: primary stain
  - Gram's iodine: mordant/fixative
  - Acetone-ethanol: decolorizer
  - Safranin: counterstain

Direct Fluorescent Antibodies

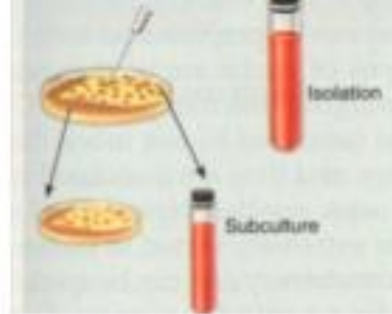


Indirect Fluorescent Antibodies





Specimen collection



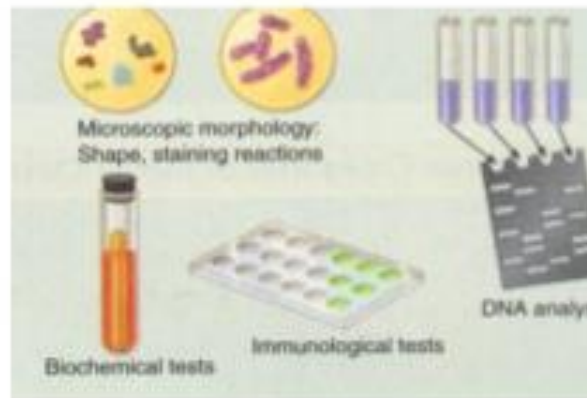
isolation

inoculation

inspection

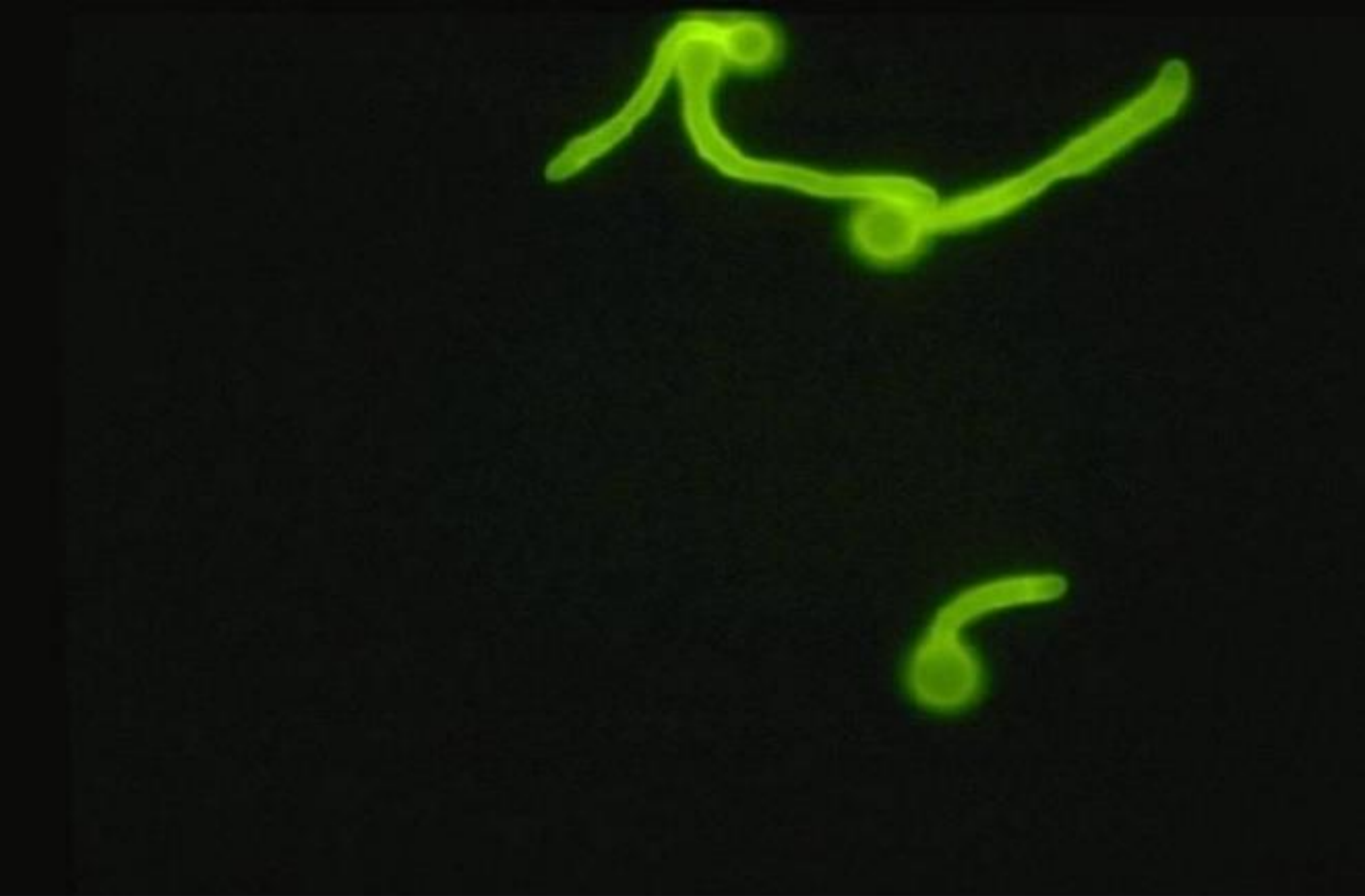
5 “I” s

incubation

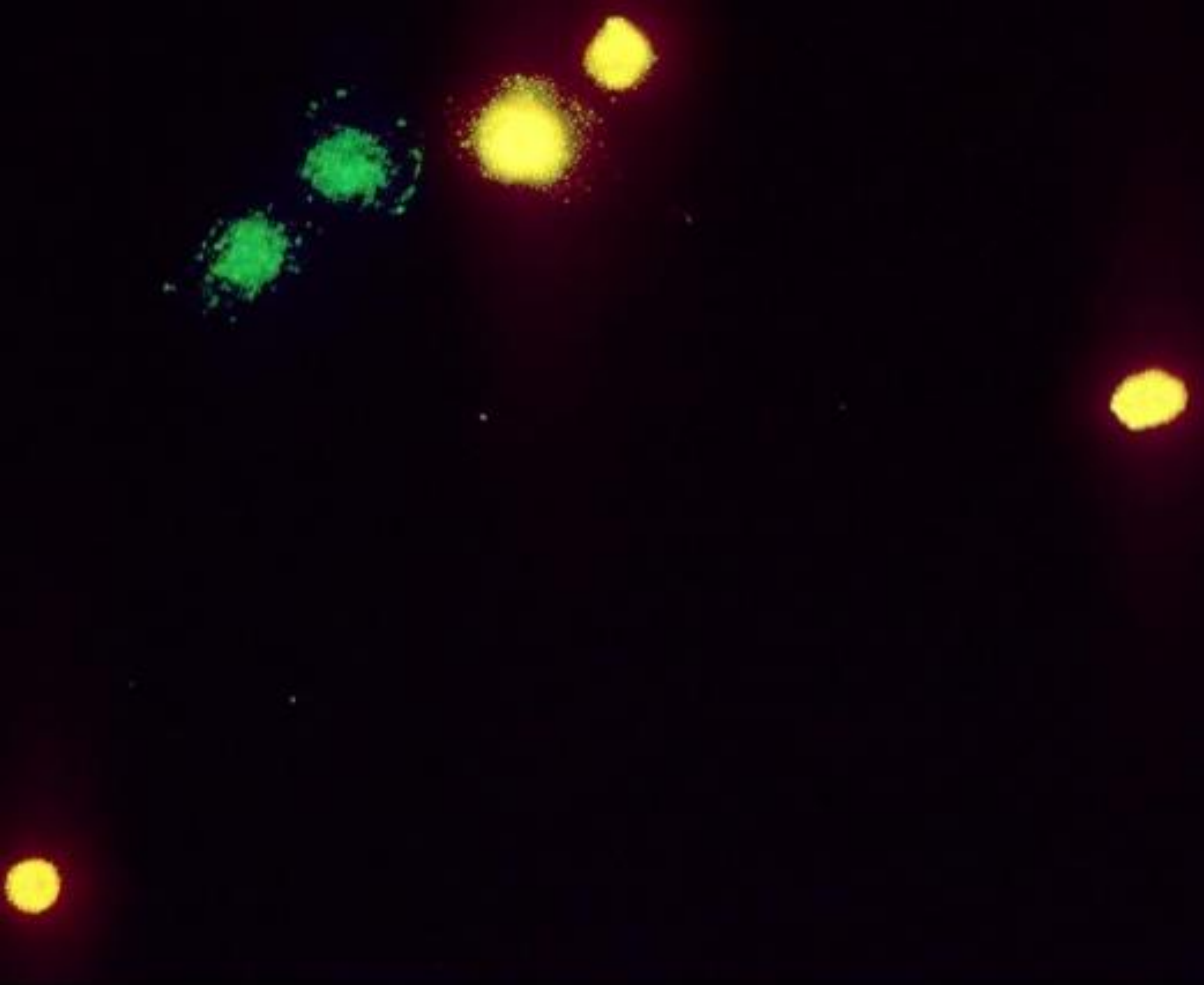


identification

Mould—calcofluor white



Yeast—calcofluor white
http://www.med.sc.edu:85/mycology/mycology-3.htm



Influenza virus infected cells, fluorescent antibody stain

Mycobacterium – auramine stain

Better Use Microscopy

- Phase Contrast Microscopy
  - shift in light allows visualization of organism; can visualize viable organisms
- Fluorescent Microscopy
  - certain dyes (fluorochromes) give off light when excited (fluorescence)
  - color of light depends on the dye and the filters used
  - Staining techniques
    - Fluorochroming: direct chemical interaction with organism
      - Acridine orange: stains nucleic acid; useful for cell-wall deficient organisms
      - Auramine-rhodamine: bind to mycolic acids in nearly all *Mycobacteria*
      - Calcoflour white: binds to chitin in cell walls of fungi
    - Immunofluorescence: fluorochrome is bound to an antibody; can detect/identify specific organisms

Culture and isolation of bacteria

- Principles of Cultivation
  - Nutritional requirements
    - General concepts
      - non-fastidious: simple requirements for growth
      - fastidious: complex, unusual, or unique requirements for growth
    - Phases of growth media
      - solid → agar; boil to dissolve, solidifies at 50ºC
      - liquid, broth

- **Media classifications and functions**
  - **Enrichment**
    - used to enhance growth of specific organisms
  - Supportive
    - support growth of most non-fastidious organisms
  - **Selective**
    - contains agents that inhibit the growth of all agents except that being sought (dyes, bile salts, alcohols, acids, antibiotics)
  - Differential
    - contains factor(s) that allow certain organisms to exhibit different metabolic characteristics

According to Use

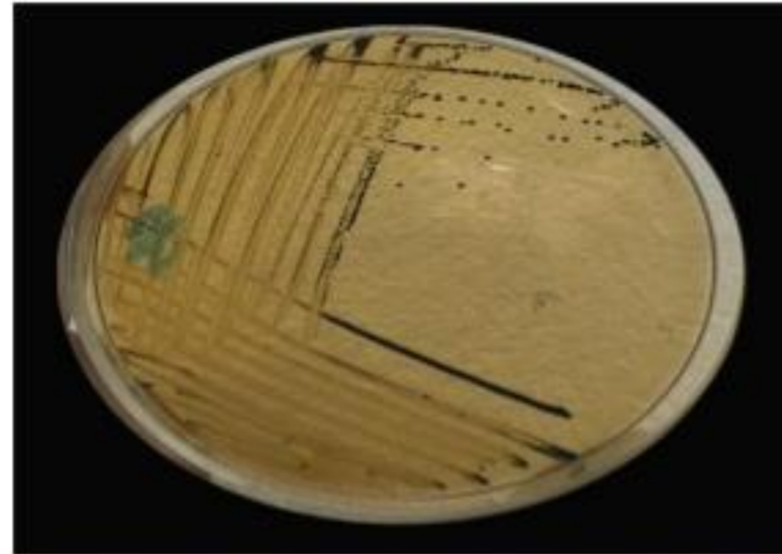
- Enriched Medium – broth or solid, contains rich supply of special nutrients that promotes growth of a particular organism while not promoting growth of other microbes that may be present (e.g BAP & chocolate agar)

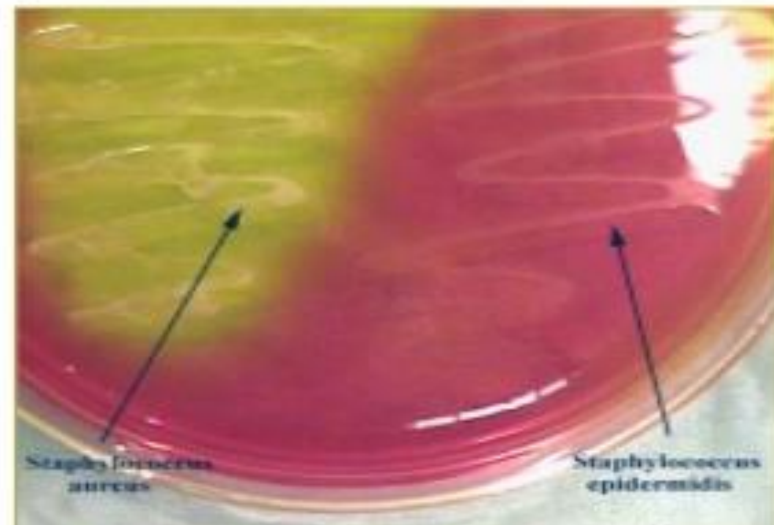
• Types of artificial media

- Brain-heart infusion
  - nutritionally rich supportive media used in broths, blood culture systems and susceptibility testing
- Sheep blood agar
  - supportive media containing 5% sheep blood for visualization of hemolysis
- Chocolate agar
  - same as sheep blood agar except blood has been "chocolatized" RBCs lysed by heating; releases X (hemin) and V (NAD) factors for *Neisseria* and *Haemophilus*
- MacConkey agar
  - selective for Gram-negative rods (GNRs) because of crystal violet and bile salts; differential due to lactose, fermenters lower pH changing neutral red indicator pink/red

According to Use

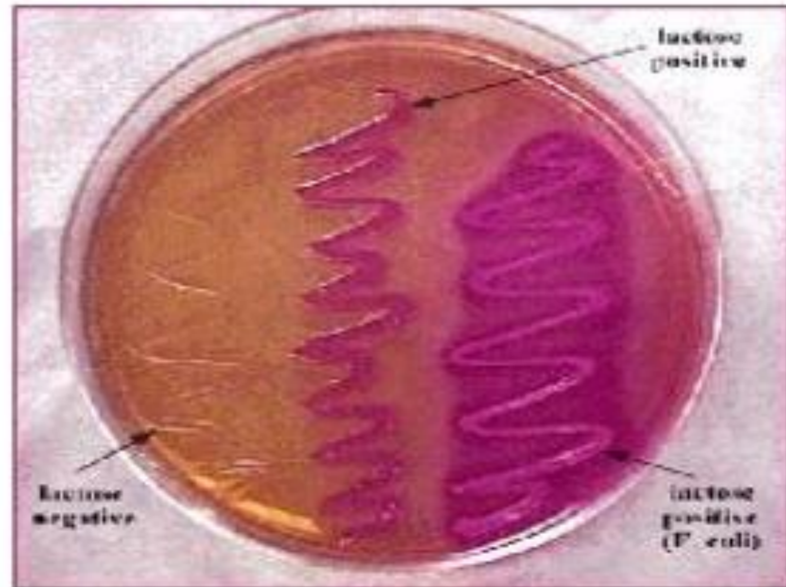
- Selective Medium - contains inhibitors that discourage the growth of certain organisms & enhances the growth of the microbe sought (e.g. SSA, Mannitol Salt Agar)

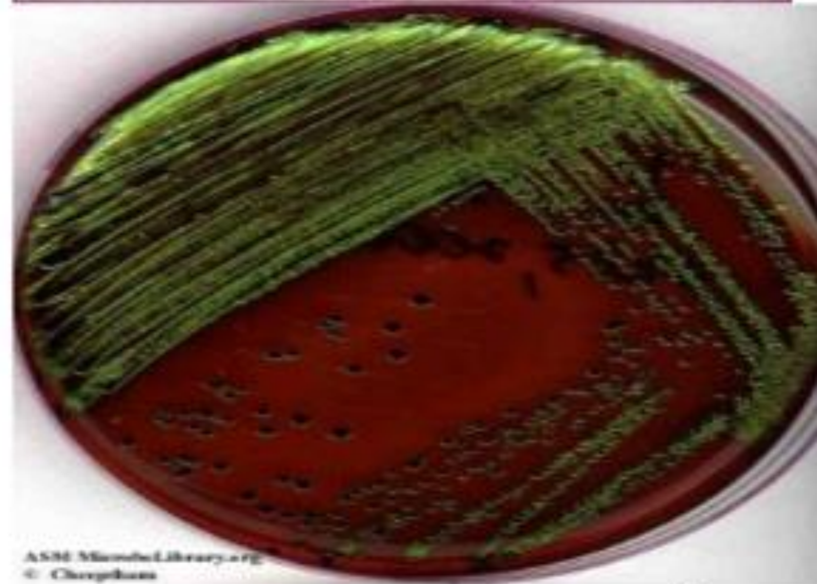




According to Use

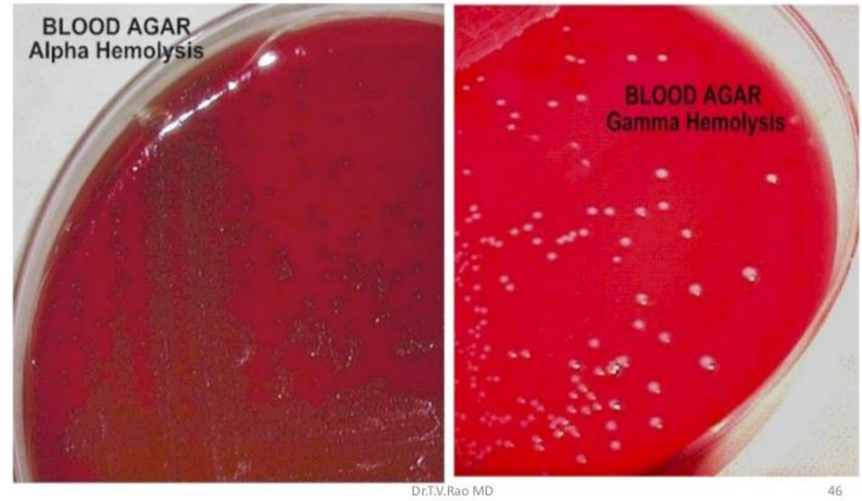
- Differential Medium - contains dyes, indicators or other constituents that give colonies of particular organisms distinctive and easily recognizable characteristics (e.g. McConkey Agar)



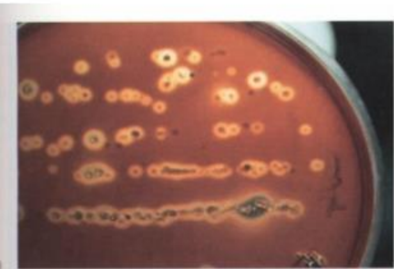


Hemolysis a guiding factor

Hemolysis a guiding factor

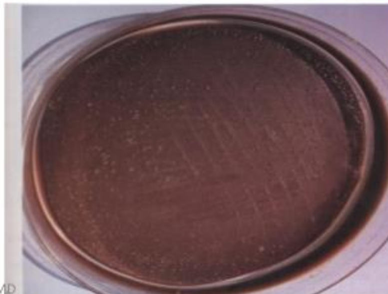


Enriched Media

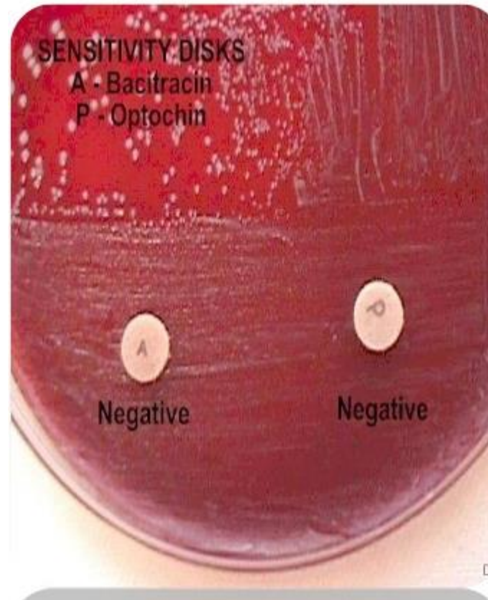


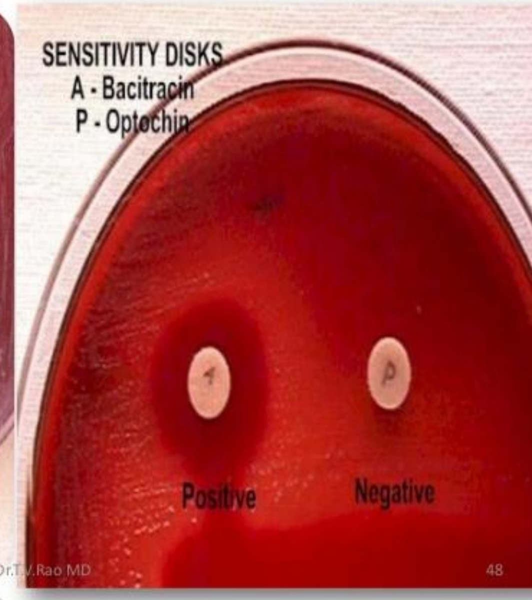
Blood agar plate with bacteria from human throat. This media differentiates among different colonies by appearance

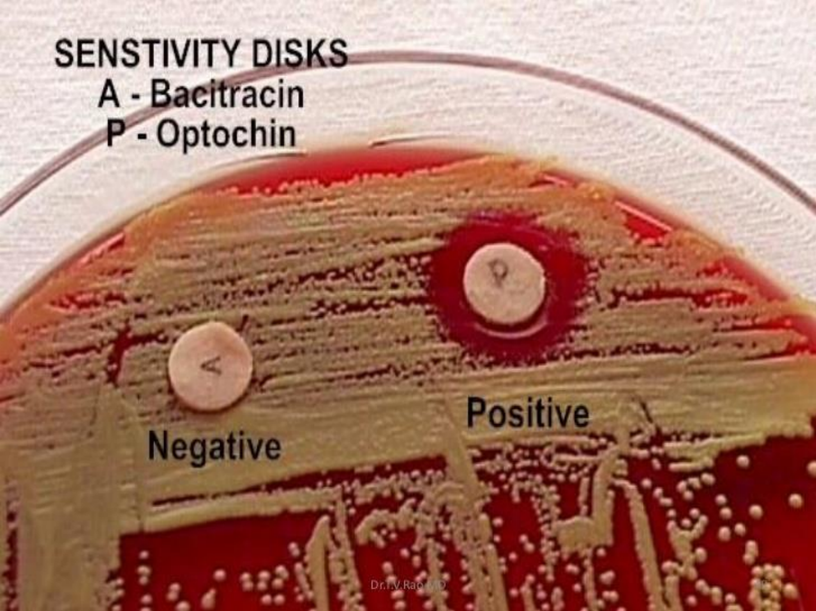
Chocolate agar, a medium that gets brown from heated blood. Used for isolation of *N. gonorrhea*.



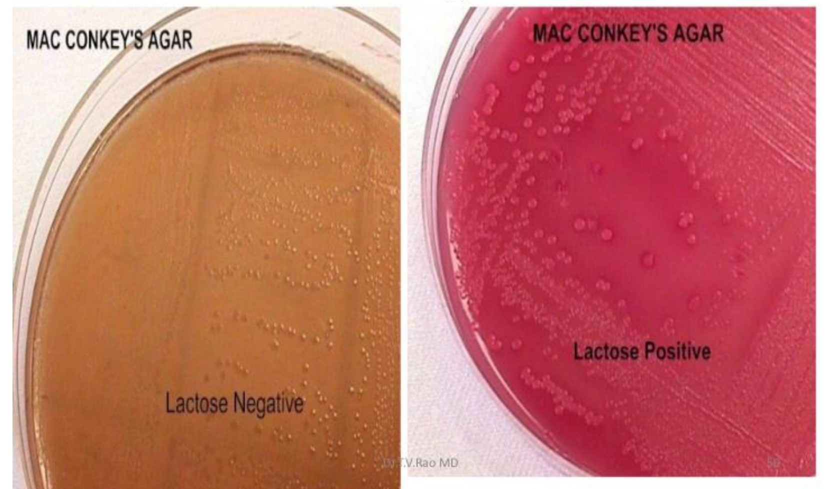
Testing for Bacitracin and Optochin Sensitivity





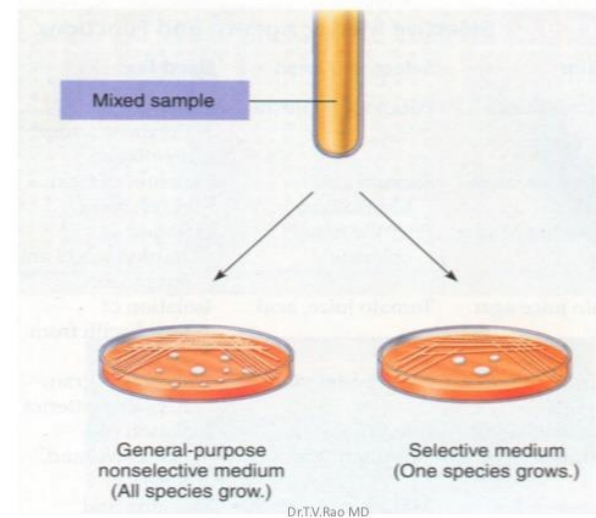


Mac Conkey Agar a Minimal differentiating Medium

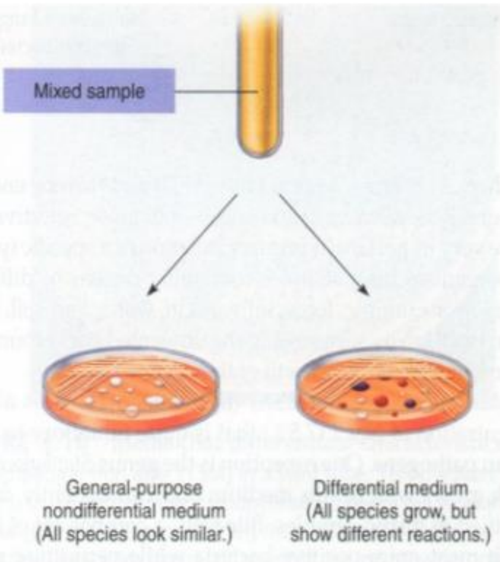


MacConkey Agar

General vs Selective Media

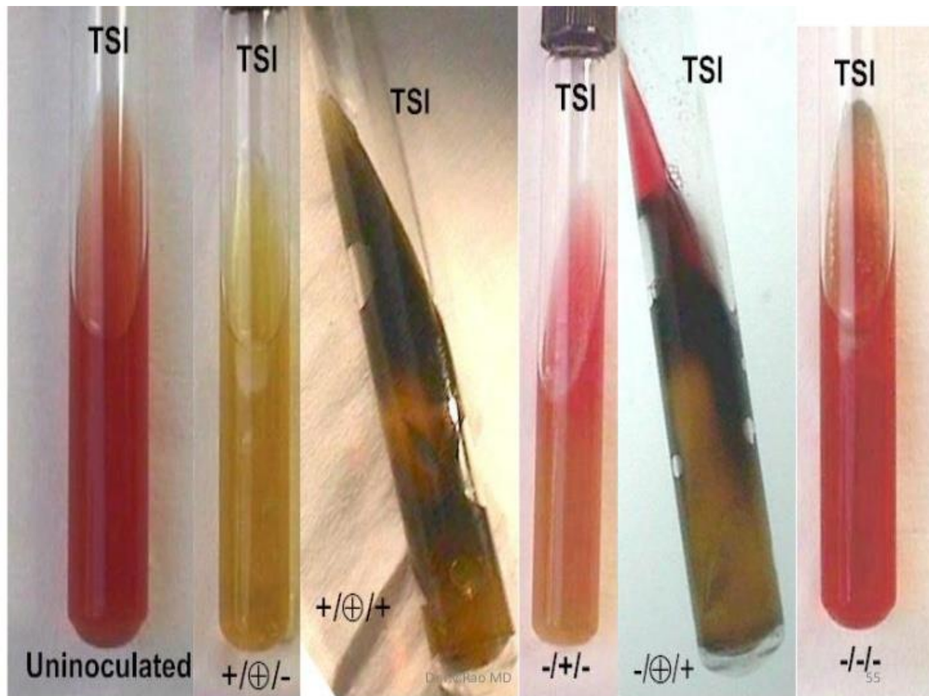


Differential Media



- **Differential media** grow several types of organisms and **display visible differences** among organisms.
- Differences may show up as colony size, media colour, gas bubble formation and precipitate formation.

Salmonella-Shigella Agar



- **Types of artificial media**

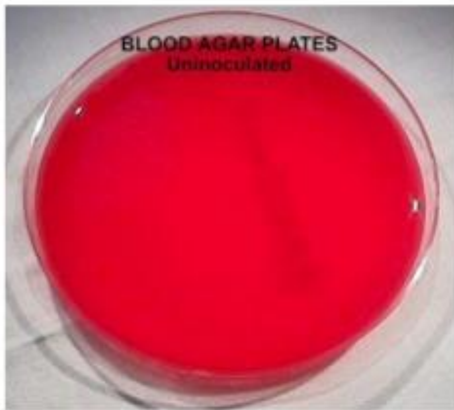
– Hektoen enteric agar
  - contains bile salts and dyes (bromothymol blue and acid fuchsin) to inhibit non-pathogenic GNRs; non pathogens ferment lactose changing BTB to orange; pathogens *Salmonella* and *Shigella* are clear; ferric ammonium citrate detects H_2S production of *Salmonella* (black colonies)

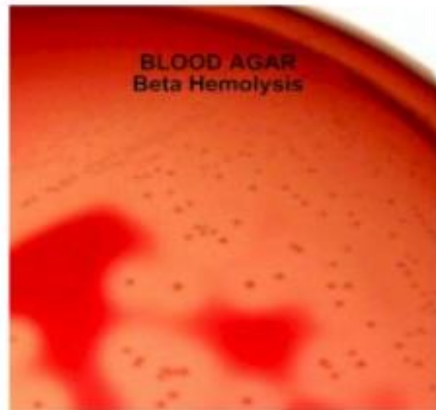
– Columbia colistin-nalidixic acid (CNA) agar
  - Columbia agar base, sheep blood, colistin and nalidixic acid; selective isolation of gram-positive cocci

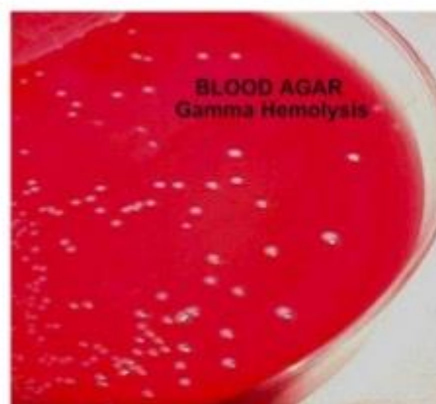
Types of artificial media

- Thayer-Martin agar
  - CAP with antibiotics (colistin inhibits gram neg, vancomycin inhibits gram pos, nystatin inhibits yeast); for *N. gonorrhoeae* and *N. meningitidis*; Martin-Lewis has similar function but different antibiotics

- Preparation of artificial media
  - Sterilization
    - autoclave: pressurized steam at 121ºC for 15-30 min.







http://science.nhmccd.edu/biol/wellmeyer/media/media.htm

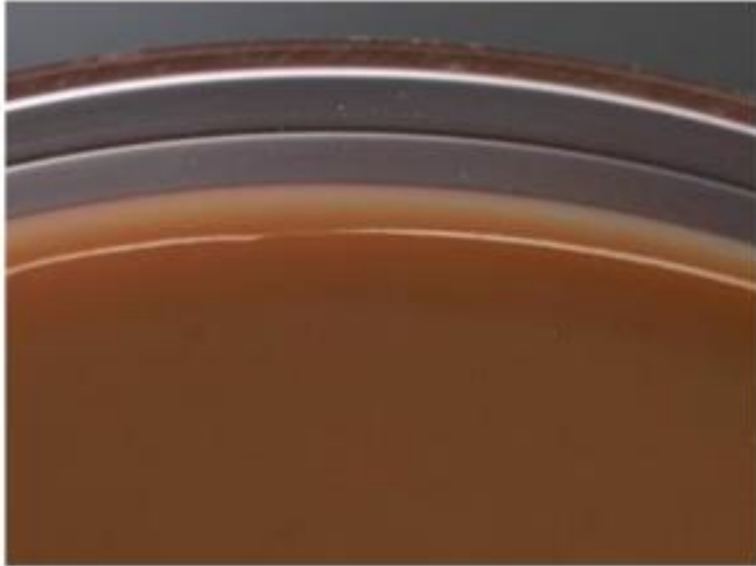
• Environmental requirements

- Oxygen and Carbon dioxide availability
  - aerobic: room air
  - facultative: aerobic or anaerobic
  - microaerophilic: reduced oxygen tension
  - anaerobic
    - strict or aerotolerant
  - capnophilic: increased $C0_2$ (5-10%)
- Temperature
  - 35-37ºC
  - 30ºC
  - cold
  - 42ºC

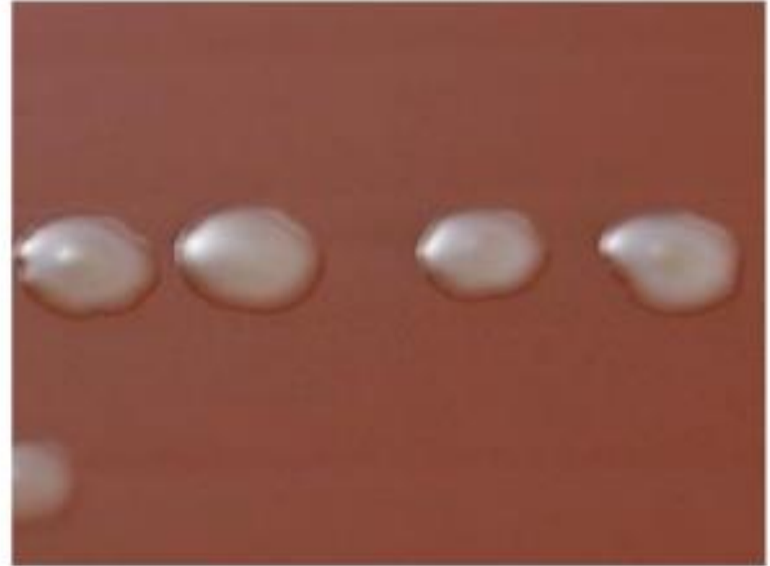
• Bacterial Cultivation

- Isolation of bacteria from specimens
  - streaking for isolation
  - streaking for quantitation
- Evaluation of colony morphologies
  - Type of media supporting growth
  - Relative quantities of each colony type
  - Colony characteristics
    - colony form: pinpoint, circular, filamentous, irregular
    - colony elevation: flat, raised, convex
    - colony margin: smooth, irregular
  - Gram stain and subcultures
    - sterile loop, isolated colonies

Chocolate agar



Uninoculated



Haemophilus

MacConkey agar

Hektoen agar

Enterobacter produces acid on HE agar and turns the medium orange.

Salmonella growing on HE agar produces colonies with black centers (produces hydrogen sulfide).

- Nutritional requirements and metabolic capabilities
  - Single enzyme tests
    - Catalase: H_2O_2 + catalase = O_2 and H_20; differentiates *Staphylococcus* v. *Streptococcus*, *Listeria* and Corynebacterium v. other non spore forming gram-positive bacilli
    - Oxidase: detection of cytochrome oxidase that participates in nitrate metabolism; *Pseudomonas*, *Aeromonas*, *Neisseria*
    - Indole: tryptophanase degrades tryptophan into pyruvic acid, ammonia, and indole; indole is detected by aldehyde indicator; presumptive id for *E. coli*
    - Urease: hydrolyzes urea into ammonia, water and CO_2; increase pH changes causes bright pink color of indicator
    - PYR: hydrolysis of PYR, indicator turns pink; Group A Strep and enterococci are +

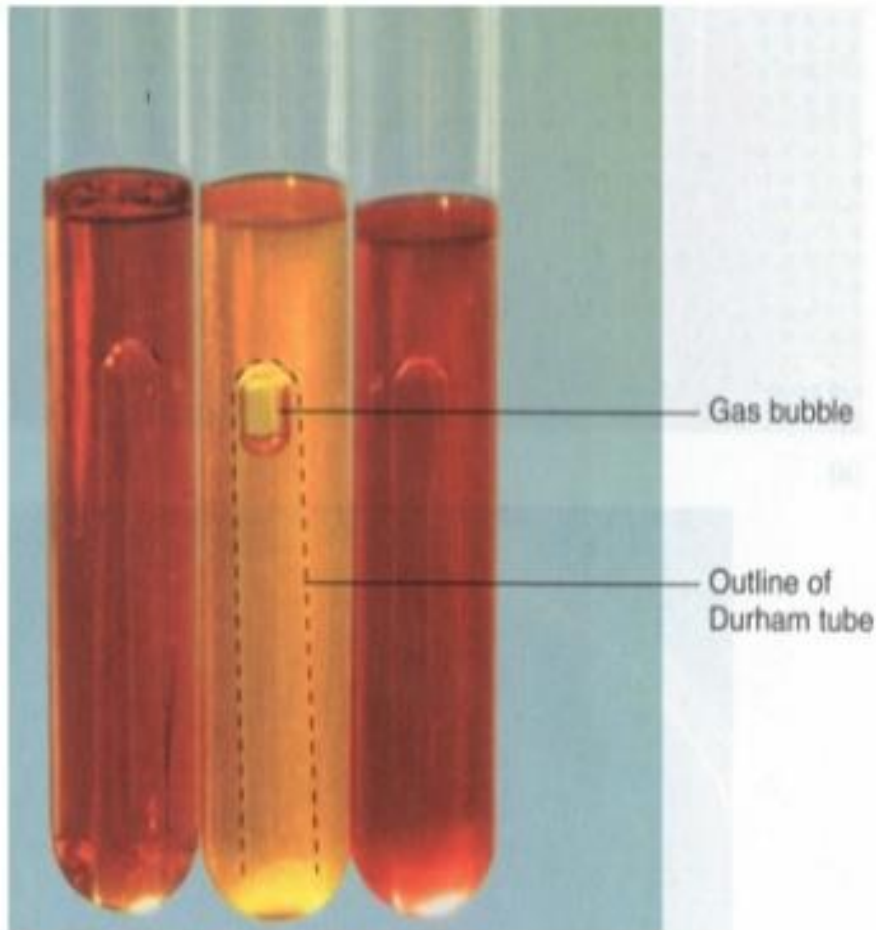
Biochemical Tests

- The microbe is cultured in a media with a **special substrate and tested for an end product**.
- Prominent biochemical tests include **carbohydrate fermentation, acid or gas production and the hydrolysis of gelatin or starch.**
- Many of these test in rapid miniaturized system that can detect for 23 characteristics in small cups called **Rapid test**.
- The info from the rapid test are input into a computer to help in identification of the organisms.

Biochemical Tests

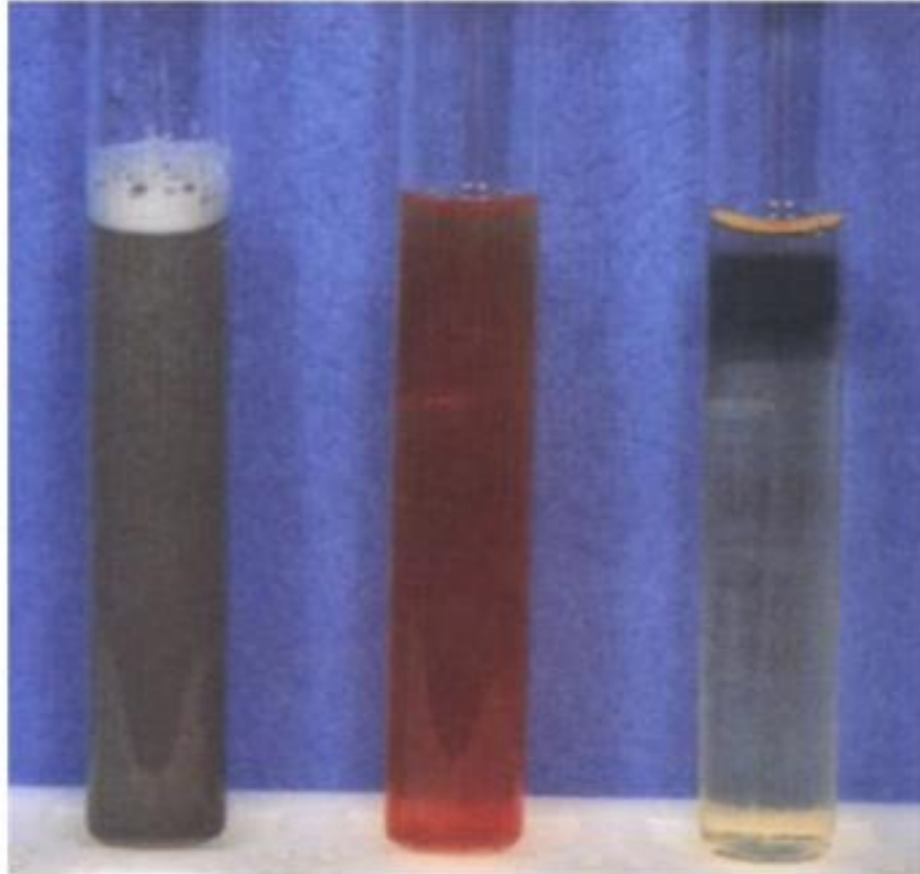
- Other biochemical tests of interest include:
  - H_2S production
  - Indole test
  - Oxidase test
  - Oxidation fermentation
  - Phenylalanine deaminase test
  - Antibiotic susceptibility tests
- Principle, procedure, most common use.

Carbohydrate Fermentation



- . This medium show **fermentation** (**acid production**) and **gas formation**.
- The small **Durham tube** for collecting gas bubbles.
- Left- right:
  - Uninoculated negative control
  - Centre, positive for acid (yellow) and gas (open space).
  - Growth but no gas or acid.

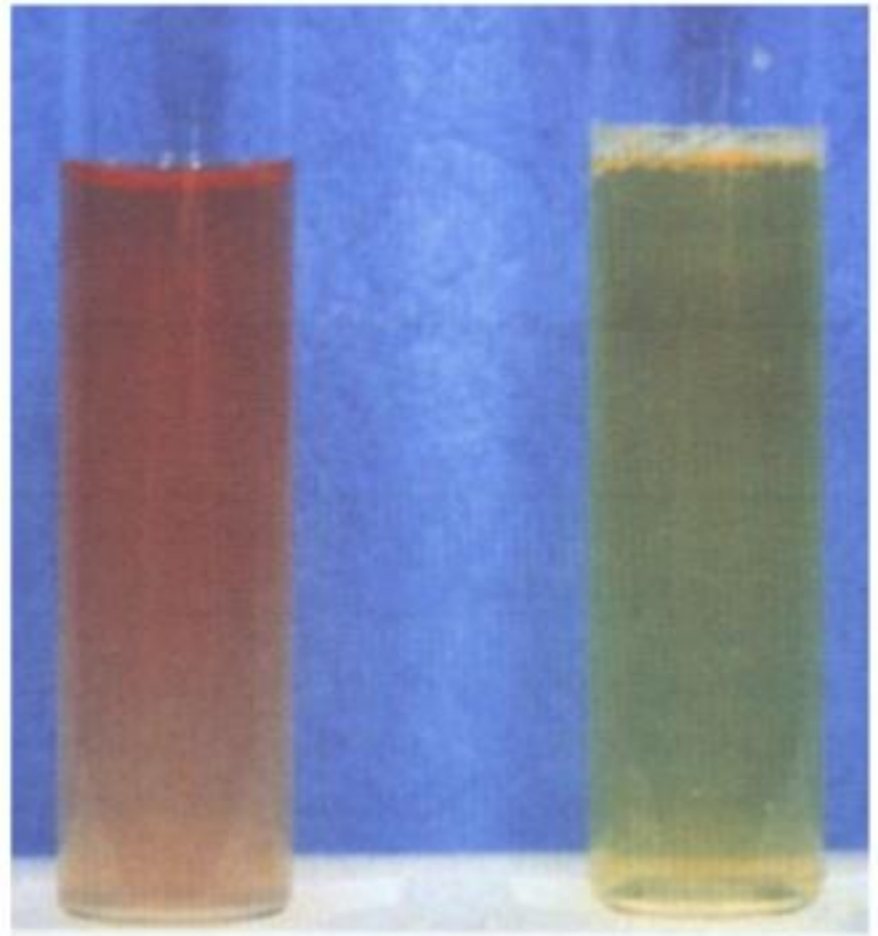
Nitrate Reduction



- After 24-48 hrs of incubation, **nitrate reagents** are added.
- Left to right:
  - Gas formation (positive for nitrate reduction).
  - positive for nitrate reduction to nitrite (red colour).
  - Negative control

Methyl Red Test

- This is a qualitative test for **acid production**.
- The bacteria is grown in MR-VP broth.
- After addition of several drops of **methyl red solution** a bright red colour is positive and yellow-orange negative.



CATALASE TEST

Negative

Positive

COAGULASE TEST

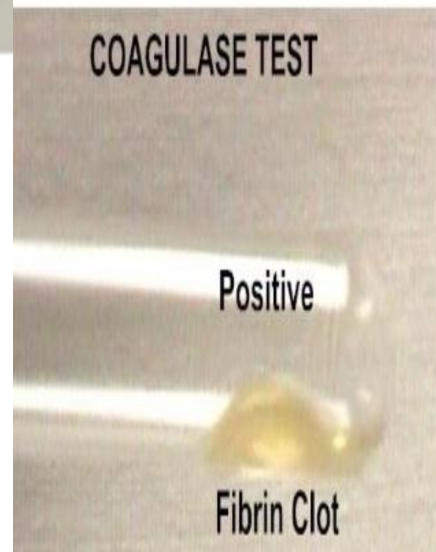
Rabbit Plasma

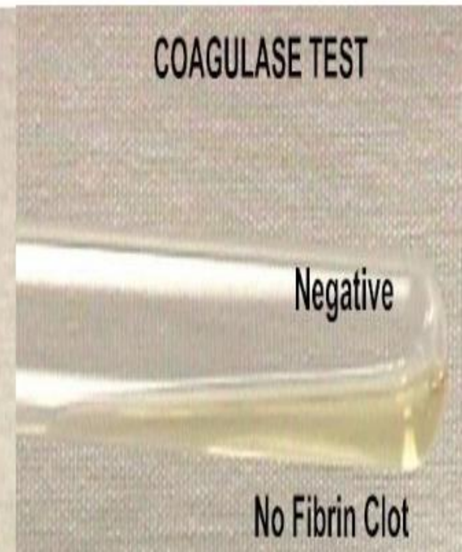
Nutrient Broth

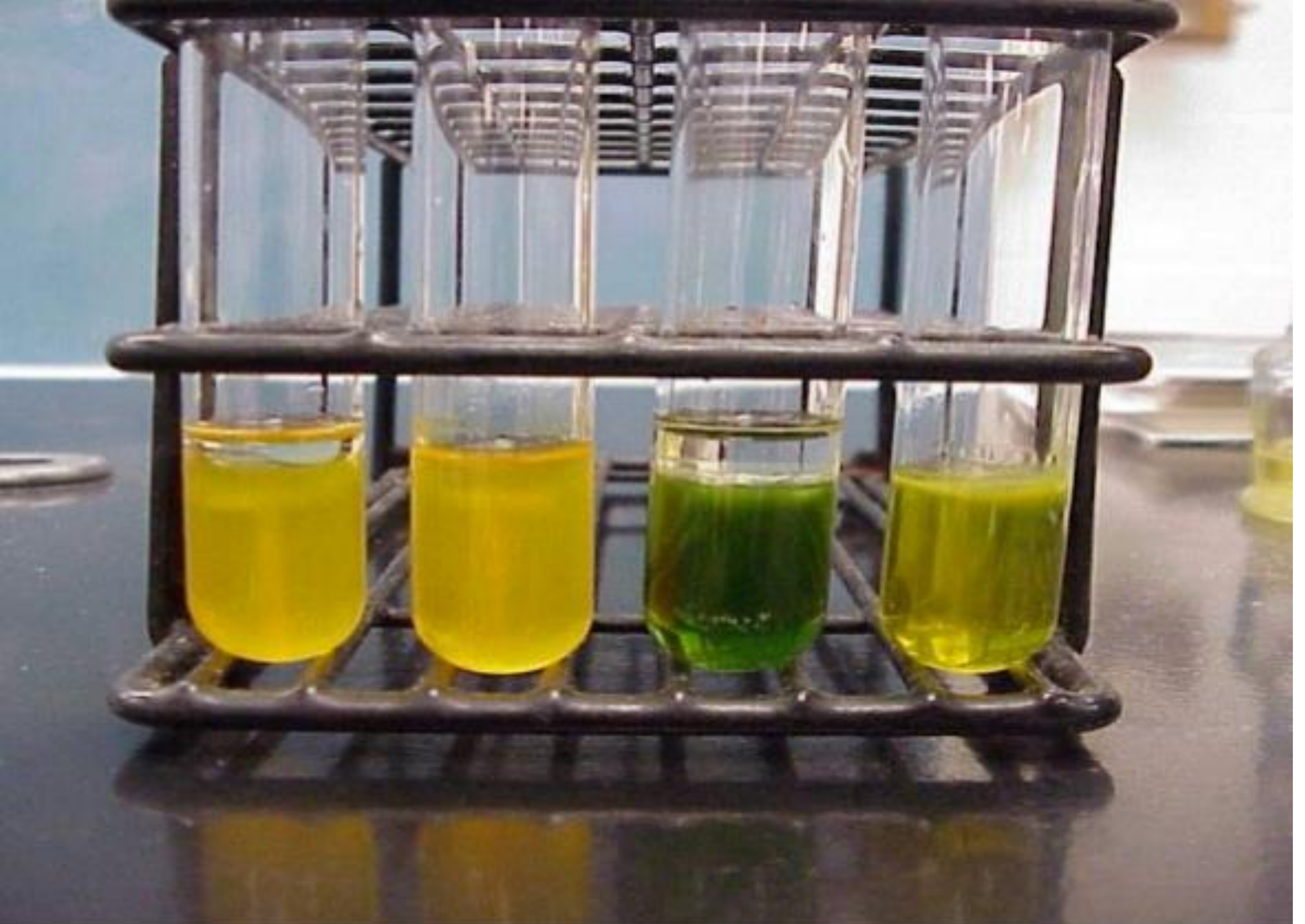
Pipette

Dr.T.V.Rao MD

Coagulase Test







O-F glucose media

Rapid Tests

- **Rapid test**: a biochemical system for the identification of ***Enterobacteriaceae*** and other Gram –ve bacteria.
- It consist of **plastic strips with 20 µl of dehydrated biochemical substrates** used to detect biochemical characteristics.
- The biochemical **substrates are inoculated** with pure cultures and suspended in physiological saline.
- After 5 hrs-overnight the 20 tests are converted to **7-9 digital profile.**

–Tests for presence of metabolic pathways

- Oxidation and fermentation: oxidation of glucose requires oxygen, fermentation does not; pH decreases causing yellow color
- Amino acid degradation: detection of amino acid decarboxylase enzymes

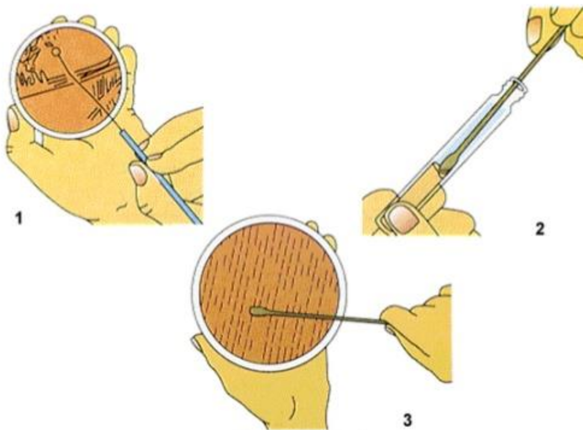
Differential Media Chromagar

- Chromagar orientation uses colour-formation to distinguish at least 7 common urinary pathogens.
- This allow for rapid identification and treatment.
- The bacteria were streaked as to spell their names.

Bacteriophage Typing

- **What are bacteriophages?**
- Bacteriophage typing is based on the **specificity of phage surface receptor** for the cell surface receptor.
- Only those phages that can **attach to the surface receptors** can cause lysis.
- The procedure involves:
- A plate is **heavily inoculated** so that there is no uninoculated areas.
- The plate is **marked off** in squares (15-20 mm) and each square **inoculated** with a drop of suspension for **different phages**.

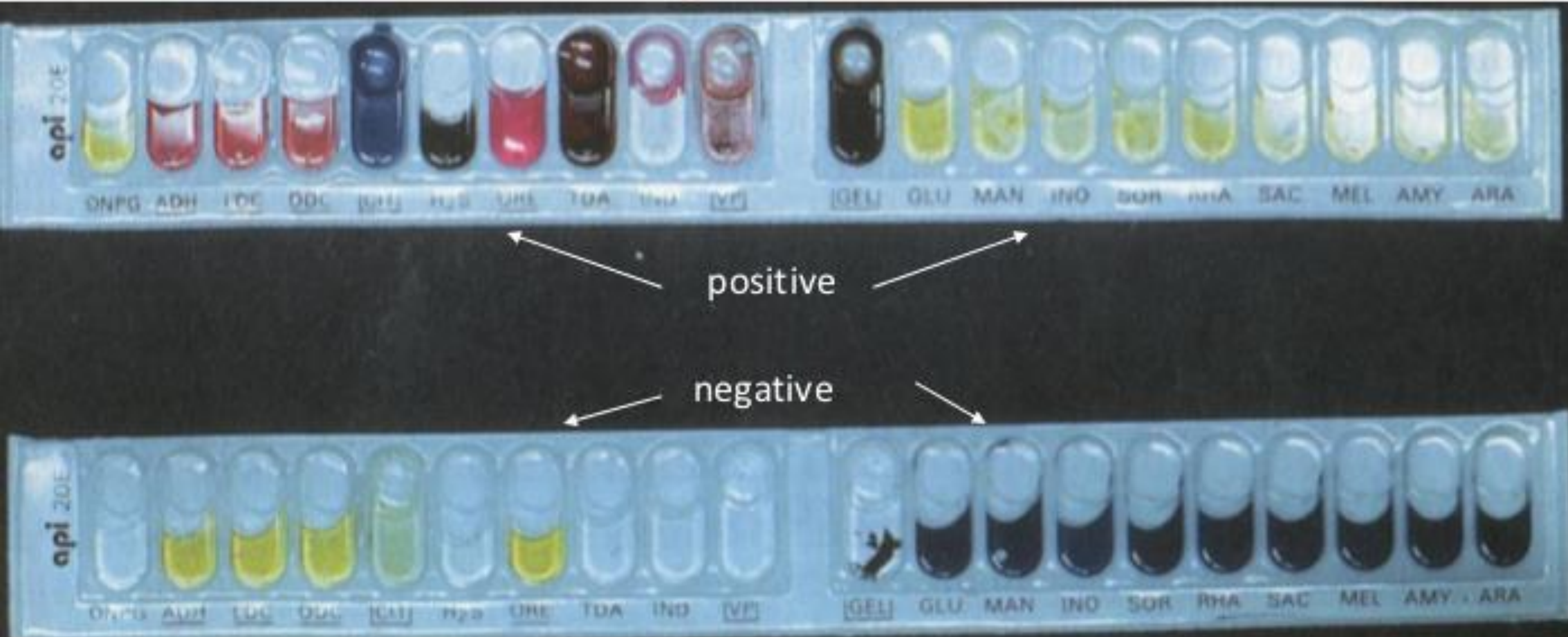
Heavily Inoculated Plate



Bacteriophage Typing

- The plate is incubated for 24 hrs then observed for plaques.
- The phage type is reported as a specific **genus and species** followed by **the types** that can infect the bacterium.
- E.g. 10/16/24 means that the bacteria is sensitive to phages 10, 16 and 24.
- Phage tying remain a **tool for research** and **reference labs**.

Rapid Tests



ONPG (β galactosidase); ADH (arginine dihydrolase); LDC (lysine decarboxylase); ODC (ornithine decarboxylase); CIT (citrate utilization); H_2S (hydrogen disulphide production); URE (urease); TDA (tryptophan deaminase); IND (indole production); VP (Voges Proskauer test for acetoin); GEL (gelatin liquefaction); the fermentation of glucose (GLU), mannitol (MAN), inositol (INO), sorbitol (SOR), rhamnose (RHA), sucrose (SAC); Melibiose (MEL), amygdalin (AMY), and arabinose (ARA); and OXI (oxidase).

Flow Cytometry

- Classical techniques are not successful in identification of those microorganisms that cannot be cultured.
- What do is the name of microorganisms that cannot be cultured?
- **Flow cytometry** allows single or multiple microorganisms detection an easy, reliable and fast way.
- Inflow cytometry microorganisms are identified on the basis of the **cytometry parameters** or by means of certain dyes called **fluorochromes** that can be used independently or bound to specific antibodies.

Flow Cytometry

- The cytometer forces a suspension of cells through a **laser beam** and measures the light they scatter or the fluorescence the cell emits as they pass through the beam.
- The cytometer also can measure the cell's shape, size and the content of the DNA or RNA.

Immunology

Immunological Methods

- Immunological methods involve the interaction of a microbial **antigen with an antibody** (produced by the host immune system).
- Testing for microbial antigen or the production of antibodies is **often easier** than test for the microbe itself.
- **Lab kits** based on this technique is available for the identification of many microorganisms.

Principles of Serologic Test Methods

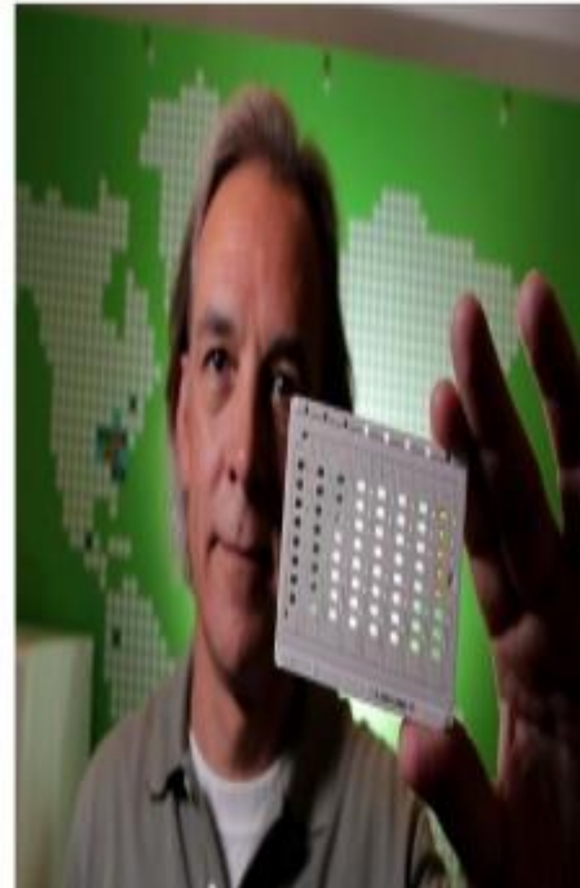
- Methods for antibody detection
  - Direct whole pathogen agglutination assays
    - pos patient sera causes organism to clump
  - Particle agglutination tests
    - latex beads or RBCs coated with Ag
  - Flocculation tests
    - RPR – precipitation of soluble Ag with Ab
      - charcoal particles coated with cardiolipin-lecithin binds reagin
  - ELISAs
  - IFAs
    - organism/antigen on slides; patient Ab detected with fluorescent secondary Ab
  - Western blots

Commercial ID systems

– Advantages and examples of commercial systems
  - ARIS (Trek)
  - MicroScan (Dade-Behring/Seimens)
  - Phoenix (Becton-Dickinson)
  - **Vitek (bioMérieux)**

Immunochemical methods for ID

- Principles of immunochemical methods
  - Particle agglutination
    - Latex agglutination
  - Immunofloresecent assays
    - Direct immunofluorescence assay (DFA)
    - Indirect immunofluorescence assay (IFA)
    - Enzyme immunoassays
  - Solid-phase immunoassays
    - Membrane bound immunoassays
    - Immunochromatographic assays
  - Optical immunoassays



Serologic methods for diagnosis

- Features of the Immune Response
  - Characteristics of antibodies
    - Features of immune response useful in diagnostic testing
      - Acute v. anamnestic response
      - IgM v. IgG
      - IgM can't cross placenta
      - immunocompetent v. immunocompromised
    - Interpretation of serologic tests
      - single v. paired sera; rare pathogen
      - 4-fold rise in titer v. qualitative testing
      - cross reactivity (herpes viruses, heterophile Abs, pregnancy)

Principles of Serologic Test Methods

- Methods for antibody detection
  - Direct whole pathogen agglutination assays
    - pos patient sera causes organism to clump
  - Particle agglutination tests
    - latex beads or RBCs coated with Ag
  - Flocculation tests
    - RPR – precipitation of soluble Ag with Ab
      - » charcoal particles coated with cardiolipin-lecithin binds reagin
  - ELISAs
  - IFAs
    - organism/antigen on slides; patient Ab detected with fluorescent secondary Ab
  - Western blots

Genotypic methods

- Genotypic methods of microbe identification include the use of :
  - ✓Nucleic acid probes
  - ✓PCR (RT-PCR, RAPD-PCR)
  - ✓Nucleic acid sequence analysis
  - ✓rRNA analysis
  - ✓RFLP
  - ✓Plasmid fingerprinting.

Genotypic Methods

- Genotypic methods involve examining the genetic material of the organisms and has **revolutionized bacterial identification** and classification.
- Genotypic methods include PCR (RT-PCR, RAPD-PCR),use of nucleic acid probes, RFLP and plasmid fingerprinting.
- Increasingly genotypic techniques are becoming the **sole means of identifying** many microorganisms because of its **speed and accuracy**.

RAPD-PCR

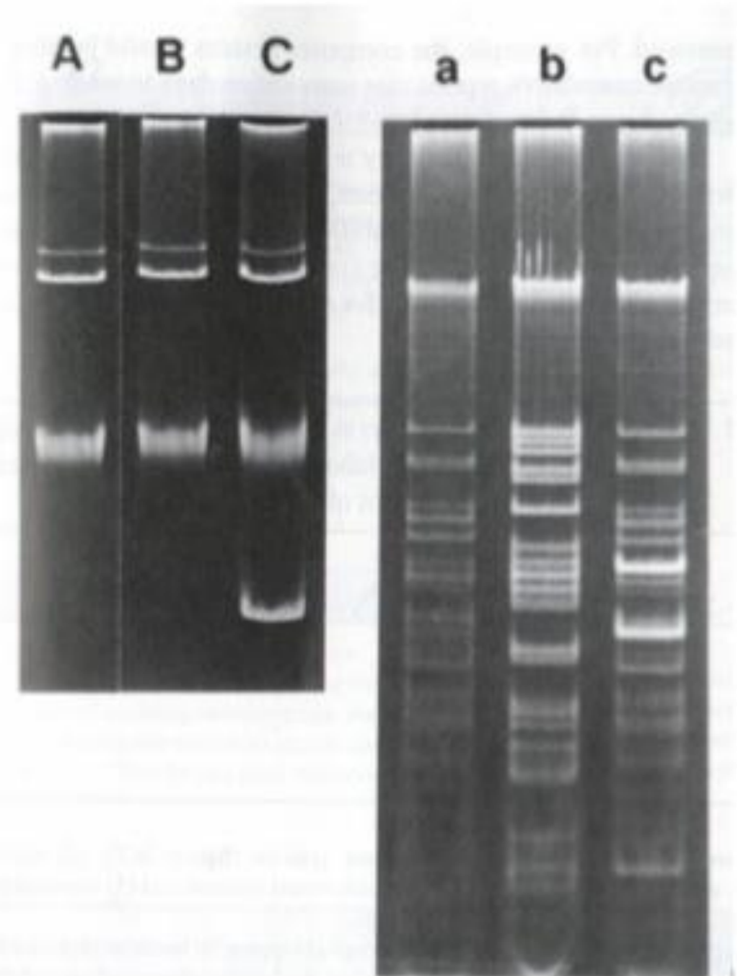
- Random amplified polymorphic DNA PCR uses **a random primer** (10-mer) to generate a DNA profile.
- What are random primers?
- **The primer** anneals to several places on the DNA template and generate a DNA profile which is used for microbe identification.
- RAPD has many advantages:
  - Pure DNA is not needed
  - Less labour intensive than RFLP.
  - There is not need for prior DNA sequence data.
- RAPD has been used to fingerprint the outbreak of *Listeria monocytogenes* from milk.

Real Time PCR and RT-PCR

- Currently many PCR tests employ **real time PCR**.
- This involves the use of **fluorescent primers**.
- The PCR machine **monitors the incorporation of the primers** and display an **amplification plot** which can be viewed continuously thru the PCR cycle.
- Real time PCR **yields immediate results**.
- Another application of PCR is **RT-PCR** (reverse trancriptase PCR).
- During RT-PCR an RNA template is used to **generate cDNA and from this dsDNA** is generated.
- The enzyme used is **reverse transciptase**.
- RT-PCR is used to detect for HIV and to monitor the progress of the disease.

Plasmid fingerprinting

- The procedure involves:
- The bacterial strains are **grown**, the cells lysed and harvested.
- The plasmids are **separated** by agarose gel electrophoresis
- The gels are **stained with EtBr** and the plasmids located and compared.



Computer and Bacteria Identification

- Computers improve the efficiency of the lab operations and increase the speed and clarity with which results can be reported.
- Computers are also important for the result entry, analysis and preparation.