Bacterial Classification and Identification

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URGENT!!!!

- It is important for you to review the powerpoint lectures on Bacterial Cell Structure and Bacterial Metabolism from first year before coming to class for this lecture.

Objectives

- Review the criteria for bacterial classification and identification
- Discuss the principles underlying the biochemical, staining and molecular techniques used for classification, identification and diagnosis
- Illustrate the clinical applications of these diagnostic techniques
- Emphasize the clinical implications of proper identification in the diagnosis, source monitoring of bacterial diseases and antibiotic resistance

MICROBIAL DIVERSITY

- Taxonomy (science of classsification)
- Classification (evolutionary relatedness)
- Nomenclature (naming systems)
- Binomial System (Genus / species)
- Identification (for correct diagnosis and treatment)

BACTERIAL NOMENCLATURE

- Bacterial nomenclature is a system of assigning names to similar groups of bacteria according to international codes and rules.
- Binomial nomenclature : Genus and species, e.g *Pseudomonas aeruginosa*
- *species* represents strains with a high degree of overall similarities that differ from other strains
- *Genus* represents collection of similar species.
- Binomials may indicate morphology (*Streptococcus, Staph, Bacillus*) , discoverer (Escherichia, Dr Escherich), metabolic feature (*Staph aures)* or disease association (*Klebsiella pneumonia.)*
- Bacterial nomenclature allows scientists to communicate efficiently and accurately

BACTERIAL CLASSIFICATION

- Classification is a systematic arrangement of organisms into groups or taxa according to a set of criteria
- Medically important bacteria (~300 species) are classified according to phenotypic, analytic and genotypic characteristics
- Bacterial classification :
  - a .Facilitates proper laboratory identification of clinical isolates
  - b. Necessary for determining etiology of infectious diseases during epidemiological investigations
  - c. essential to bacterial nomenclature

DNA homology compares DNA sequences among bacteria using molecular probes and hybridization studies to determine genetic relatedness

Phylogenetic relatedness is determined by comparisons of 16srRNA sequences among bacteria

Classification Criteria

- Include:
  - Cell wall structure (peptidoglycan, mycolic acid)
  - Cell membrane structure (phospholipid, lipid A)
  - DNA base composition (guanine, cytosine, adenine, thymidine)

Review of Bacterial Structure & Function

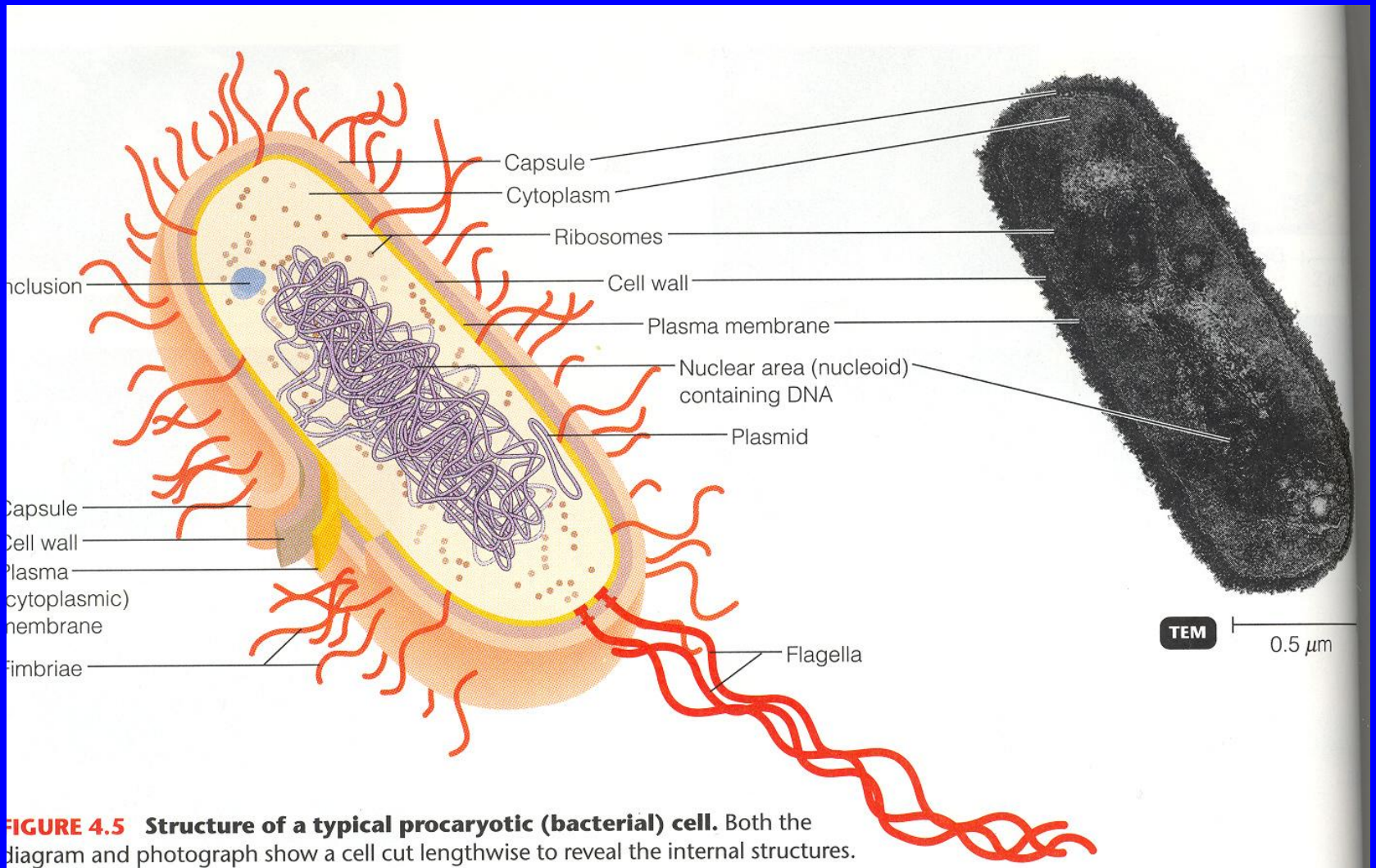


FIGURE 4.5 Structure of a typical procaryotic (bacterial) cell. Both the diagram and photograph show a cell cut lengthwise to reveal the internal structures.

METHODS FOR BACTERIAL CLASSIFICATION AND IDENTIFICATION

METHOD	CLASSIFICATION	IDENTIFICATION
• Morphological Characteristics (cocci, rods)	No	Yes
• Differential Staining – Gram Stain – Acid fast stain	Yes (cell wall type)	Yes
• Biochemical Tests – Lactose fermentation – H_2S production	No	Yes
• Serology – Slid Agglutination – Serological Testing for serotypes – ELISA – Western blotting	Yes	Yes
• Phage Typing	No	Yes
• Amino Acid Sequencing	Yes	No
• Fatty Acid Profiles	No	Yes
• Flow Cytometry (for Pseudomonas and Listeria)	No	Yes
• DNA Base Composition	Yes	No
• Plasmid Fingerprinting	No	Yes
• Ribosomal RNA (rRNA) Sequencing	Yes	No
• Nucleic Acid Hybridization	Yes	Yes
• Polymerase Chain Reaction (PCR)	Yes	Yes

Most Clinically Relevant Methods for ID and Diagnosis

- Gram Stain (cell wall)
- Acid Fast Stain (cell wall)
- Biochemical Tests (cell macromolecules)
- Serology & Latex Agglutination (surface agns)
- Western Blot (cell proteins)
- ELISA (cell proteins, CHOs)
- Plasmid Fingerprinting (plasmid DNA)
- Nucleic Acid Hybridization (DNA, RNA)
- Polymerase Chain Reaction (PCR) (DNA)

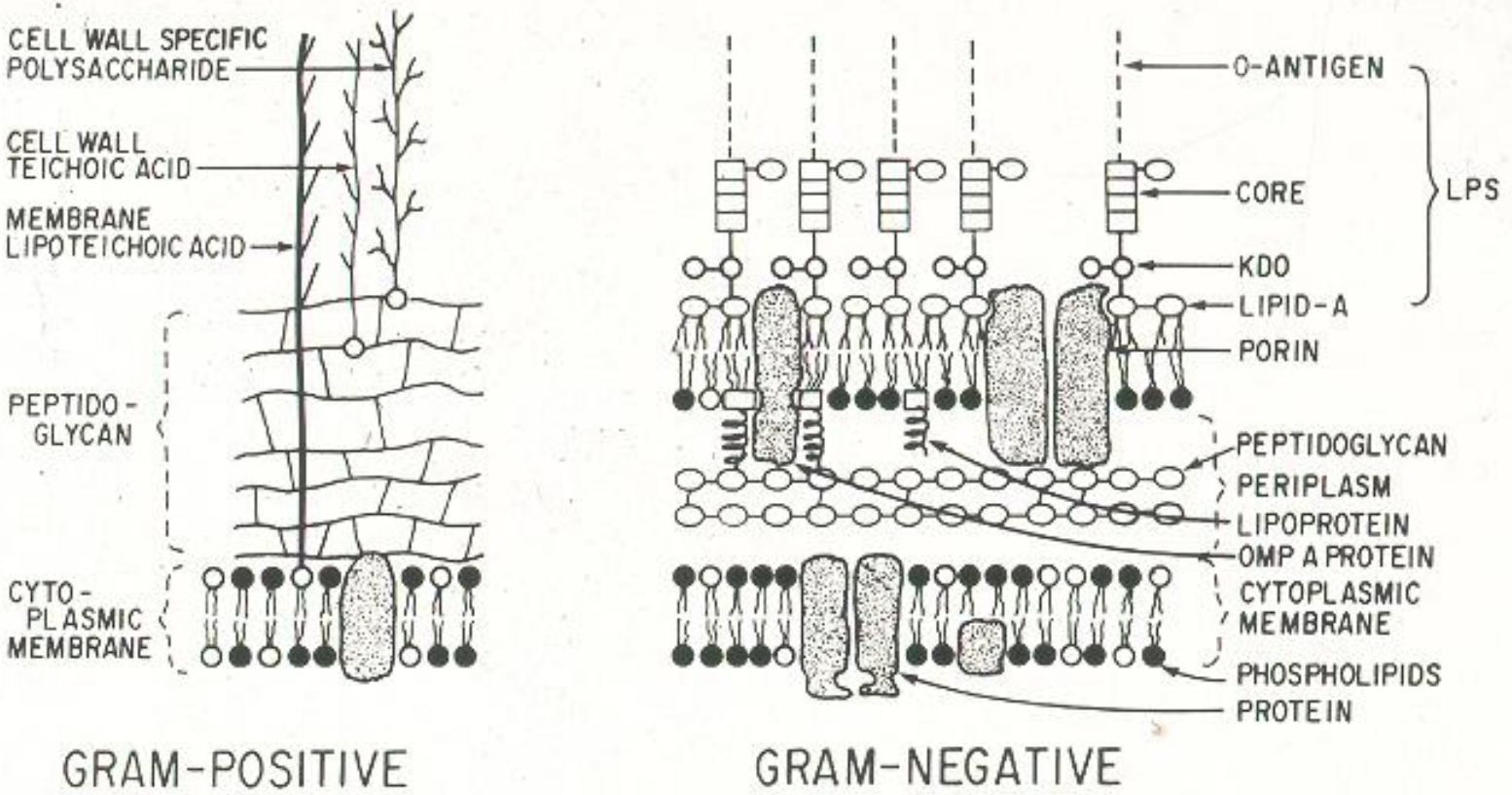


Fig. 6–6. Diagram comparing some major envelope structures of gram-positive and gram-negative eubacterial cells. The peptidoglycan layer of typical gram-positive cells is much thicker than that of gram-negative cells. Gram-positive cells often have polysaccharides covalently linked to peptidoglycan (represented by straight lines ending at peptidoglycan layer), as well as lipoteichoic acids that penetrate the peptidoglycan layer from the cytoplasmic membrane (represented by single feathered lines). Gram-positive bacteria do not have an OM. In contrast, gram-negative cells do have an OM and often exhibit a periplasmic space between the cytoplasmic membrane and the OM, in which is found the relatively thin peptidoglycan layer. Helical lipoproteins, covalently linked to the peptidoglycan anchor the OM. No polysaccharides are bound to the peptidoglycan of gram-negative bacteria. Lipopolysaccharides are found in the outer leaflet of the OM. Transmembrane proteins (OmpA and porins) occur only in OM.

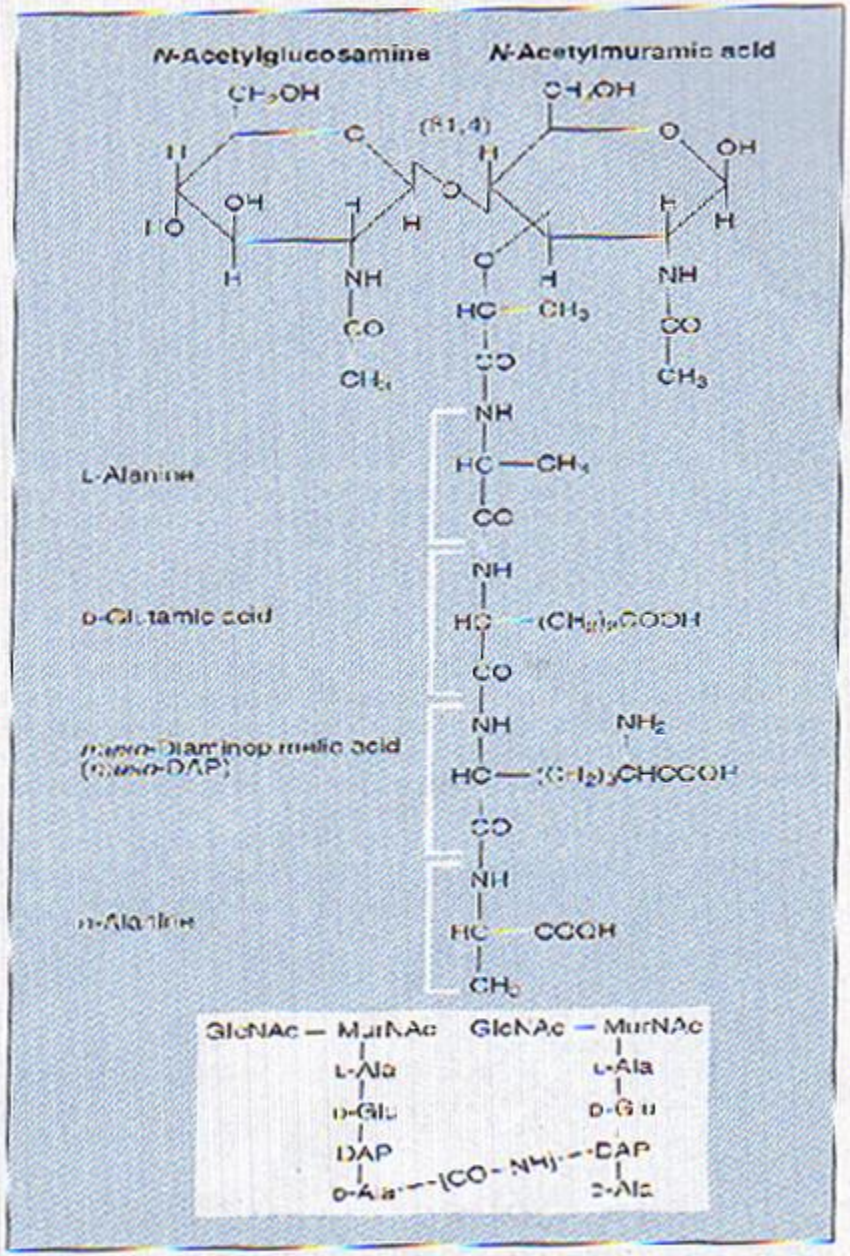
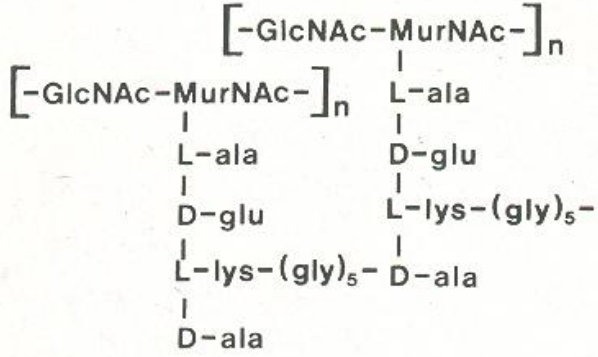


FIGURE 1–8. The fundamental unit of peptidoglycan, consisting of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc). Each disaccharide GlcNAc-MurNAc unit is attached to other disaccharide units by β1,4-glycosidic bonds, and the MurNAc peptides are cross-linked with the peptides of other MurNAcs via a peptide bond.

GRAM⁺



GRAM⁻

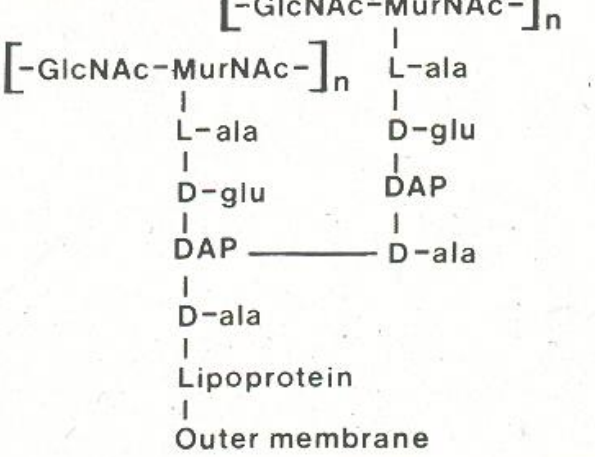


Figure 2.4. Typical structure of murein in Gram positive and Gram negative bacteria. In the Gram positives, peptide chains are cross-linked through a peptide bond between the free amino group of lysine and the terminal carboxyl group of a D-ala residue. In the Gram negatives, the cross-link is between diaminopimelic acid and D-ala. Other D-ala residues are linked to a lipoprotein that is attached to the outer membrane.

Gram Stain

- Based on cell wall composition and peptidoglycan thickness
- Gram positive cell wall

- Gram negative cell wall

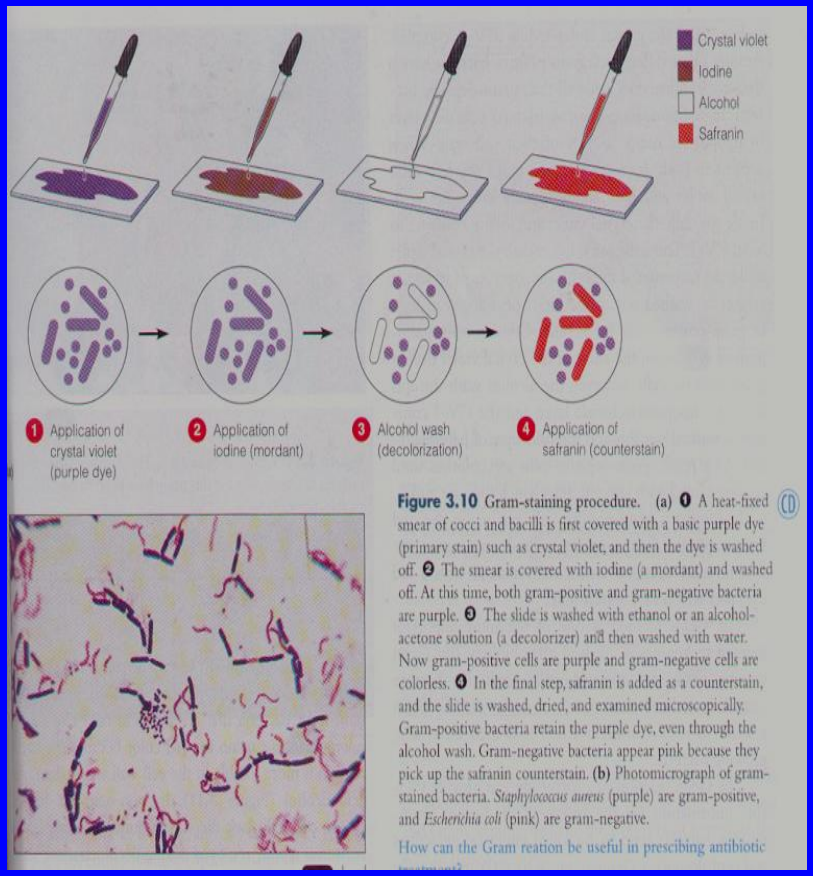
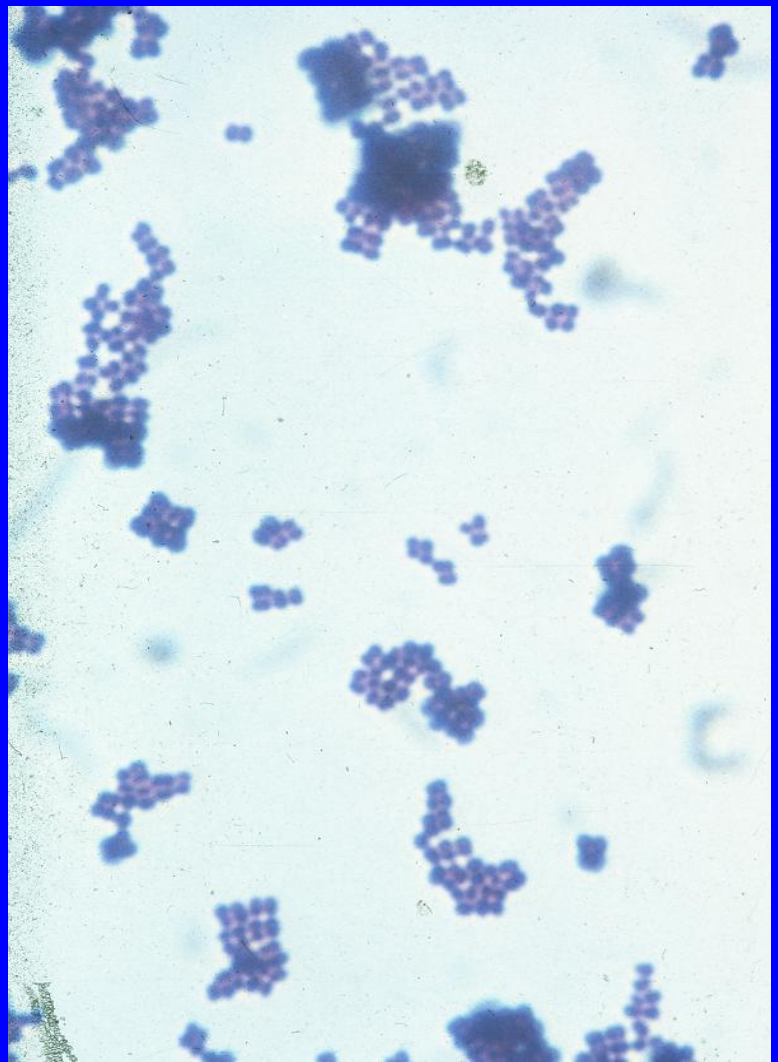


Figure 3.10 Gram-staining procedure. (a) ❶ A heat-fixed smear of cocci and bacilli is first covered with a basic purple dye (primary stain) such as crystal violet, and then the dye is washed off. ❷ The smear is covered with iodine (a mordant) and washed off. At this time, both gram-positive and gram-negative bacteria are purple. ❸ The slide is washed with ethanol or an alcohol-acetone solution (a decolorizer) and then washed with water. Now gram-positive cells are purple and gram-negative cells are colorless. ❹ In the final step, safranin is added as a counterstain, and the slide is washed, dried, and examined microscopically. Gram-positive bacteria retain the purple dye, even through the alcohol wash. Gram-negative bacteria appear pink because they pick up the safranin counterstain. **(b)** Photomicrograph of gram-stained bacteria. *Staphylococcus aureus* (purple) are gram-positive, and *Escherichia coli* (pink) are gram-negative.

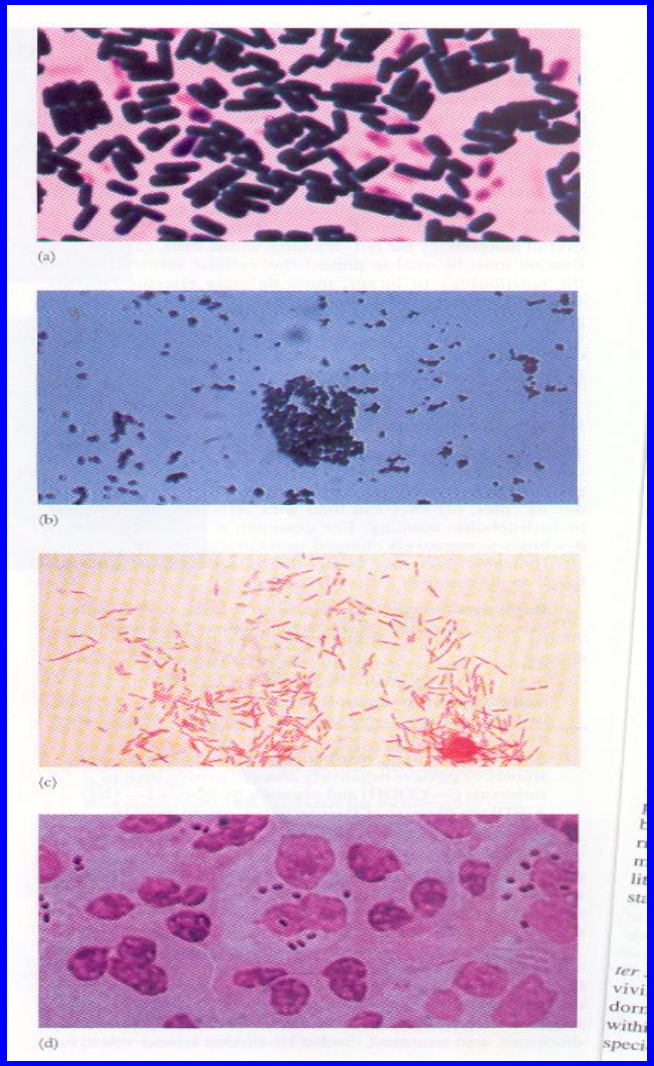
How can the Gram reation be useful in prescibing antibiotic

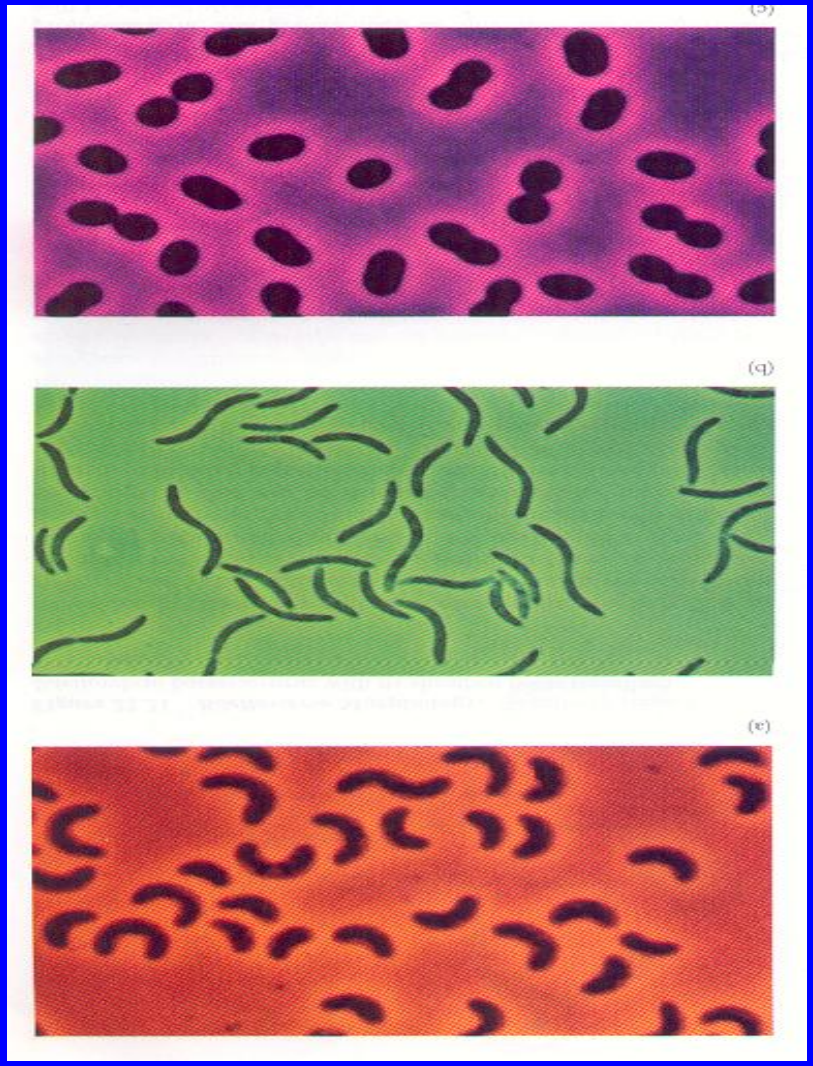
Morphological Characteristics

Colony Isolation & Gram Stain



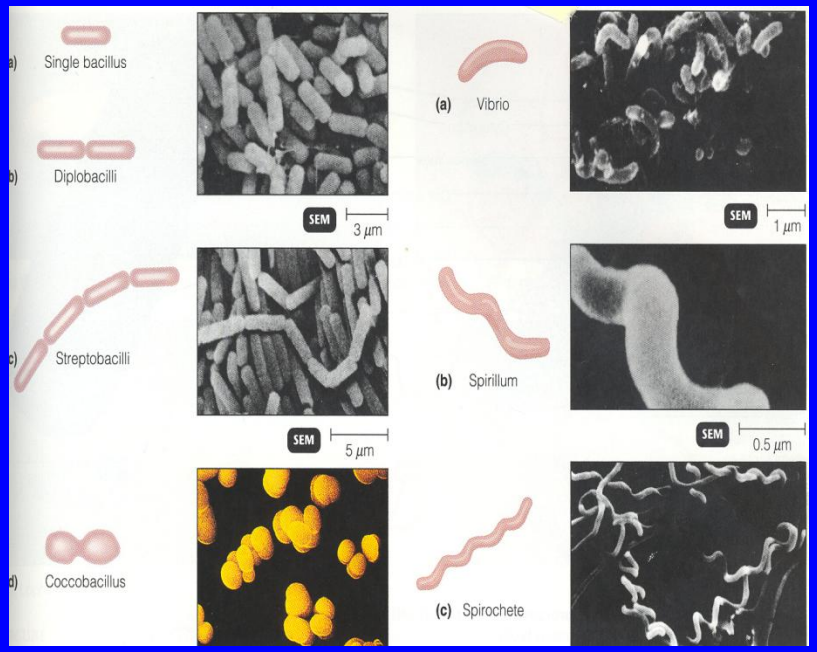
Gram- Stained Rods and Cocci





Morphologies of Bacilli

- Diplobacillus
- Streptobacillus
- Coccobacillus
- Vibrio
- Spirillum
- Spirochete



Bacterial Nomenclature (Genus / species)

- Streptococcus pyogenes

  pharyngitis, impetigo, cellulitis
- Streptococcus pneumoniae

  pneumonia, meningitis, otitis media
- Streptococcus viridans

  dental caries, acute endocarditis

Streptococcus viridans

- Streptococcus mutans
  - tooth enamel, dental caries
- Streptococcus mitis
  - pharyngeal epithelium
- Streptococcus salivarius
  - surface of tongue

Acid Fast Stain

- Also called Ziehl_Neelsen stain
- Used for :
  - Mycobacterium tuberculosis
  - Mycobacterium leprae
  - Nocardia species
  - Actinomyces species
  - Cryptosporidium species

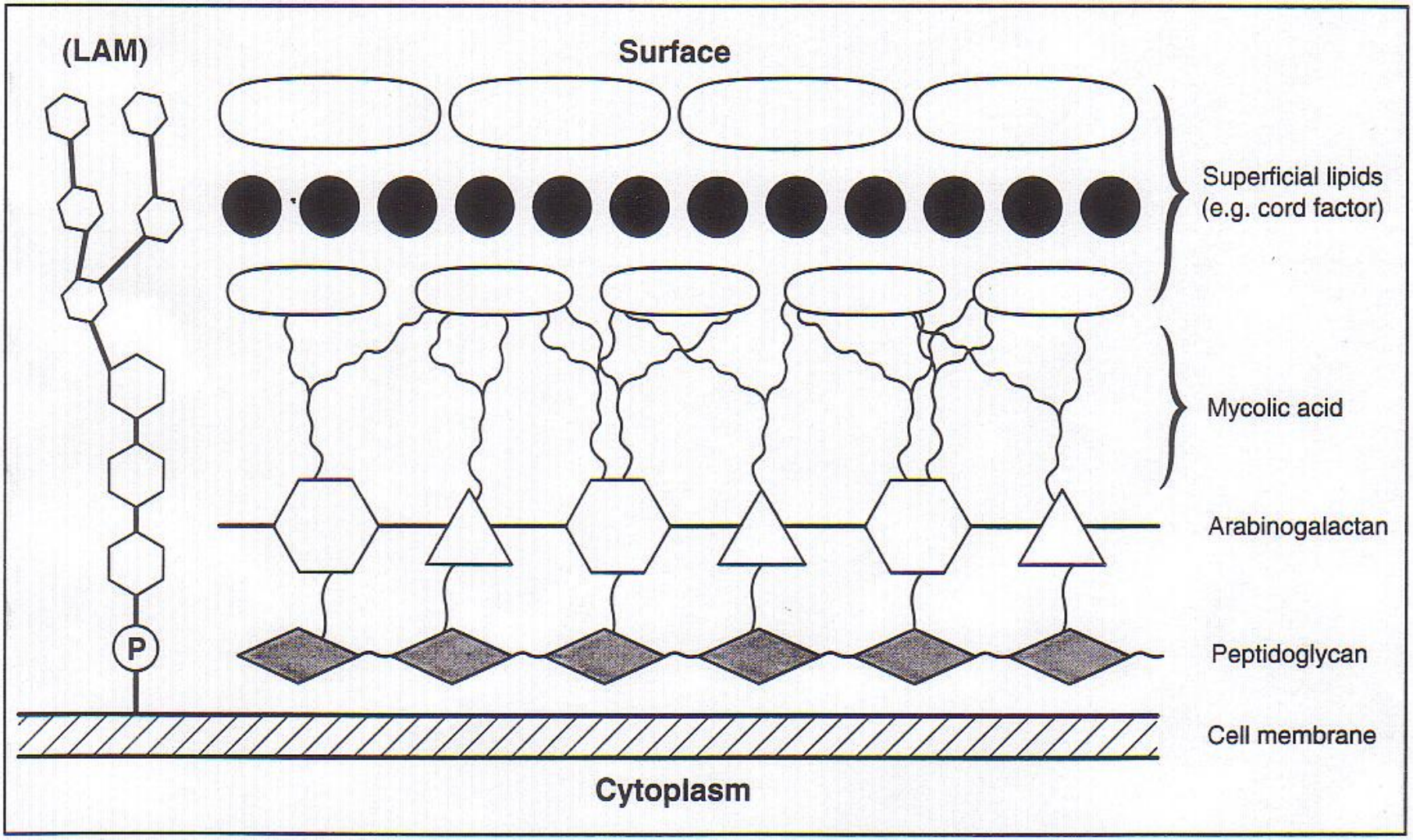


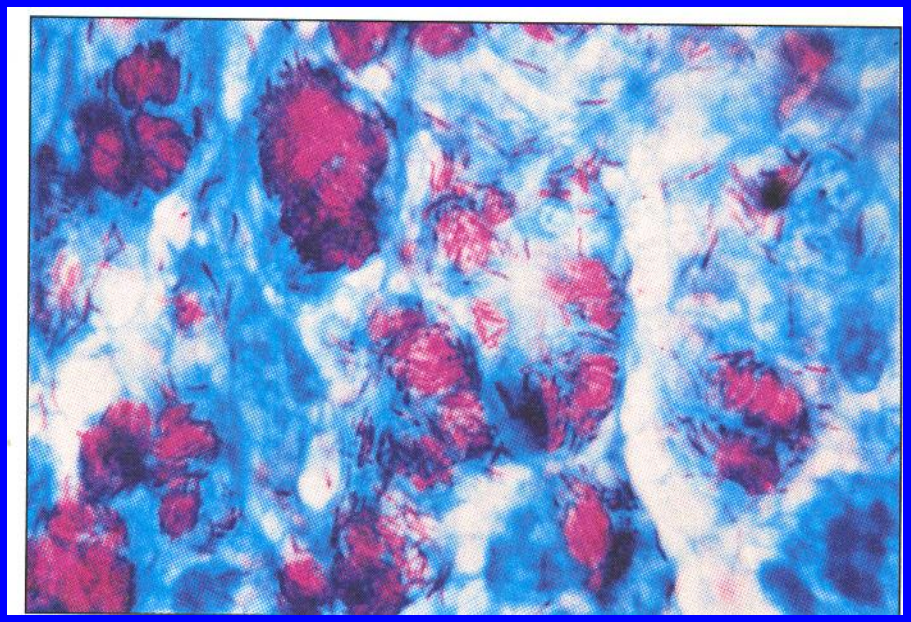
FIGURE 33-1 Complex cell wall structure of mycobacteria.

CELL WALL OF ACID-FAST BACTERIA

- *Mycobacterium tuberculosis, Corynbacteria diptheriae, and Norcardia asteroides* contain complex lipids in their cell walls (mycolic acid, lipoarabinomanan, arabinogalactan). These bacteria respond poorly to the Gram stain. They resist the action of acid alcohol due to their complex lipids (acid-fastness)
- The complex glycolipid allows *M. tuberculosis* to survive the degradative effects of the phagolysosomes in unactivated macrophages. They also render the bacterium difficult to study by molecular biology techniques
- The glycolipid is also the active ingredient in Freund's Adjuvant

Acid Fast Stain

- Red dye basic carbolfuchsin is the principal stain
- Background is counterstained with methylene blue
- Stain based on the mycolic (glycolipid) acid content of the cell wall
- Mycobacterium species is stained red, while background is stained blue



Other types of Stain

- Capsule stain with India ink
- Endospore stain with Schaeffer-Fulton stain
- Flagella stain with carbolfuchsin dye
- Giemsa stain for protozoan pathogens

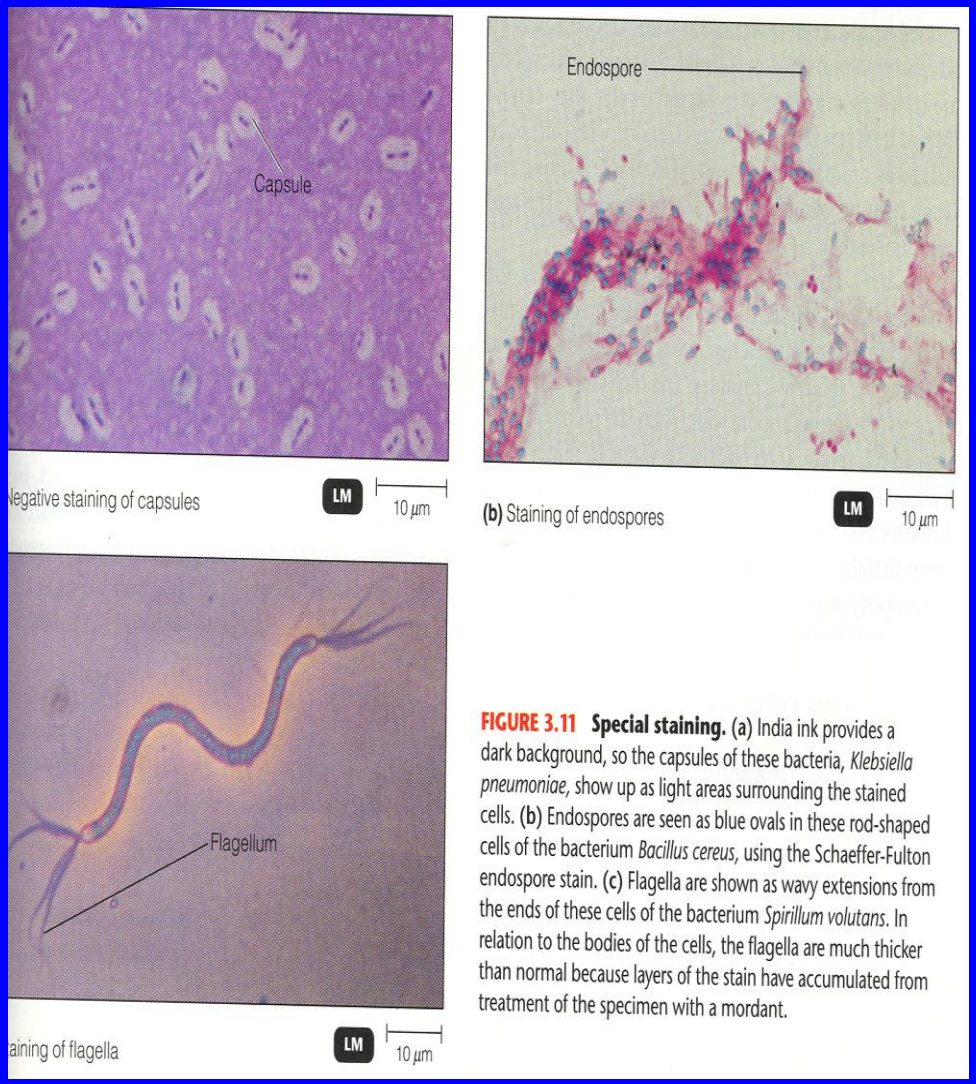


FIGURE 3.11 Special staining. (a) India ink provides a dark background, so the capsules of these bacteria, *Klebsiella pneumoniae*, show up as light areas surrounding the stained cells. (b) Endospores are seen as blue ovals in these rod-shaped cells of the bacterium *Bacillus cereus*, using the Schaeffer-Fulton endospore stain. (c) Flagella are shown as wavy extensions from the ends of these cells of the bacterium *Spirillum volutans*. In relation to the bodies of the cells, the flagella are much thicker than normal because layers of the stain have accumulated from treatment of the specimen with a mordant.

Biochemical Tests

- Coagulase enzyme production /incubation of bacteria with plasma / (+) if plasma coagulates

  Staphylococcus aureus vs Staph epidermidis
- Oxidase enzyme production

  (cytochrome c oxidase activity)

  aerobics (+), anaerobics (-)
- Nitrate reductase production

  gram neg enterics (+), nonenterics (-)

Oxidase and Nitrate Tests Derived from ETS

- Oxidase for presence of cytochrome oxidase enzyme
- Nitrate test for presence of functional nitrate reductase enzyme

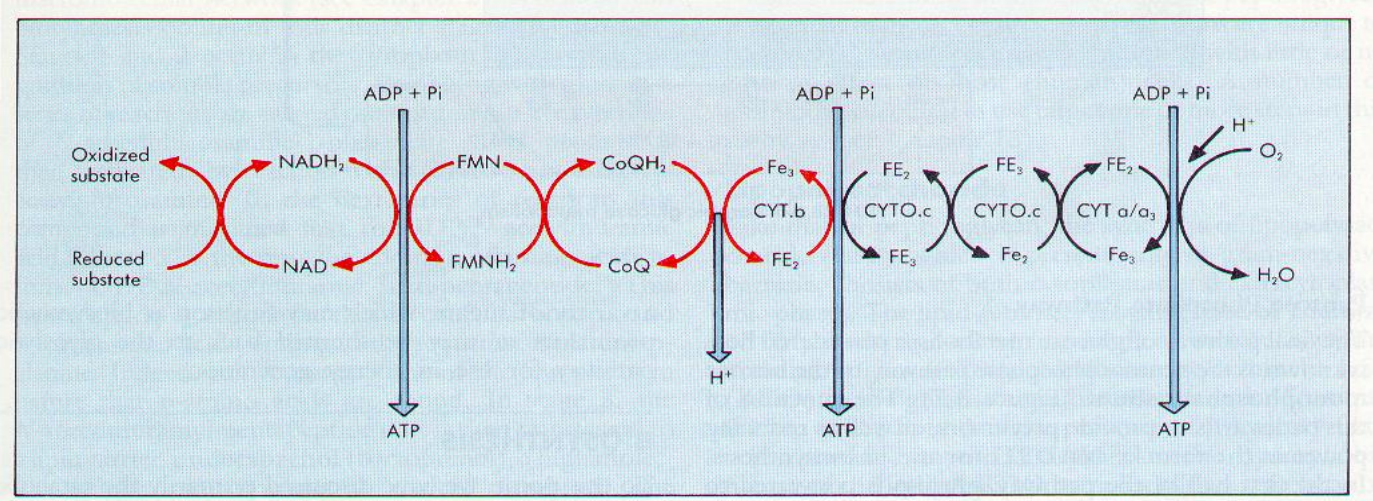


FIGURE 3-4 Electron transport chain, showing sequential oxidation steps and energy-generating steps. Electron transfer is accompanied by a flow of protons (H^+) from $NADH_2$ through coenzyme Q but not in later steps involving cytochromes. Three ATPs are formed per molecule of $NADH_2$ reoxidized but only two ATPs are formed per molecule of $FADH_2$ reoxidized. (Redrawn from Slots J, Taubman MA, editors: *Contemporary oral microbiology and immunology,* St. Louis, 1992, Mosby.)

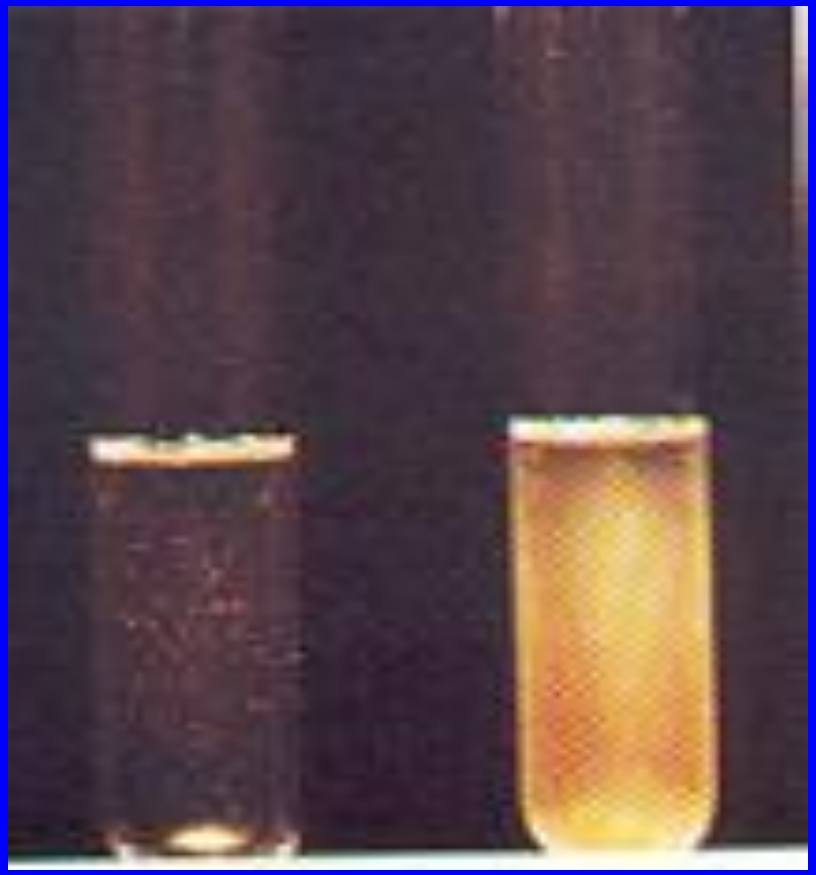
Catalase Test

- Hydrogen peroxide reduced to oxygen bubbles
- Gram positive cocci
- Left (+)

  Staphylococcus sp
  Right (-)

  Streptococcus sp

Bile (deoxycholate) solubility test



- Left tube (+) lysis of Strep pneumoniae due to autolysins activated by bile (sodium deoxycholate)
- Right tube (-) alpha Streptococcus (no lysis)

Fermentation /mannitol test

- Yellow (+)

  Acid production

  E. coli, Staph aureus

- Pink (--)

Staph epidermidis

Pseudomonas

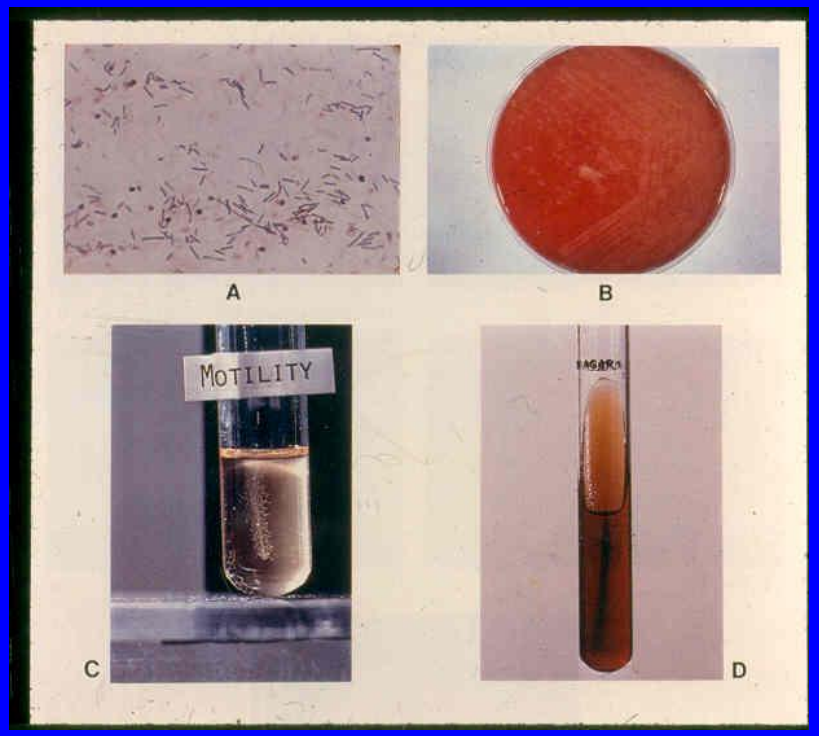
Motility Test for Flagella

Motile

- Salmonella typhi
- Proteus mirabilis

Non-motile

- Shigella dysenteria
- E. coli



Entero tube carry 12 biochemical tests for ID of gram negatives

ID Value	Organism	Atypical Test Results	Confirmatory Test
32143	*Enterobacteria cloacae*	Sorbitol$^-$	–
	Enterobacter sakazakii	Urea$^+$	+
32161	*Enterobacter cloacae*	None	VP$^+$
32162	*Enterobacter cloacae*	Citrate$^-$	

Microtiter plate for bacterial ID and antibiotic sensitivities

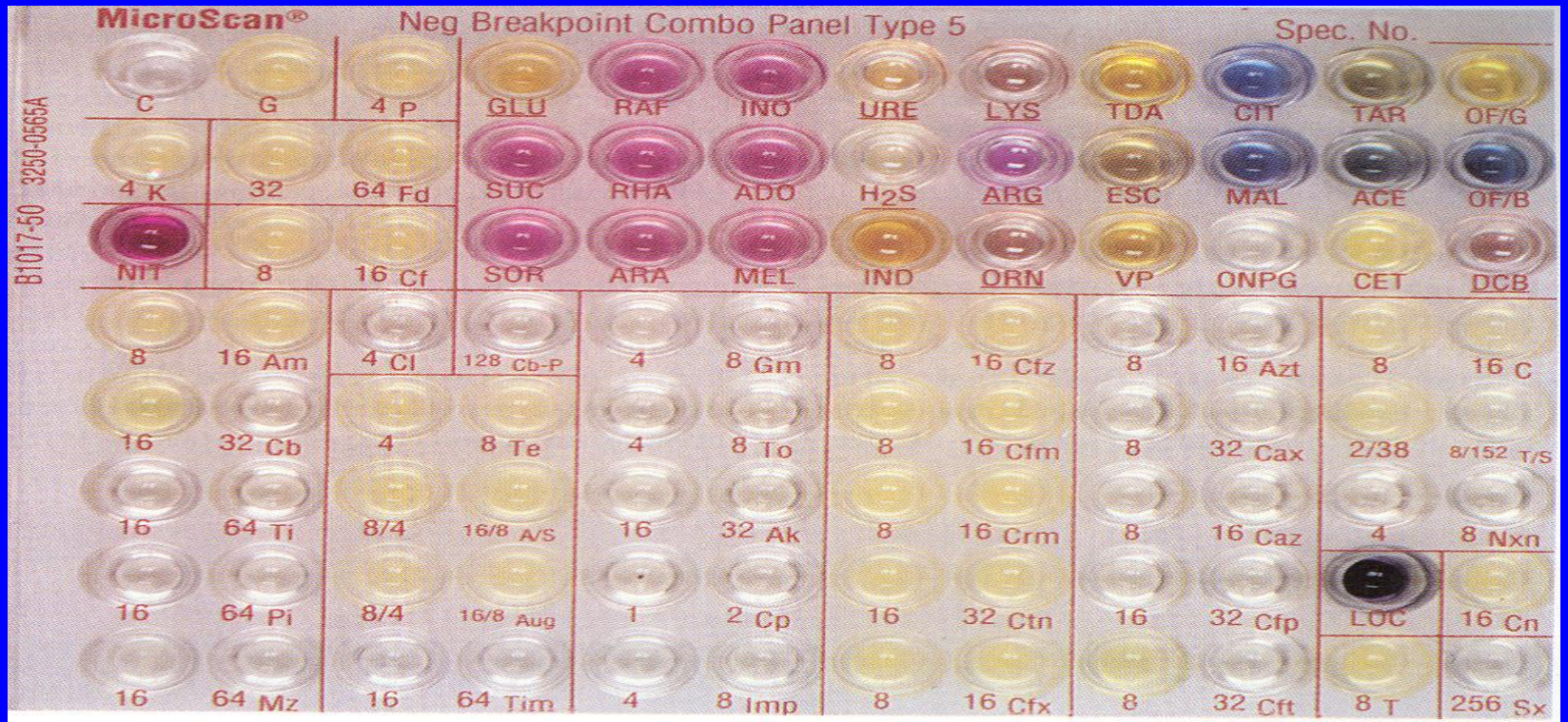


FIG. 2-30 Microtiter plate used in clinical microbiology laboratories for determination of metabolic characteristics of isolated bacteria. The color reactions indicate utilization of specific substances.

Triple Sugar Fermentation by Gram Negatives

- Glucose
- Sucrose
- Lactose
- Ferric chloride
- Hydrogen sulfide
- Black precipitate
- E. coli, Salmonella, Shigella

ELISA Procedure

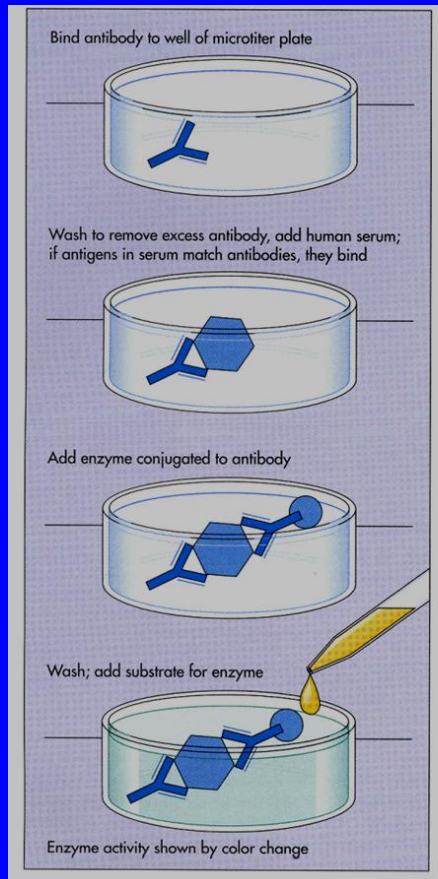


FIG. 11-27 The direct ELISA procedure uses antibody bound to the walls of a microtiter plate to trap antigen. A second antibody molecule with an attached ligand, typically a substrate for an enzymatic reaction, is added. When the enzyme is added, activity is shown by a color change indicating the reaction of the enzyme with its substrate.

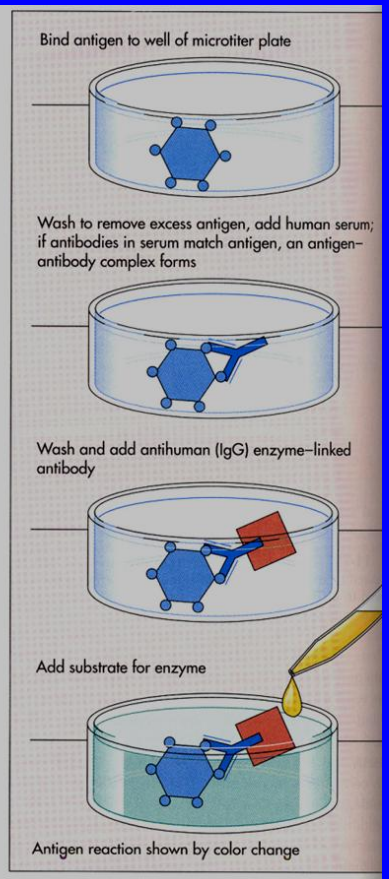


FIG. 11-28 The indirect ELISA procedure uses anti bound to the walls of a microtiter plate to trap human body if it is present in serum. A second antihuman-antib IgG molecule with an attached enzyme is added. When enzyme substrate is added, activity is shown by a c change indicating the reaction of the enzyme with its strate.

ELISA Readings

DYNATECH MR5000

TEST NO. : 40
TEST NAME : 410NM H11 12
PLATE : SCHIGM60

W/L MODE : SINGLE
TEST FILTER : 410 nm
REF. FILTER : *

DATE : 07.29.94
TIME : 05:02p
OPERATOR : Hollowell

Maternal / Child Serum

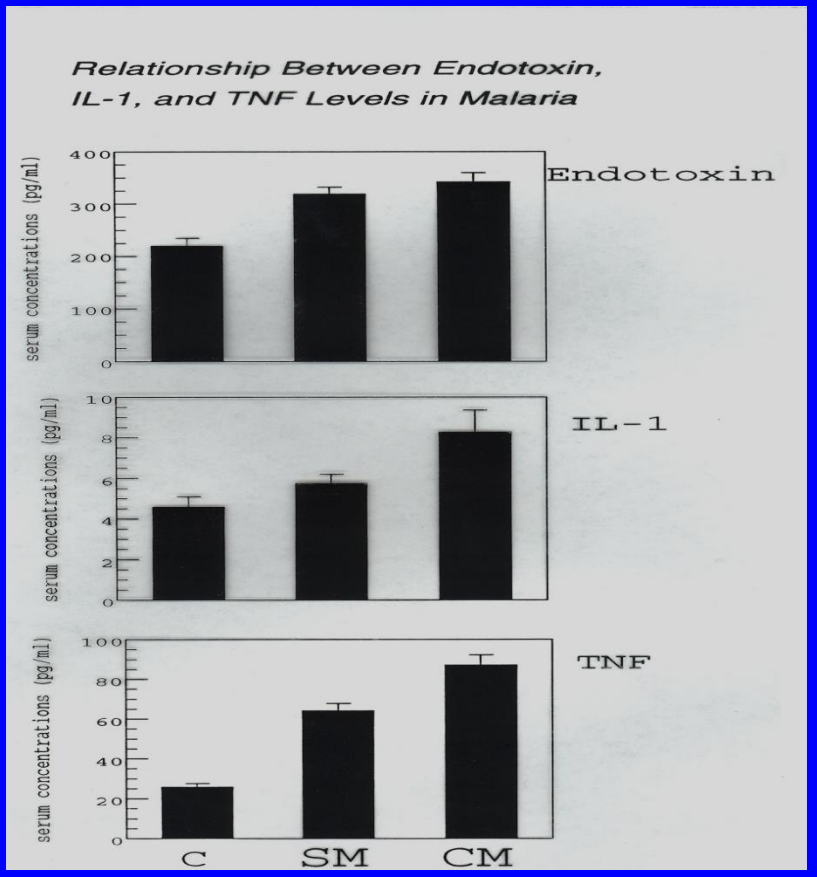
Schizont ag = 200X
Serum = 200X
(IgM) Conjugate = 200X
Incubation = 1 hr

AVERAGE BLANKS:
H11 = 0.040 H12 = 0.039

AVERAGE BLANK = 0.040

	1 M	2	3	4	5	6	7	8	9	10	11	12
A	0.517	0.335	0.673	0.391	0.307	0.578	0.283	0.380	0.415	0.428	0.561	0.486
B	0.609	0.189	0.493	0.282	0.308	0.305 MS	0.346	0.392	0.383	0.492	0.514	0.602
C	0.285	0.170	0.132 CS	0.363	0.315	0.257	0.190	0.211	0.383	0.230 N	0.165	0.264
D	0.328	0.137	0.115	0.144	0.151	0.154	0.150	0.215	0.222	0.374	0.165	0.256
E	0.530	0.144	0.259	0.167	0.254	0.130	0.294	0.302	0.360	0.311	0.273	0.289
F	0.341	0.227	0.141	0.186	0.259	0.326	0.225	0.238	0.292	0.371	0.287	0.213
G	0.434	0.168	0.210	0.346	0.277	0.207	0.402	0.336	0.228	0.364	0.203	0.286
H	0.277	0.424	0.195	0.189	0.243	0.193	0.190	0.229	0.269	0.467	BLANK	BLANK

ELISA Applications



Western Blot

- Includes:
  - gel electrophoresis
  - electroblotting with nitrocellulose paper
  - incubating with antigen-specific or patient's antisera
  - additional incubation with enzyme-conjugated secondary antibody and enzyme substrate for color production and antigen identification
- Used for diagnosis of HIV and other microbial infections

Western Blot

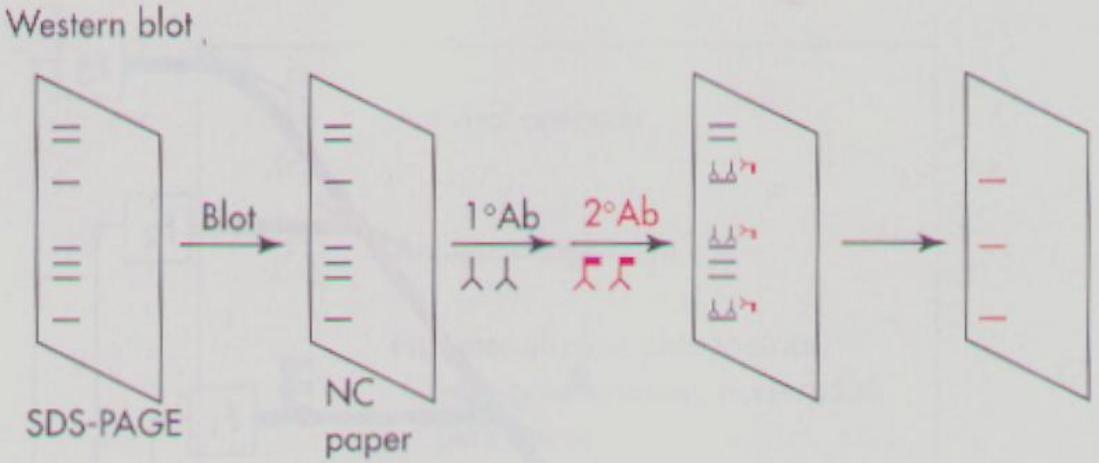


FIGURE 18-6 Western blot. Proteins are separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis *(SDS-PAGE)*, electroblotted onto nitrocellulose *(NC)* paper, and incubated with antigen-specific or patient's antisera *(1°Ab)* and then enzyme-conjugated antihuman serum *(2°Ab)*. Enzyme conversion of substrate identifies the antigen.

Western Blot / HIV Diagnosis

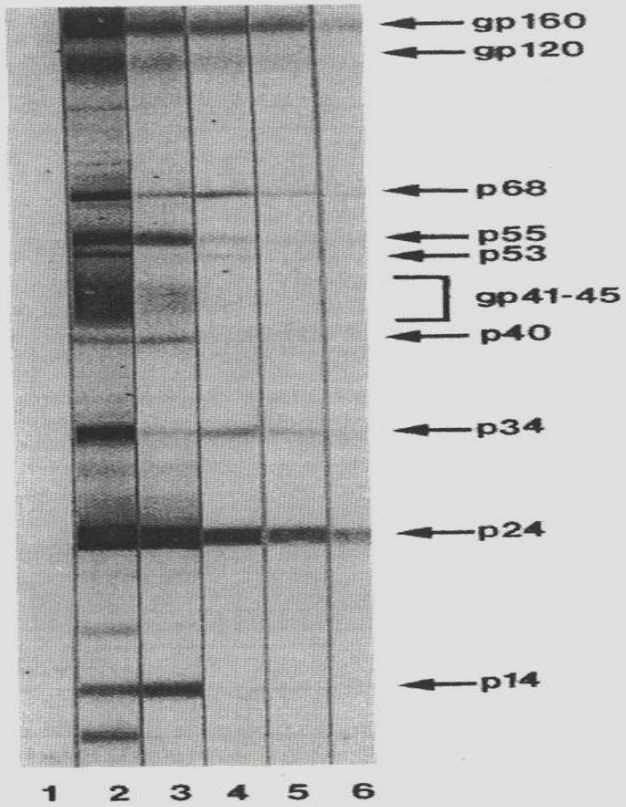


Fig. 35-11 Western blot for identification of HIV antibodies. HIV-1 was solubilized with sodium dodecyl sulfate (SDS), subjected to polyacrylamide gel electrophoresis (PAGE) to separate the constituent proteins on the basis of M_r, and the bands transferred by electrophoresis to a nitrocellulose membrane. After blocking unoccupied sites, the membrane was cut into strips and each strip incubated with a 1:100 dilution of serum from different subjects, then rinsed and treated with an enzyme-labeled anti-human immunoglobulin, rinsed again, and treated with a substrate that produces an insoluble precipitate in the presence of the enzyme. Lane 1, Negative control; lane 2, strong known positive control; lane 3, subject under test (positive); lanes 4–6, quality control, namely, twofold dilutions from 1:12,800 of a known positive standard. Note that not all infected persons produce antibody against all HIV proteins, and that the titers of antibody against certain proteins are consistently higher than others. Certain proteins (gp160, p55, p40) are precursors or intermediates in the cleavage pathways that produce the final products (gp120, gp41–45, p24, etc.). (Courtesy National HIV Reference Laboratory, Melbourne, Australia.)

Immunofluorescence

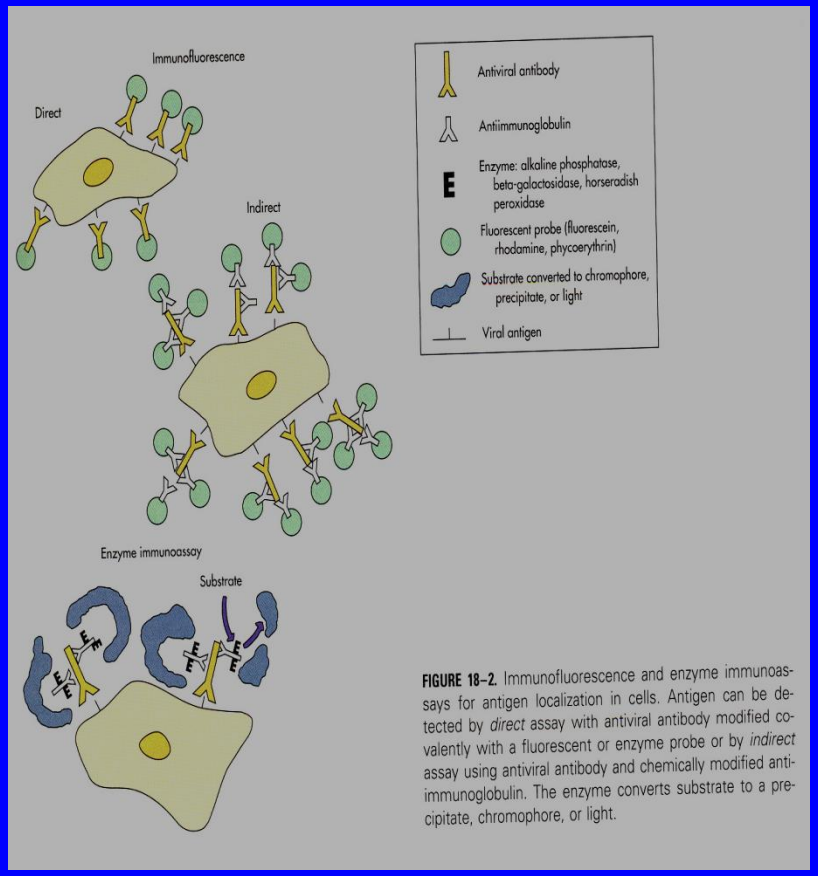
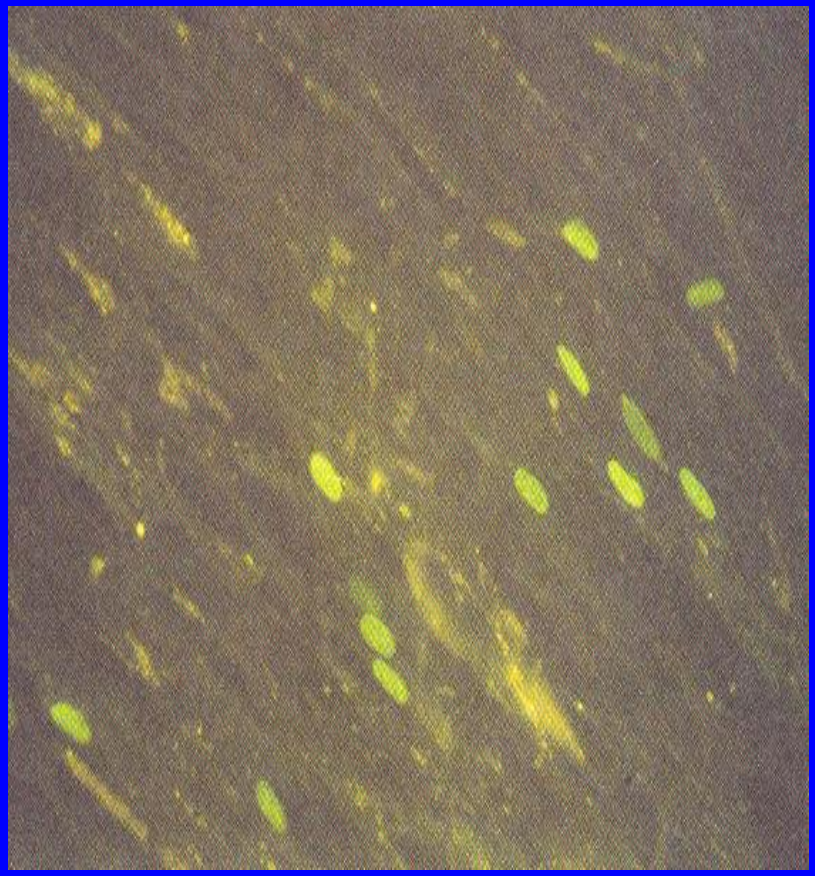


FIGURE 18–2. Immunofluorescence and enzyme immunoassays for antigen localization in cells. Antigen can be detected by *direct* assay with antiviral antibody modified covalently with a fluorescent or enzyme probe or by *indirect* assay using antiviral antibody and chemically modified anti-immunoglobulin. The enzyme converts substrate to a precipitate, chromophore, or light.

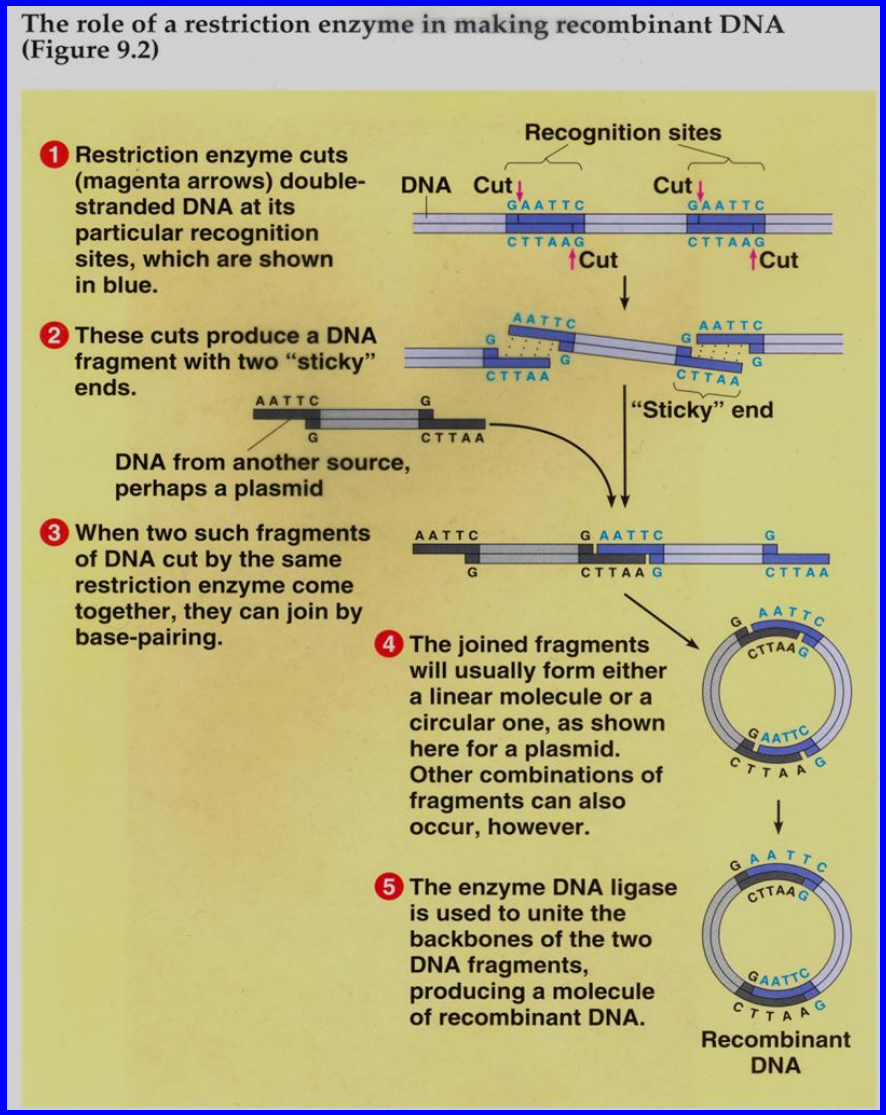


Nucleic Acid Hybridization

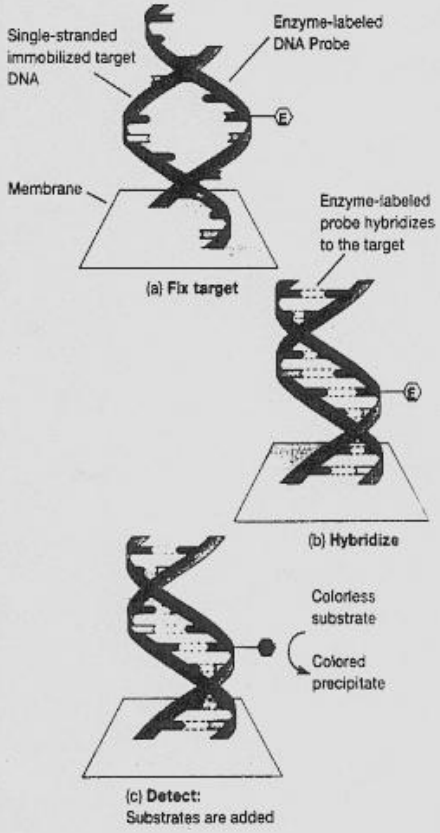
- DNA-DNA w ssDNA for closely related organisms
- DNA-RNA for distantly related organisms
- Two organisms w at least 80% homology and < 5% difference in T_m would be considered same species

Hybridization	% Homology
N. meningitis	100%
N. gonorrhea	78%
N. sicca	45%
N. flava	35%

Restriction Enzymes (BamHI, EcoRI) in DNA Digest & Hybridization



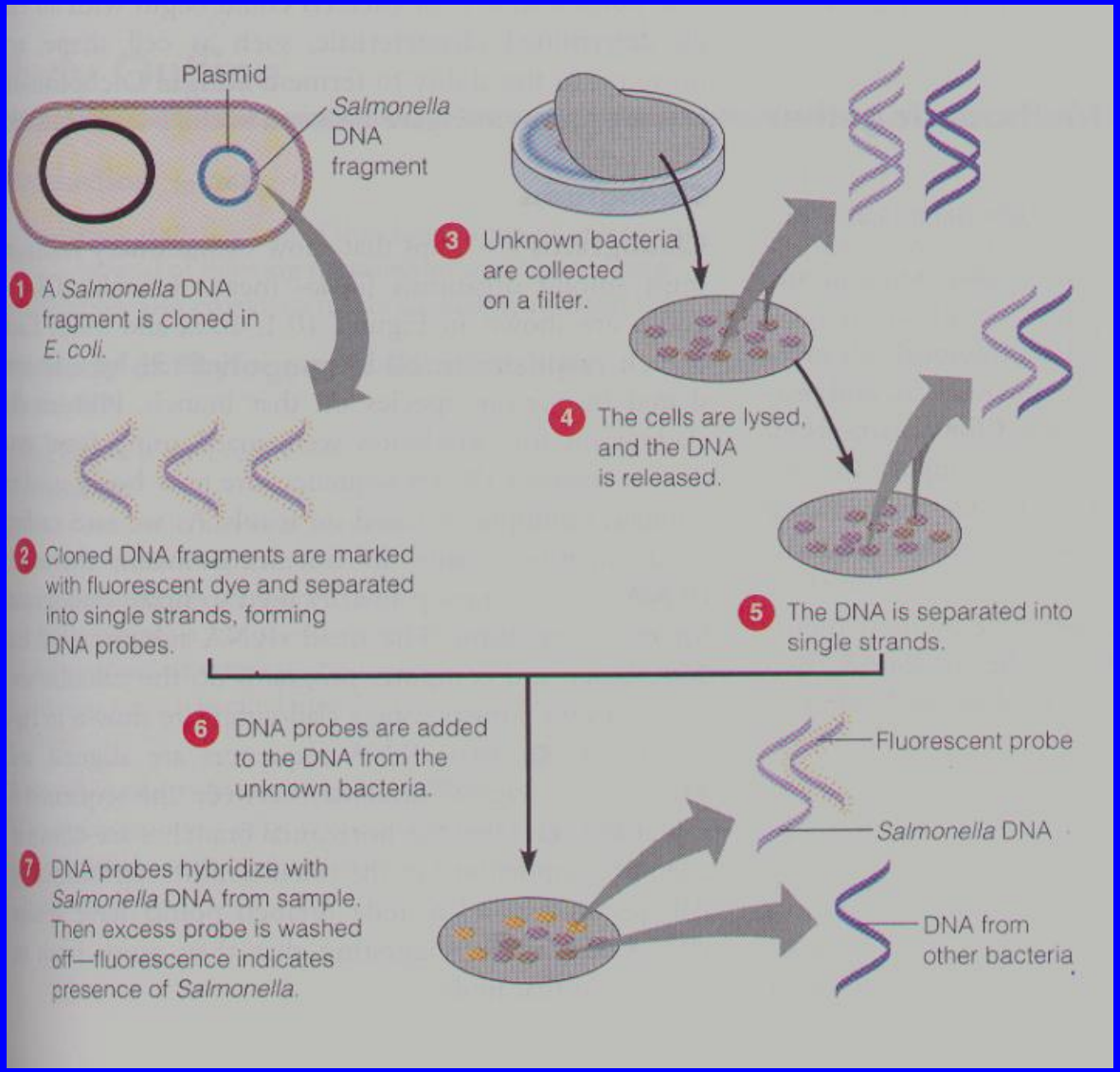
Principles of Nucleic Acid Hybridization



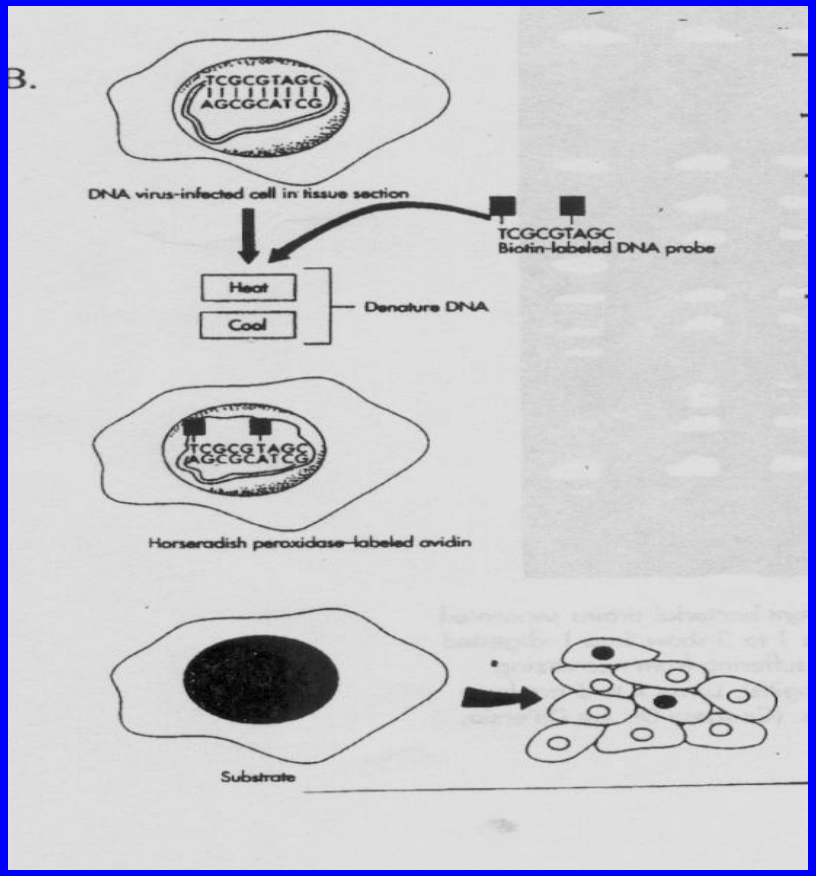
Basic Steps in A DNA Probe Hybridization

Assay. (*a*) Single-stranded target nucleic acid is bound to a membrane. A DNA probe with attached enzyme (E) also is employed. (*b*) The probe is added to the membrane. If the probe hybridizes to the target DNA, a double-stranded DNA hybrid is formed. (*c*) A colorless substrate is added. The enzyme attached to the probe converts the substrate to a colored precipitate. This detection system is semiquantitative, in that color intensity is proportional to the quantity of hybridized target nucleic acid present.

Cloning & Nucleic Acid Hybridization for Bacterial ID



DNA Probe Analysis of Virus-Infected Cells



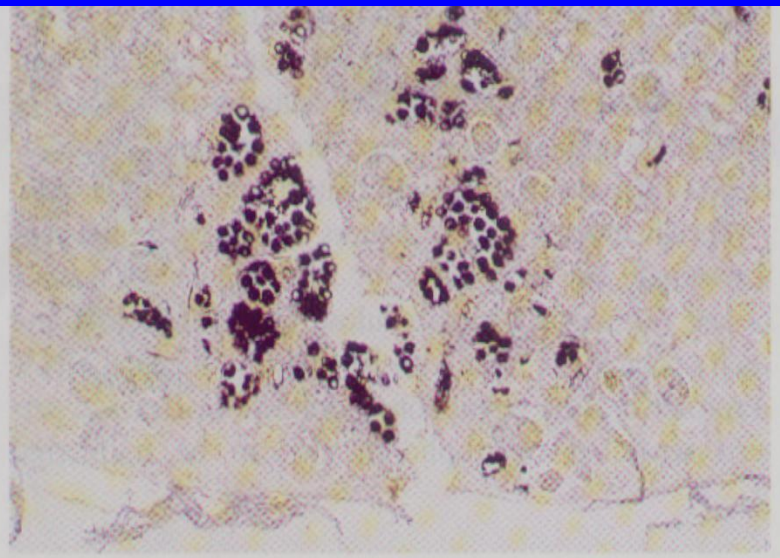
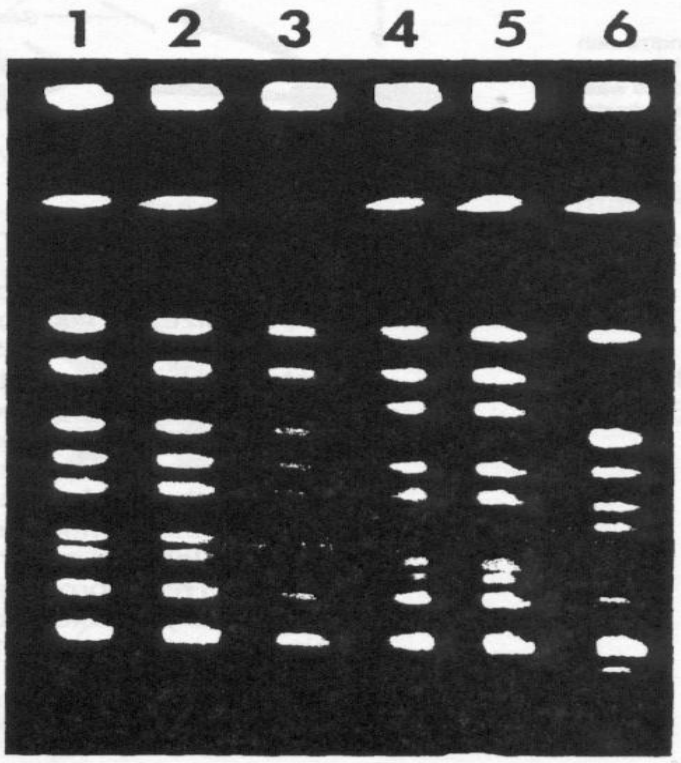


FIGURE 17-3 In situ localization of cytomegalovirus (CMV) infection using a genetic probe. CMV infection of the renal tubules of a kidney is localized with a biotin-labeled, CMV-specific DNA probe and visualized by means of the horseradish peroxidase–conjugated avidin conversion of substrate, in a manner similar to enzyme immunoassay. (Courtesy Donna Zabel, Akron, Ohio.)

Pulsed field Gel Electrophoresis: Clinical Applications

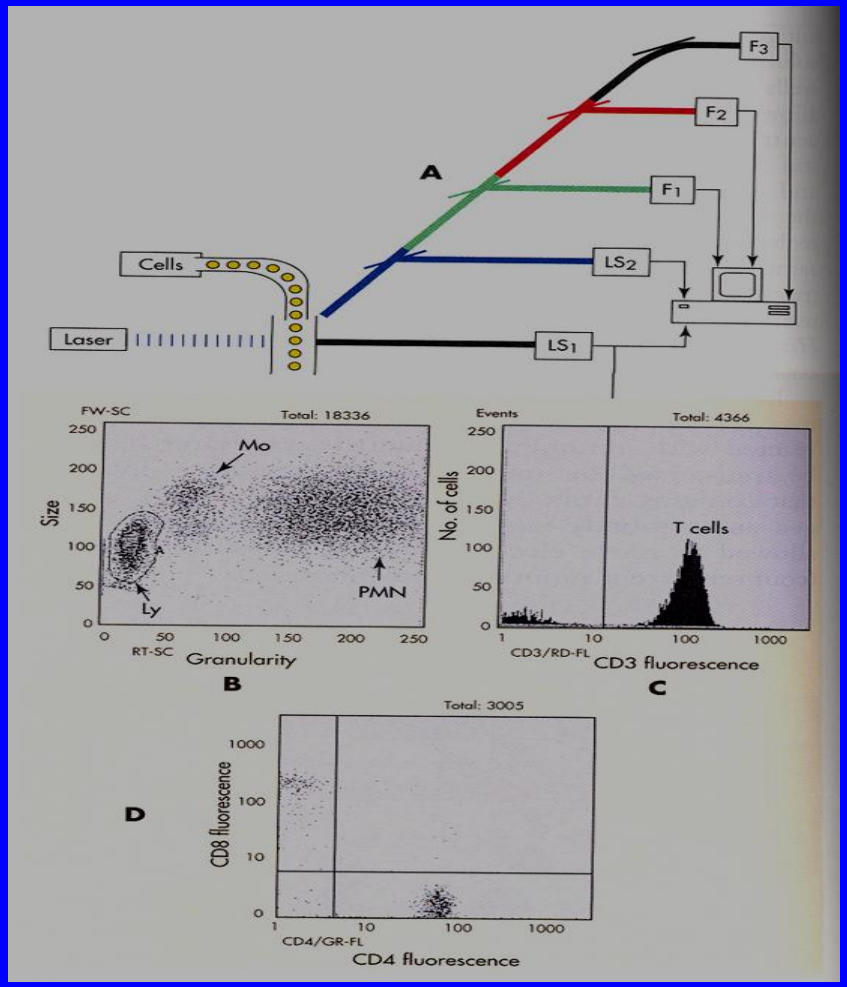
Identifying source of an infection



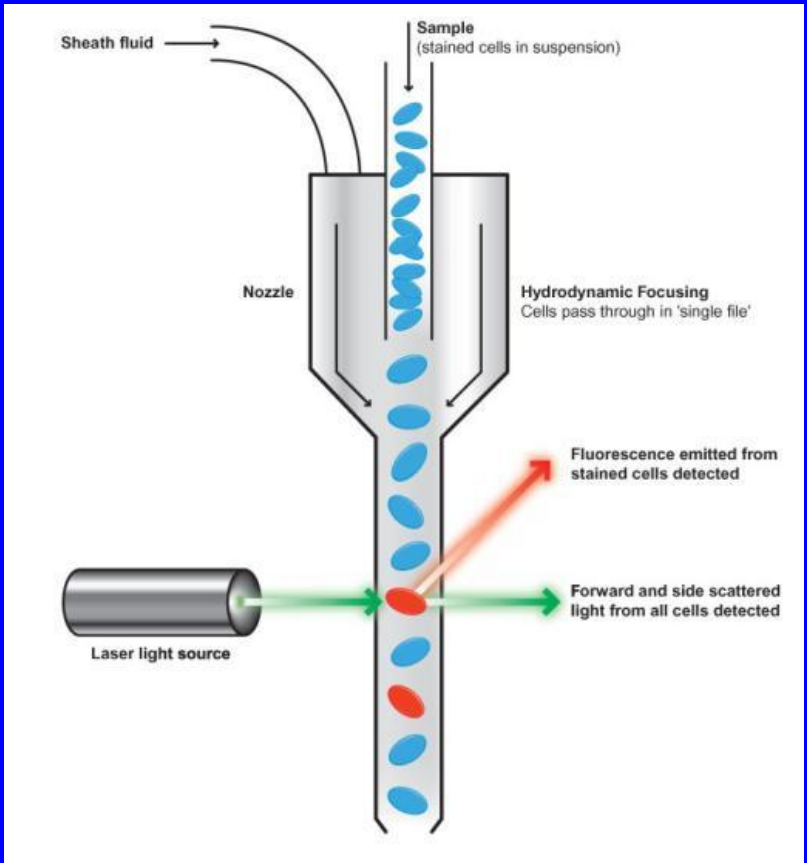
RFLP distinction of DNA from bacterial strains separated by pulsed-field gel electrophoresis. Lanes *1* to *3* show Sma I–digested DNA isolated from two family members suffering from necrotizing fasciitis and from their physician (pharyngitis). Lanes *4* to *6* are from unrelated *Streptococcus pyogenes* strains. (Courtesy Dr. Joe DiPersio, Akron, Ohio.)

Principles of Flow Cytometry

- Cells of interest are labeled (e.g. with flourescent markers) and suspended in solution.
- The cells are forced out of a small nozzle in a liquid jet stream.
- A beam of laser light of a single frequency is directed onto the stream.
- Each suspended particle passing through the beam scatters the light in some way.
- Several detectors can pick up the scattered lights and the fluctuations in brightness at each detector is analyzed.
- The data from the light scattering can be plotted on a graph to visualize different cell populations in the sample.

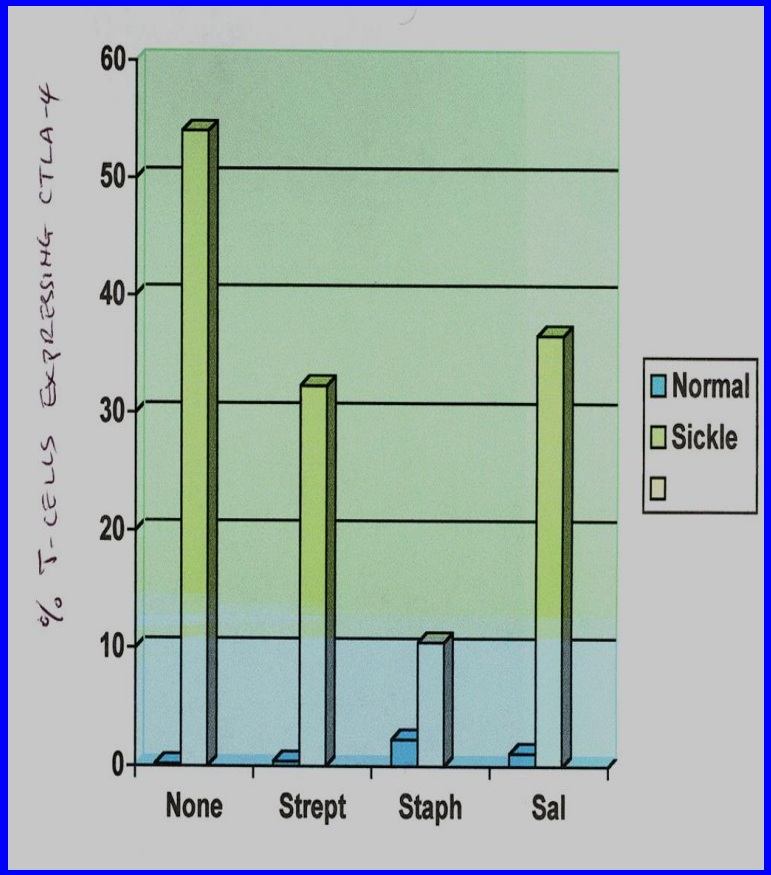


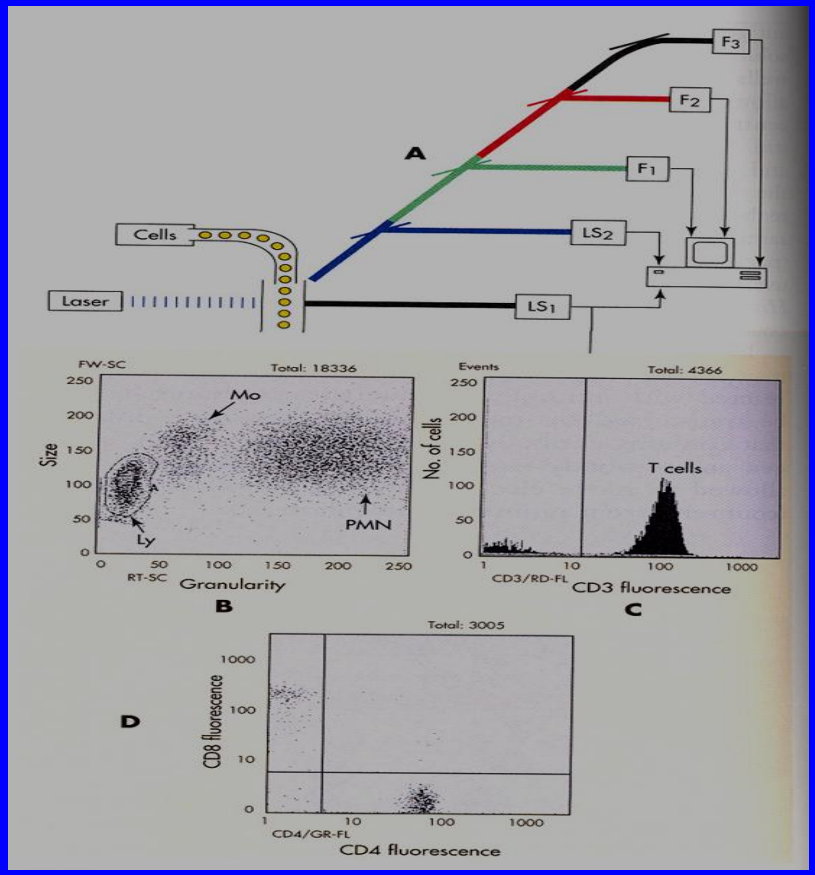
Flow Cytometry Protocol



- **<u>Used to measure:</u>**
- volume and morphological complexity of cells
- DNA and RNA
- detection of lymphomas as well as in determining the subpopulations of CD4 + helper T lymphocytes in AIDS and other diseases.
- cell surface antigens (also known as CD markers)

Flow Cytometry /CTLA-4 Expression in Sickle Cell Disease





Principles of PCR & Applications

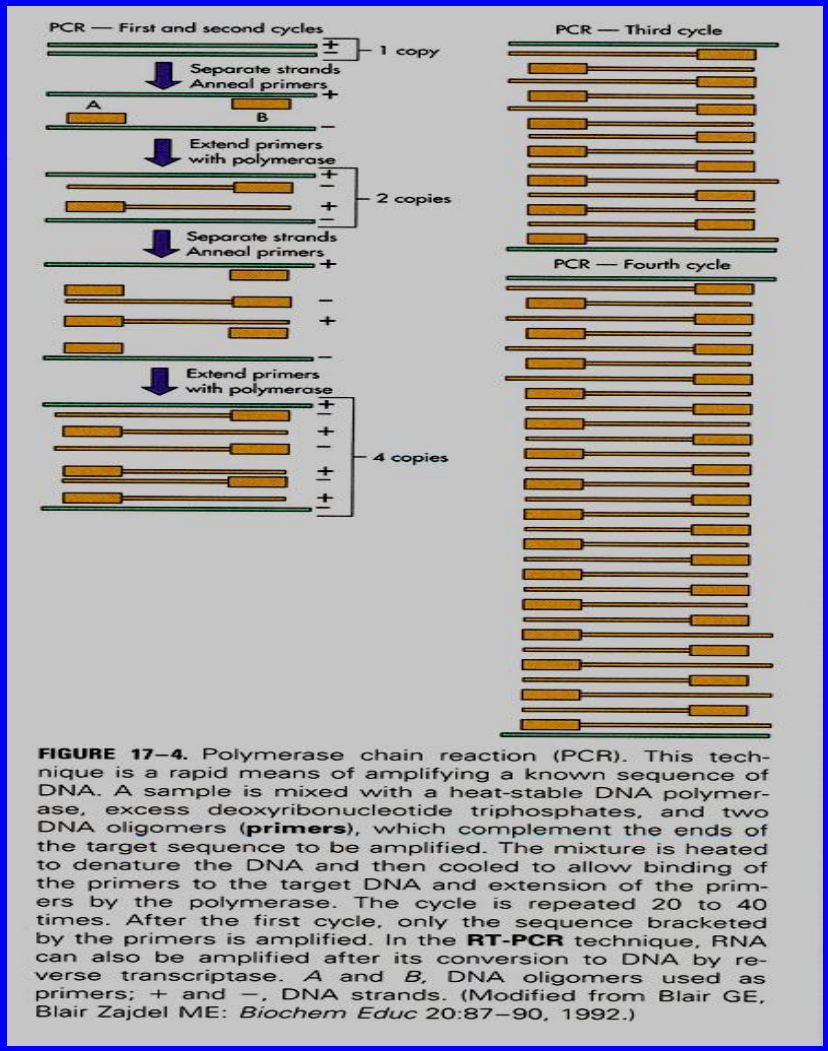
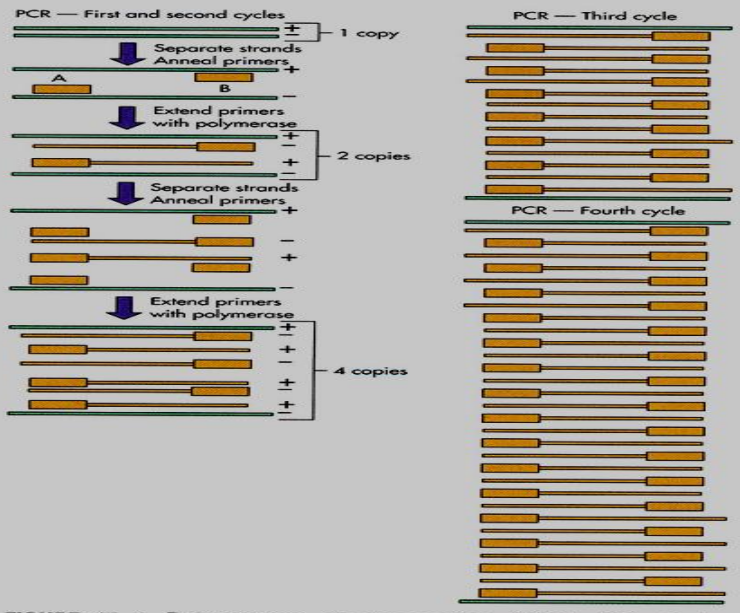


FIGURE 17–4. Polymerase chain reaction (PCR). This technique is a rapid means of amplifying a known sequence of DNA. A sample is mixed with a heat-stable DNA polymerase, excess deoxyribonucleotide triphosphates, and two DNA oligomers (**primers**), which complement the ends of the target sequence to be amplified. The mixture is heated to denature the DNA and then cooled to allow binding of the primers to the target DNA and extension of the primers by the polymerase. The cycle is repeated 20 to 40 times. After the first cycle, only the sequence bracketed by the primers is amplified. In the **RT-PCR** technique, RNA can also be amplified after its conversion to DNA by reverse transcriptase. *A* and *B*, DNA oligomers used as primers; + and −, DNA strands. (Modified from Blair GE, Blair Zajdel ME: *Biochem Educ* 20:87–90, 1992.)

- **<u>Used in:</u>**
- Forensic DNA detection
- Identify source of an infection
- Determine incidence of new infections
- Determine strains of bacteria and viruses
- Monitor antibiotic and drug resistance

PCR and DNA Fingerprinting



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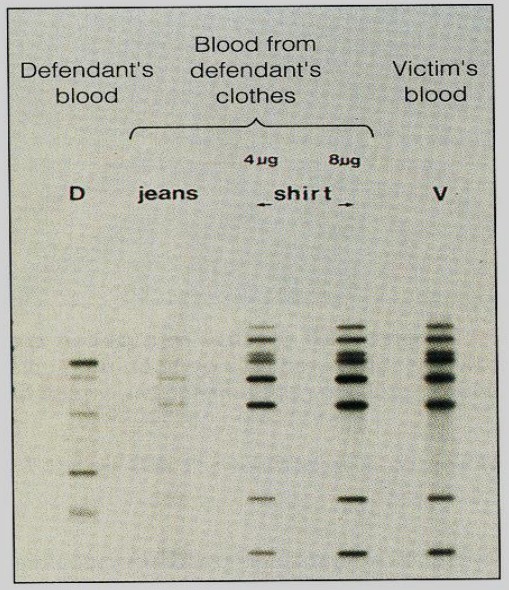
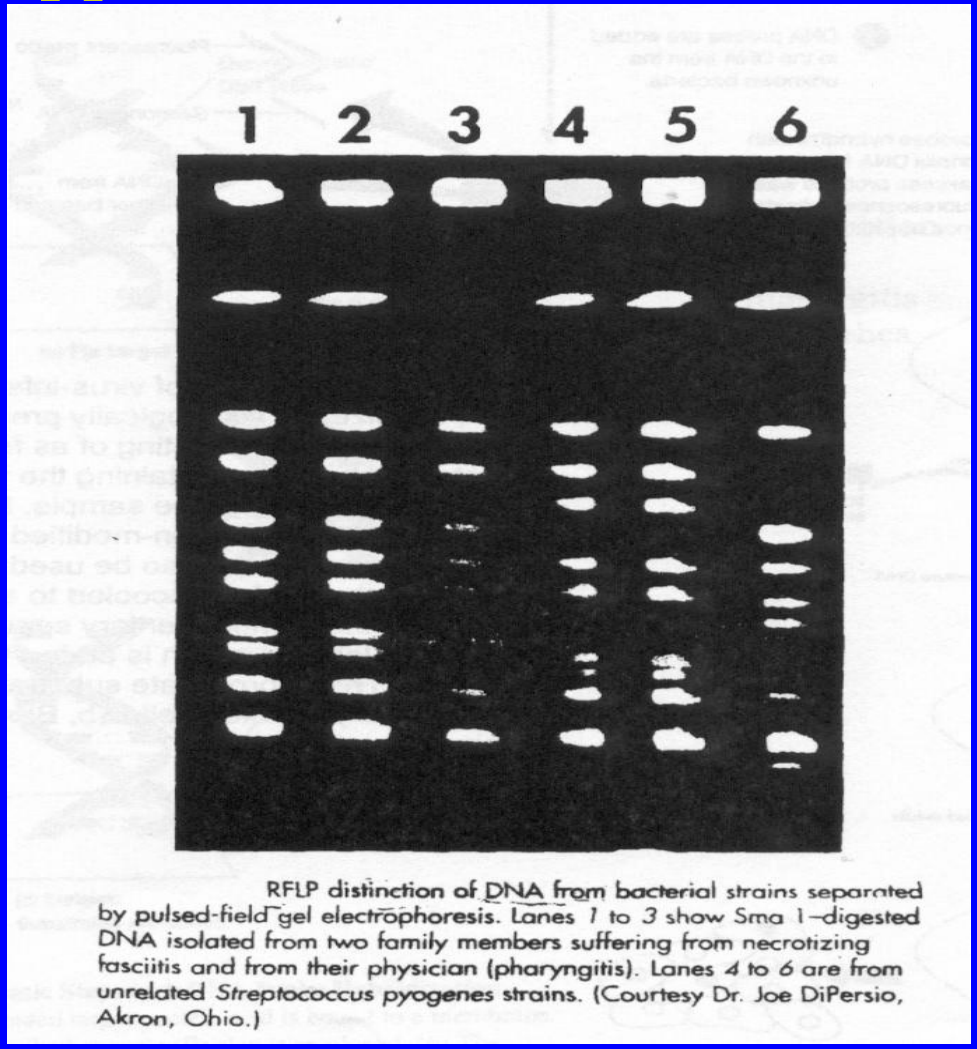


FIGURE 9.12 **DNA fingerprints from a murder case.** This is the developed film from a Southern blotting test done on blood taken from the defendant, the defendant's clothes, and the victim. Analysis of this film reveals that DNA from bloodstains on the defendant's clothes matches the DNA fingerprint of the victim but differs from the DNA fingerprint of the defendant. This is evidence that the blood on the defendant's clothes came from the victim.

PCR & Pulsed field Gel Electrophoresis: Clinical Applications

Identifying source of an infection



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Home-Work Exercise

- List organisms that may be associated with the following conditions
- 1. Bacteremia
- 2. Endocarditis
- 3. Meningitis
- 4. Pharyngitis
- 5. Pneumonia
- 6. Conjunctivitis
- 7. lntra-abdominal abscess
- 8. Gastroenteritis
- 9. Urinary Tract infections
- 10. lmpetigo
- 11. Cellulitis
- 12. Sepsis

Reading References

- Chapters 2,3,16,17 in Medical MICrobiology , 6th edition by Patrick Murray et al. Mosby Inc., 2009.
- Chapters 8 -10 in Medical Microbiology, 3rd edition by Cedric Mims et al. Mosby Inc.,2004.
- Chapters 6-9 in Mechanisms of Microbial Diseases, 3rd edition by Moselio Schaechter et al. William & Wilkins, 1998.