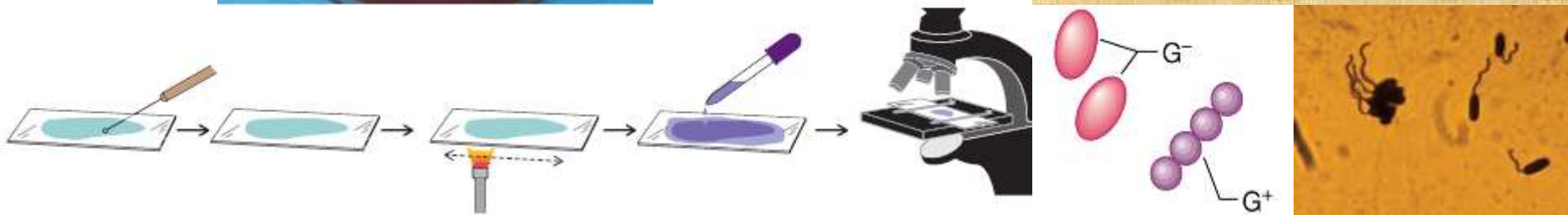


IDENTIFICATION SCHEME OF BACTERIA



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[pgt]
Dept. of Microbiology



Importance of identification

- Determining the clinical significance of particular pathogen.
- Guiding physician care of the patients.
- Determining the laboratory testing for detection of antibacterial resistance is warranted.
- Determining the type of antibacterial therapy that is appropriate.
- Determining the whether infectious organisms are risk for others patients in the hospital, the public and other laboratory workers.

Identification methods

- Traditional method/phenotypic method
- Immunochemical method/serological methods
- genotypic method/molecular method

Microbe Identification

- The **successful identification** of microbe depends on:
 - Using the proper **aseptic techniques**.
 - **Correctly** obtaining the specimen.
 - Correctly **handling the specimen**
 - **Quickly transporting** the specimen to the lab.
 - Once the specimen reaches the lab it is **cultured and identified**
 - Use care and tact to **avoid patient harm**

The specimen is the beginning. All diagnostic information from the laboratory depends upon the knowledge by which specimens are chosen and the care with which they are collected and transported.

—Cynthia A. Needham

Principles of Bacterial Cultivation

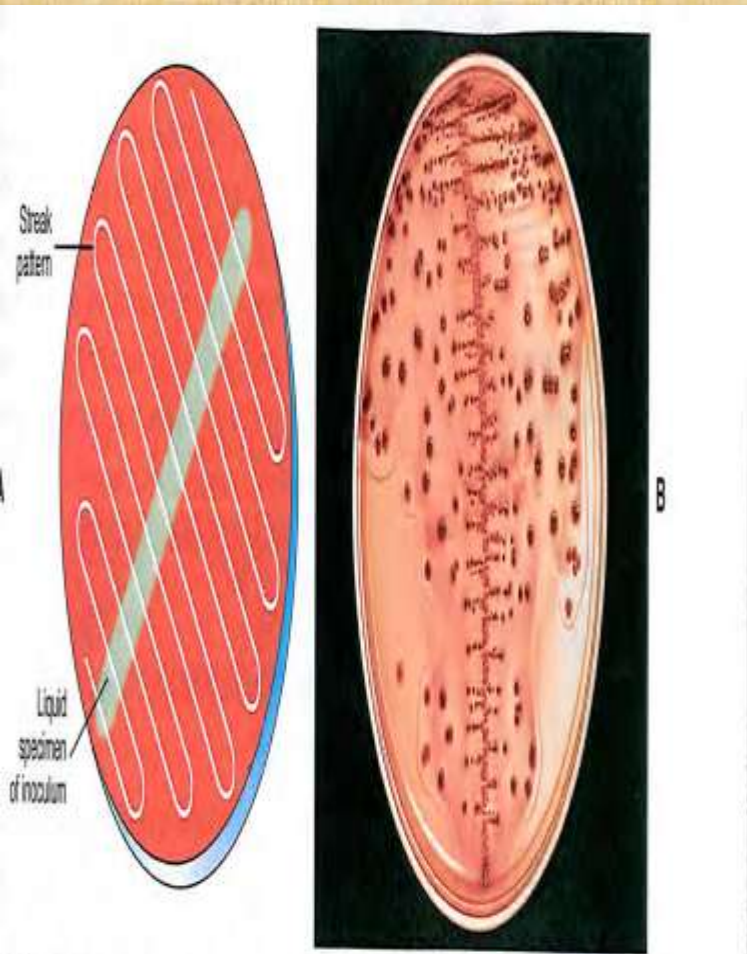


Figure 7-10 A, Streaking pattern using a calibrated loop for enumeration of bacterial colonies grown from a liquid specimen such as urine. B, Actual plate shows well-isolated and dispersed bacterial colonies for enumeration obtained with the calibrated loop streaking technique.

- to grow and isolate all bacteria present in a clinical specimen
- to determine which of the bacteria that grow are most likely causing infection and which are likely contaminants or colonizers.
- To obtain sufficient growth of clinically relevant bacteria and characterization

Culture media in diagnostic bacteriology

- Phases of growth media:

a) liquid (broth)

Enrichment media: buffered charcoal yeast extract agar for *Legionella pneumophila*

b) solid (agar)

- **Supportive media:** Nutrient agar for most non-fastidious organism
- **Selective media:** phenylethyl alcohol agar inhibit the growth of aerobic and facultative anaerobic gram (-) rods and allows gm+ cocci to grow
- **Differential media:** MacConkey agar for gram(-) bacteria.
- **CELL CULTURE for Chlamydiae, rickettsiae as they are obligate intracellular parasites.**

Phenotypic criteria

- Microscopic morphology and staining characteristics.
- Macroscopic (colony) morphology.
- Environmental requirement for growth.
- Resistance or susceptibility to antibacterials agents.
- nutritional requirement and metabolic capabilities.

Staining Reactions

- Gram stain divides bacteria into Gram positive and Gram negative
- Ziehl-Neelsen stain divides them into acid fast and non acid fast
- Special stains are necessary to bring out characteristics like flagella, capsules, spores and metachromatic granules.
- **EXCEPTION: only staining characteristics alone are used to definitively identify a bacteria species.**
- **Fluorescent dyes bring out special characteristics and fluorescent antibody technique enables to identify *Legionella pneumophila*, *Bordetella pertusis***

Unstained preparation

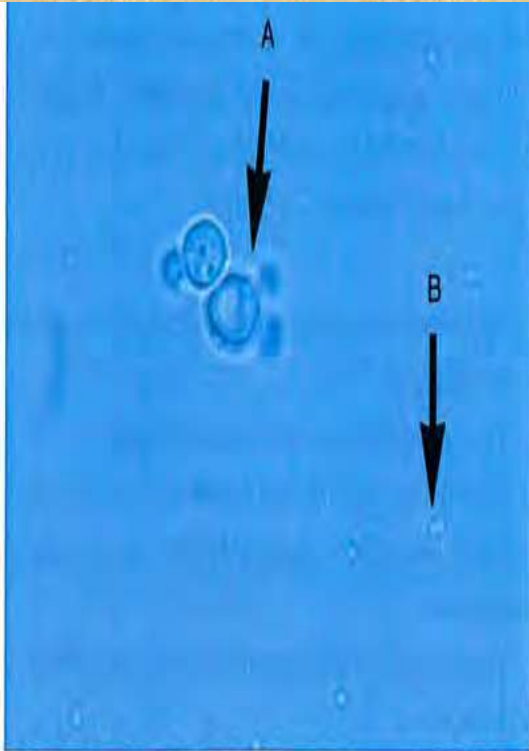


Figure 7-14 Microscopic examination of a wet preparation demonstrates the size difference between most yeast cells, such as those of *Candida albicans* (arrow A), and bacteria, such as *Staphylococcus aureus* (arrow B).

• WET PREPARATION

Alpha-hemolytic colony on blood agar reveal cocci in chains under oil immersion microscope given strong indication for streptococci.

▪ MOTILITY TEST

❖ wet mount technique

❖ hanging drop method

❖ soft-agar stabbing (tube method)

➤ *Ex-pseudomonas, proteus, E.coli*

Macroscopic morphology

- Provides additional information for the identification of the bacterium. The characters revealed in different types of media are noted.
- **While studying colonies on solid media following characteristics are observed :**
Size, Shape, Margins, Surface, Their elevations, Edge, colour, structure, consistency, pigmentation, haemolysis.
- **In fluid medium following characteristics are observed :**
Degree of growth – Absence, scanty, moderate, abundant etc.
presence of turbidity and its nature
presence of deposit and its character
Nature of surface growth and odour






Colony form	Examples
Punctiform (pinpoint)	
Circular	
Filamentous	
Irregular	
Colony elevation	
Flat	
Raised	
Convex	
Colony margin	
Entire (smooth)	
Irregular	

Figure 7-11 Colony morphologic features and descriptive terms for commonly encountered bacterial colonies.

Environmental requirement for growth

Four most critical factor

- ❖ **Oxygen and CO₂ availability**
 - **Facultative anaerobic:** most clinically significant aerobic organisms
 - **Strictly aerobic:** *pseudomonas, brucella, bordetella, francisella spp.*
 - **Anaerobic:** *peptococcus, clostridium*
 - **Capnophilic:** *neisseria spp.*
- ❖ **Temperature**
 - **35-37** degree Celsius
 - **42** degree Celsius: *Compylobacter jejuni*
 - **0** degree Celsius: *Yersinia enterocolitica, Listeria monocytogenes*
- ❖ **Neutral pH-range 6.5-7.5**
- ❖ **Moisture**
 - Essential for metabolic pathways
 - Prevent osmotic shock

Resistance or susceptibility to antimicrobial agent

The ability of an organism to grow in the presence of certain antimicrobial agents is widely used to establish preliminary identification information

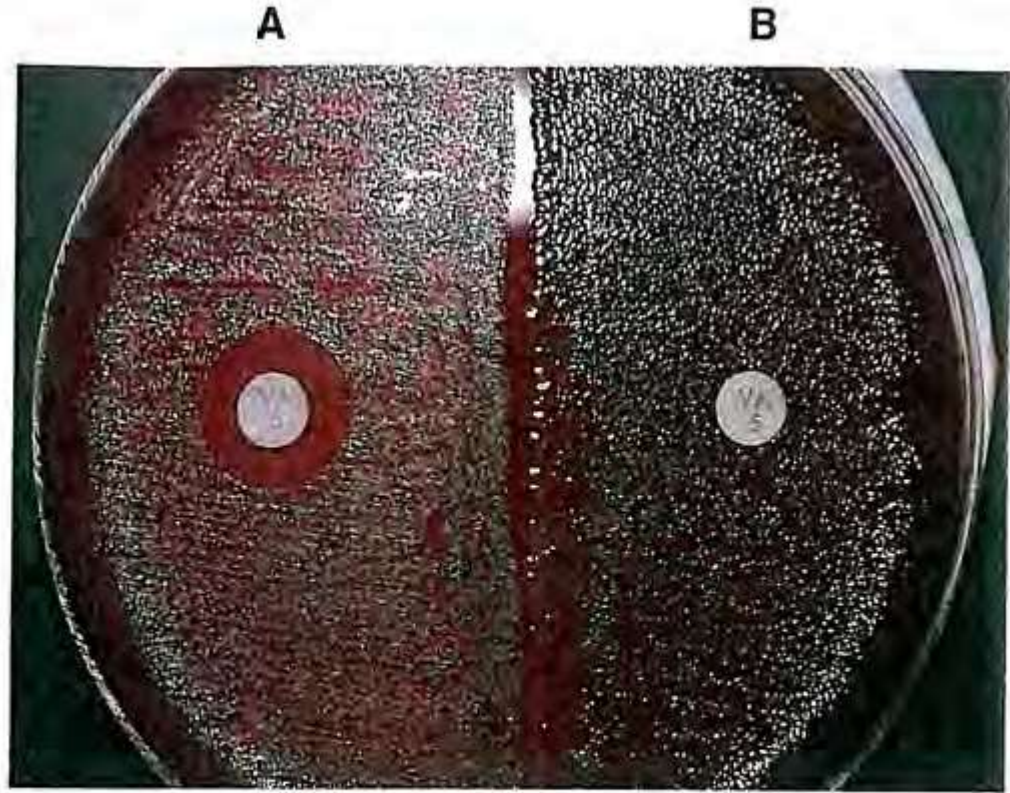


Figure 7-15 A, Zone of growth inhibition around the 5-mg vancomycin disk is indicative of a gram-positive bacterium. B, The gram-negative organism is not inhibited by this antibiotic, and growth extends to the edge of the disk.

Nutritional requirements and Metabolic pathways

- Most common approach used for determining the genus and species of an organisms
- In general all methods available are a combination of tests
 - ❖ to establish the enzymatic capabilities of a given bacterial isolate
 - and
 - ❖ Isolates ability to grow or survive the presence of certain inhibitors(e.g. salt,toxins,antibiotics)

- Nutritional requirements and metabolic capabilities

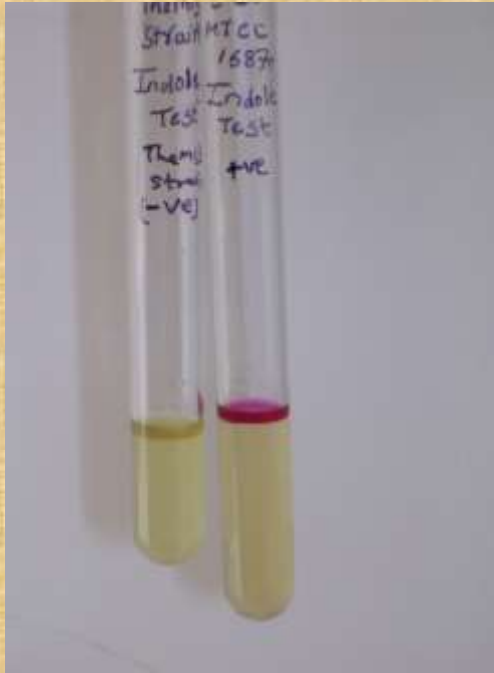
- Single enzyme tests

- Catalase: $H_2O_2 + \text{catalase} = O_2 \text{ and } H_2O$; 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 +

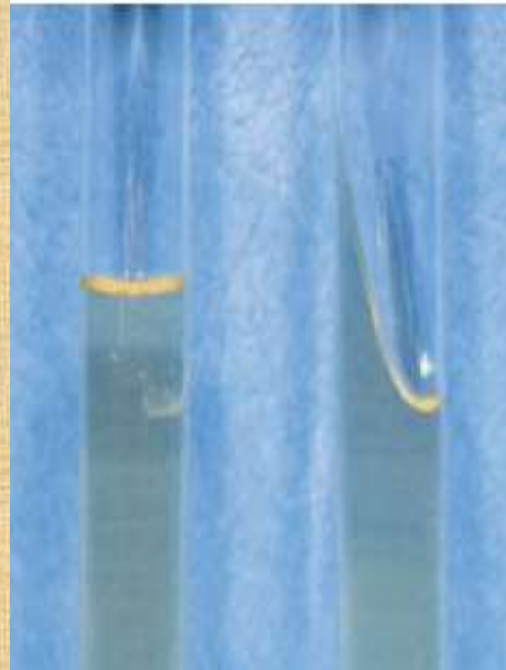
–Tests for presence of metabolic pathways

- 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

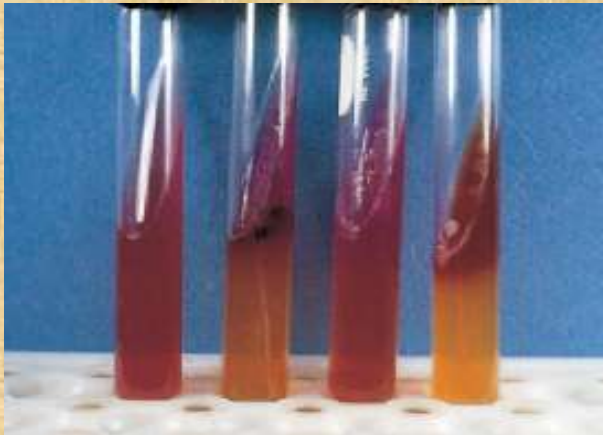
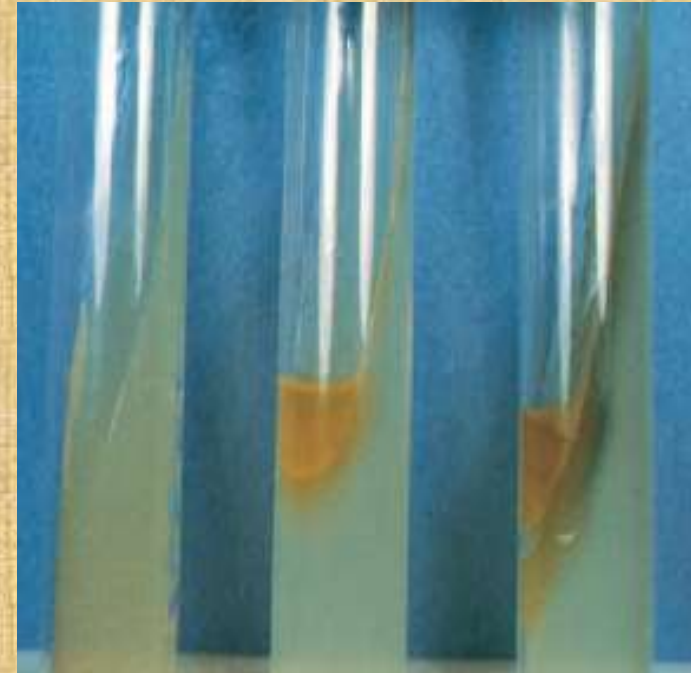
Indole test



Gelatin Hydrolysis or Liquefaction.

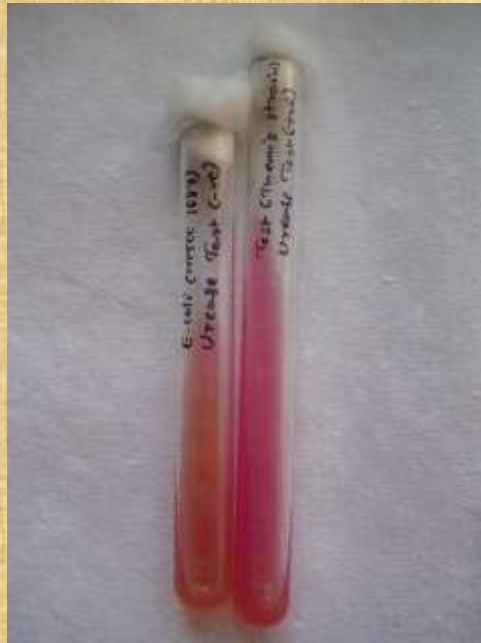


Phenylalanine Deamination Test.

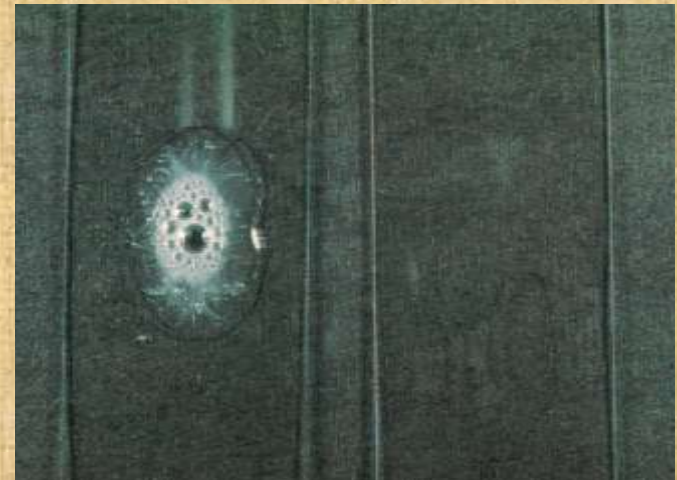
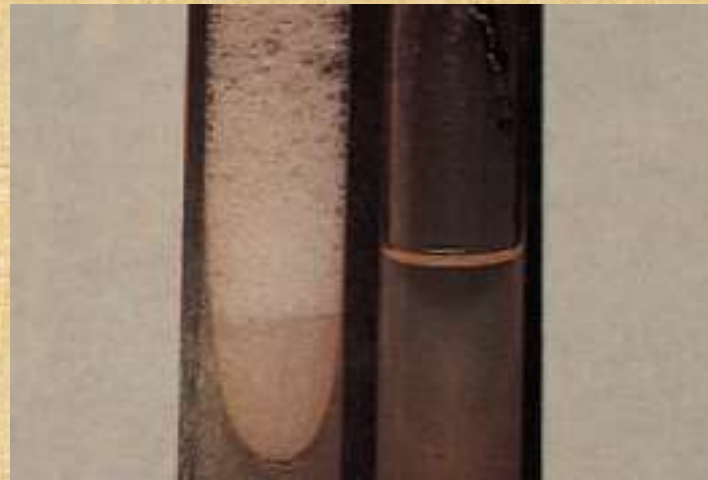
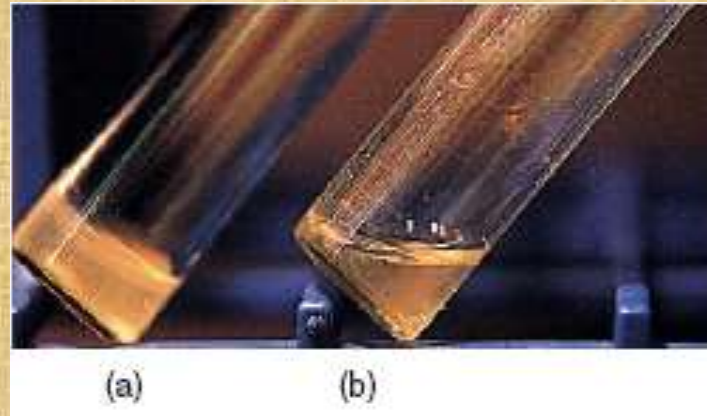


Test for Amino Acid Decarboxylase. The tube on the left is an uninoculated control; the second tube from the left is lysine decarboxylase negative; the third tube is lysine decarboxylase positive; and the tube on the right is lysine deaminase positive

Urease



cogulase test

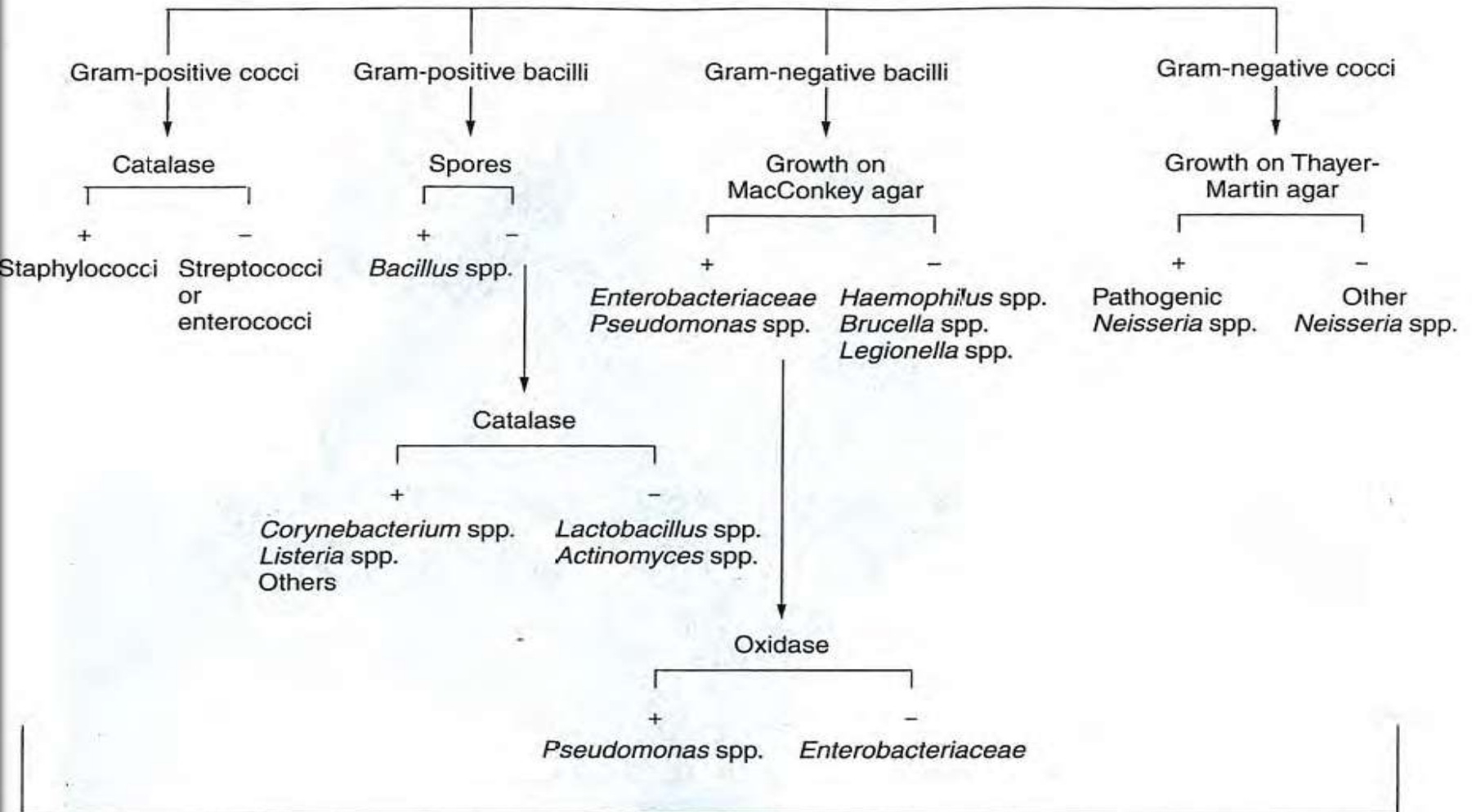


Tube and slide Catalase Test.

Establishing inhibitor profiles

- The ability to grow in the presence of one or more inhibitory substance can provide valuable identification information
- Ex- growth in the presence of high concentration NaCl (*enterococci*)
- Optochin susceptibility and bile solubility (*Streptococcus pneumoniae*)
- Ethanol survival (*Bacillus spp.*)

Gram stain morphology



Selection and performance of appropriate definitive bacterial identification schemes or systems

Limitation of phenotypic methods

- ❖ Inability to cultivate on artificial medium.

Ex-Treponema pallidum

- ❖ Fragility of organisms and failure to survive when transport.

- ❖ Fastidious nature of some microorganisms.

Ex-Bartonella, Leptospira

- ❖ Administration of antibiotic before specimen is obtained.

Immunological Methods

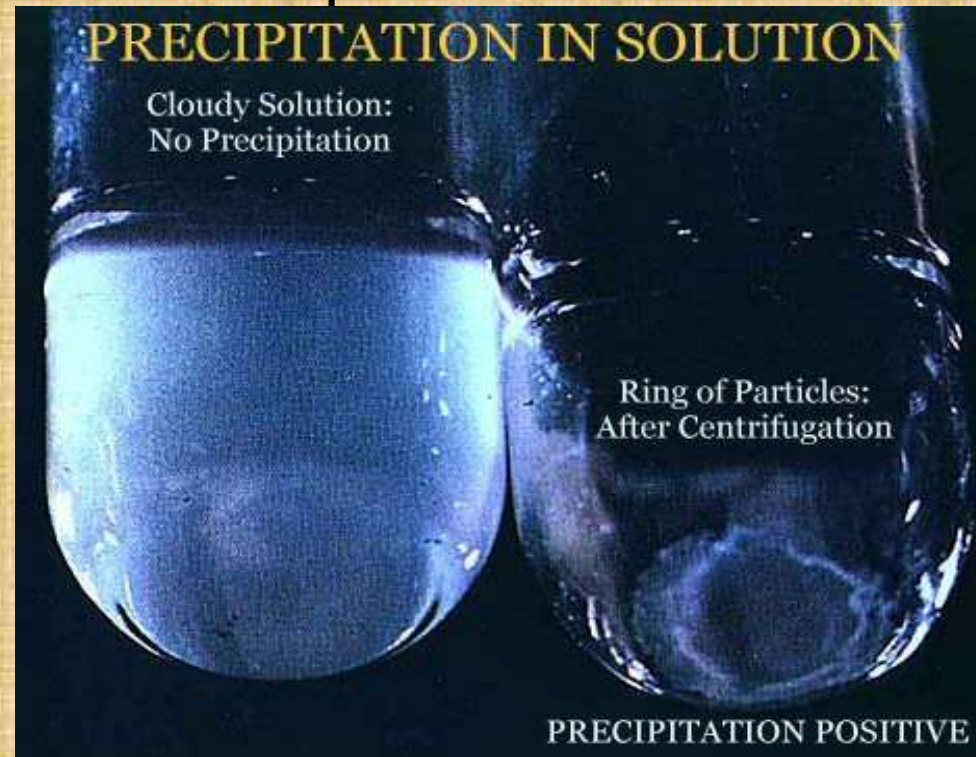
- Immunological methods involve the interaction of a microbial antigen with an antibody (produced by the host immune system).
- Testing for microbial antigen or the production of antibodies is often easier than test for the microbe itself.

Immune Testing

- **Numerous types of serologic test -differ in their speed and sensitivity.**
 - 1. Precipitation tests**
 - (a) Immuno diffusion**
 - (b) Immuno electrophoresis**
 - 2. Agglutination tests**
 - 3. Neutralization**
 - 4. Complement fixation**
 - 5. Immuno fluorescence**
 - 6. Radioimmunoassay (RIA)**
 - 7. Enzyme-Linked Immuno Sorbent Assay (ELISA)**
 - 8. Western Blotting**

Precipitation Reactions

- Precipitation is the interaction of a soluble Ag with an soluble Ab to form an **insoluble complex**.
- The complex formed is an aggregate of Ag and Ab.
- Precipitation reactions occur maximally only when the **optimal proportions** of Ag and Ab are present.
- RPR, VDRL test.



Double Immunodiffusion

- Precipitation tests - done in gels
- The precipitate is easily seen in gels yield visible **precipitin lines**
- But no visible precipitate forms in regions of Ab or Ag excess.
 - Disadvantage
- Techniques is too slow

Counterimmuno electrophoresis (CIE)

- modification of double immunodiffusion
- Speeding up migration applying electric current.

Disadvantage:

precipitin band difficult to see sometimes

Agarose gel required overnight washing

More expensive

Less sensitive to particle agglutination test. (detecting antigen approx .01 to .05 mg/ml)

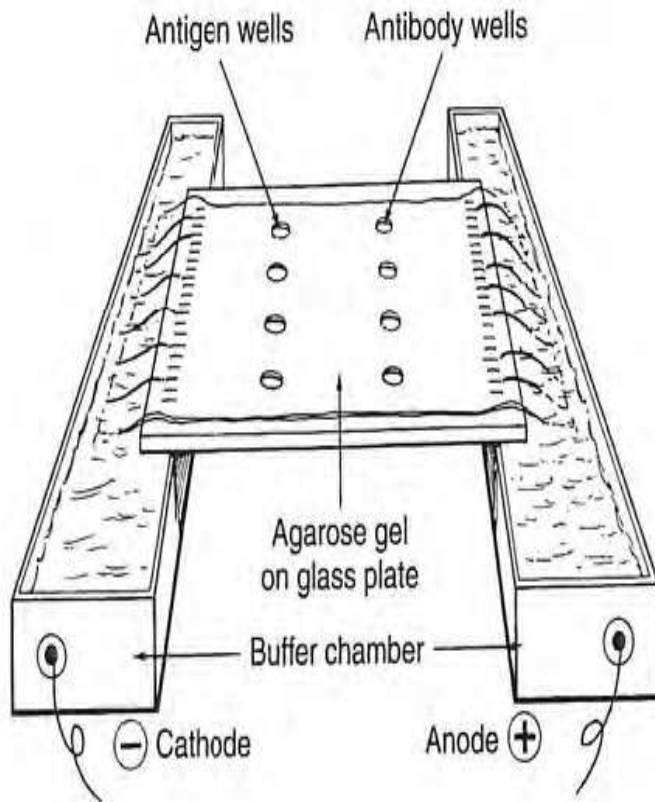


Figure 9-4 Apparatus for performing counterimmuno electrophoresis.

Agglutination Tests

- Agglutination occurs due to the cross-linking of particulate antigens by antibody molecules.
- **Agglutination is the visible clumping of insoluble particles, whereas precipitation involves the aggregation of soluble molecules**

Types of Agglutination Reactions

- (a) Direct agglutination reactions
- (b) Indirect or passive agglutination tests
- (c) Hemagglutination reactions

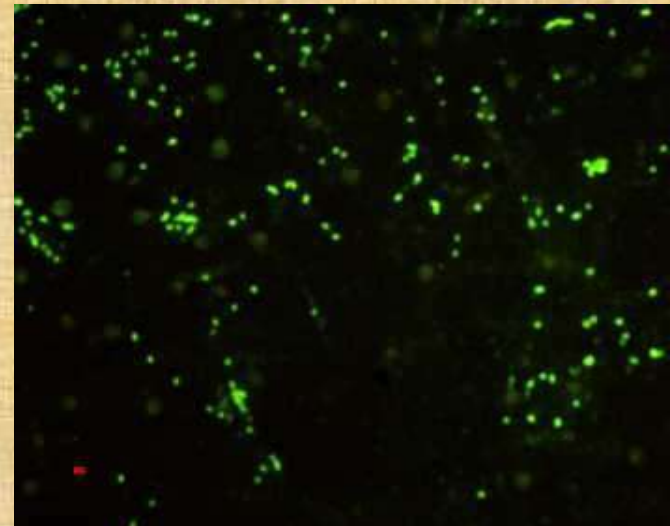
Use of agglutination test

- Bacterial agent difficult to cultivate in vitro
Ex-yersinosis, Leptospirosis, Brucellosis, Tularemia
- Microhemagglutination test for *T.pallidum*.
- Passive hemagglutination test for antibody to extracellular antigens of *streptococci*.
- CDC also perform indirect hemagglutination test for some bacteria
- *Clostridia, Burkholderia, Pseudomallei, Bacillus anthracis, Corynebacterium diphtheriae, Leptospira* etc.

Immuno-fluorescence

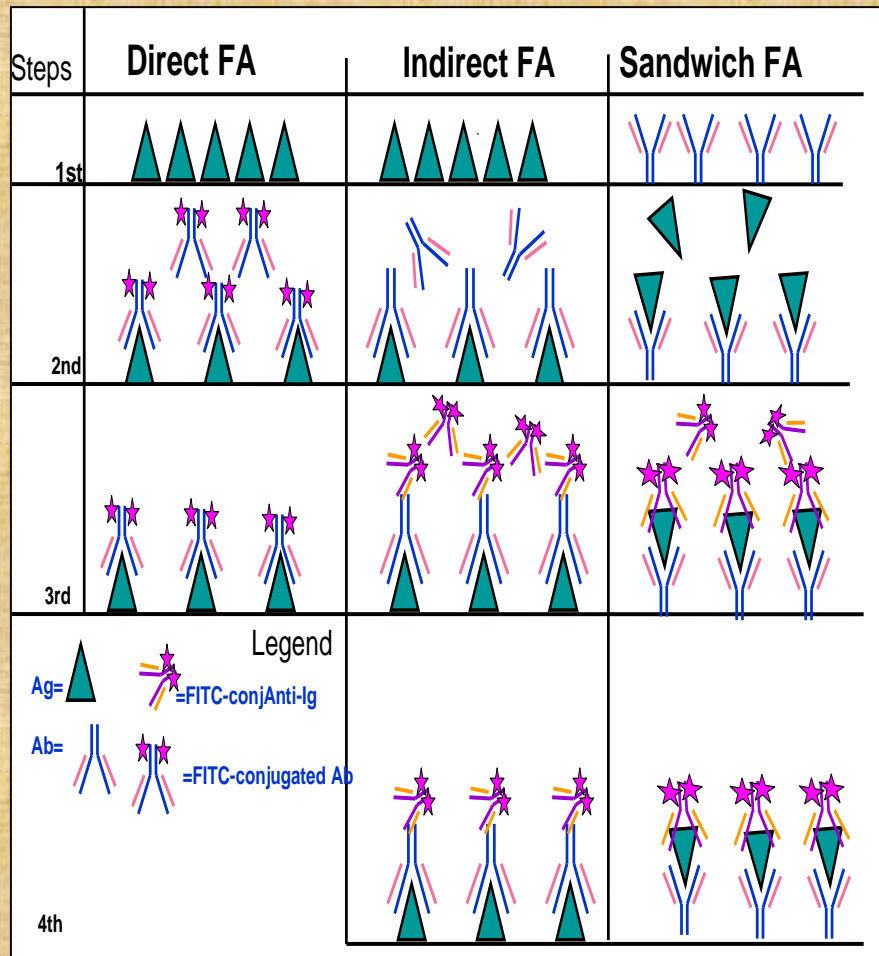
- Principle

- Use fluorescein isothiocyanate labeled-immunoglobulin to detect antigens or antibodies according to test systems
- Requires a fluorescent microscope



V. Cholerae

Types of immuno-fluorescence



- Direct immuno-fluorescence

- Used to detect antigen

- Indirect and sandwich immuno-fluorescence

- Antigen detection

- Antibody detection

Immuno-fluorescence:

Performance, applications

- **Advantages**

- Sensitive and specific
- Can be used for discrepant analysis

- **Limitations**

- Expensive (Reagents and equipment)
- Subjective
- Cross reactivity
- Non-specific immuno-fluorescence

- **Time taken**

- few minutes to few hours.

Enzyme-linked Immuno-Sorbant assay (ELISA)

Labeling technique

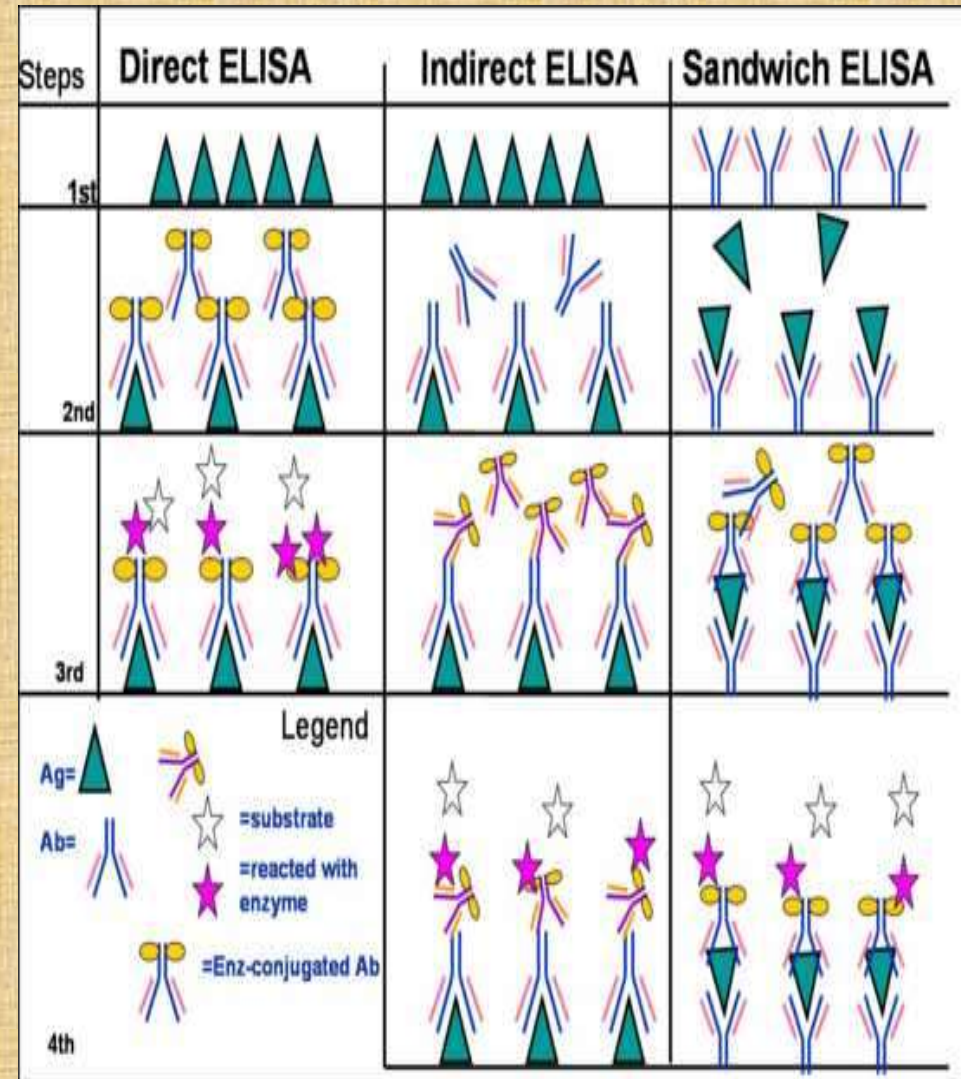
Principle

- use of enzyme-labeled immunoglobulin to detect antigens or antibodies
- signals are developed by the action of hydrolyzing enzyme on chromogenic substrate
- optical density measured by micro-plate reader

Types of ELISA (Ag Abs tests)

Competitive

- Antigen or antibody are labeled with enzyme and allowed to compete with unlabeled ones (in patient serum) for binding to the same target
- Hydrolysis signal from Ag-Ab complex (enzyme-labeled) is measured
- Antigen or antibody in serum is then calculated
- No need to remove the excess/unbound Ag or Ab from the reaction plate or tubes)



Types of ELISA used in the detection of antigens and antibodies

•Non-competitive

•must remove excess/unbound Ag or Ab before every step of reactions

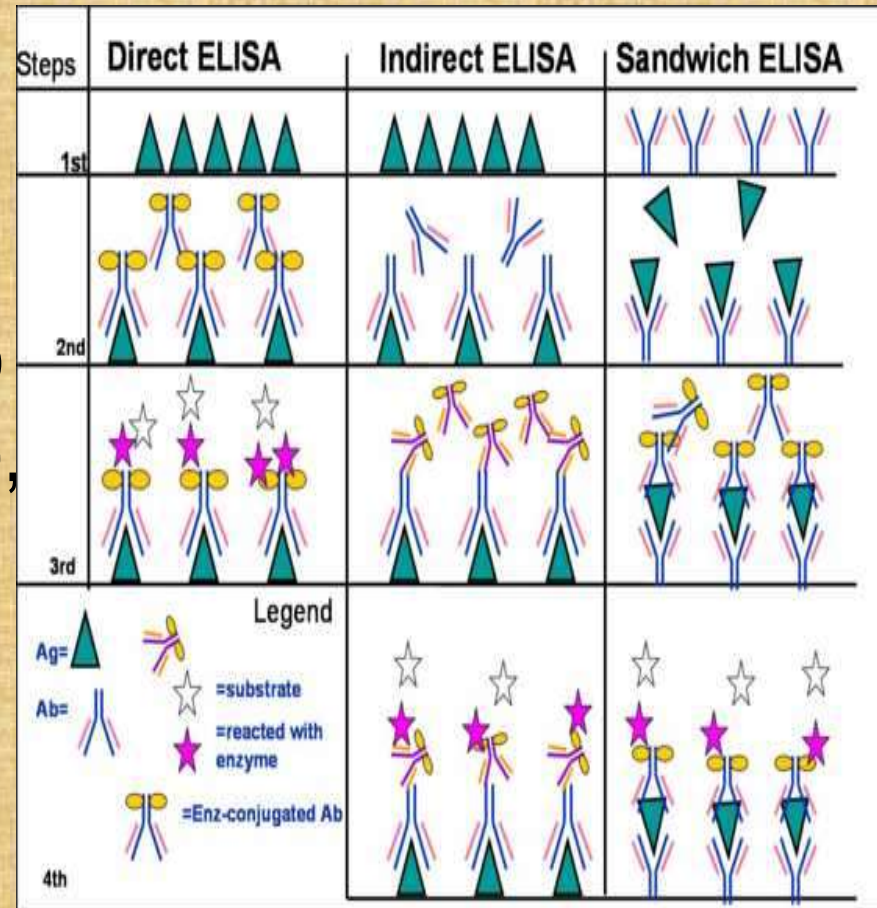
•Direct ELISA

•Indirect ELISA

•Sandwich ELISA

•Ab Capture ELISA (similar to sandwich ELISA but in 1st step, anti-Ig (M or G) is coated on the plate

•Then antibodies in patient serum are allowed to capture in next step



ELISA:

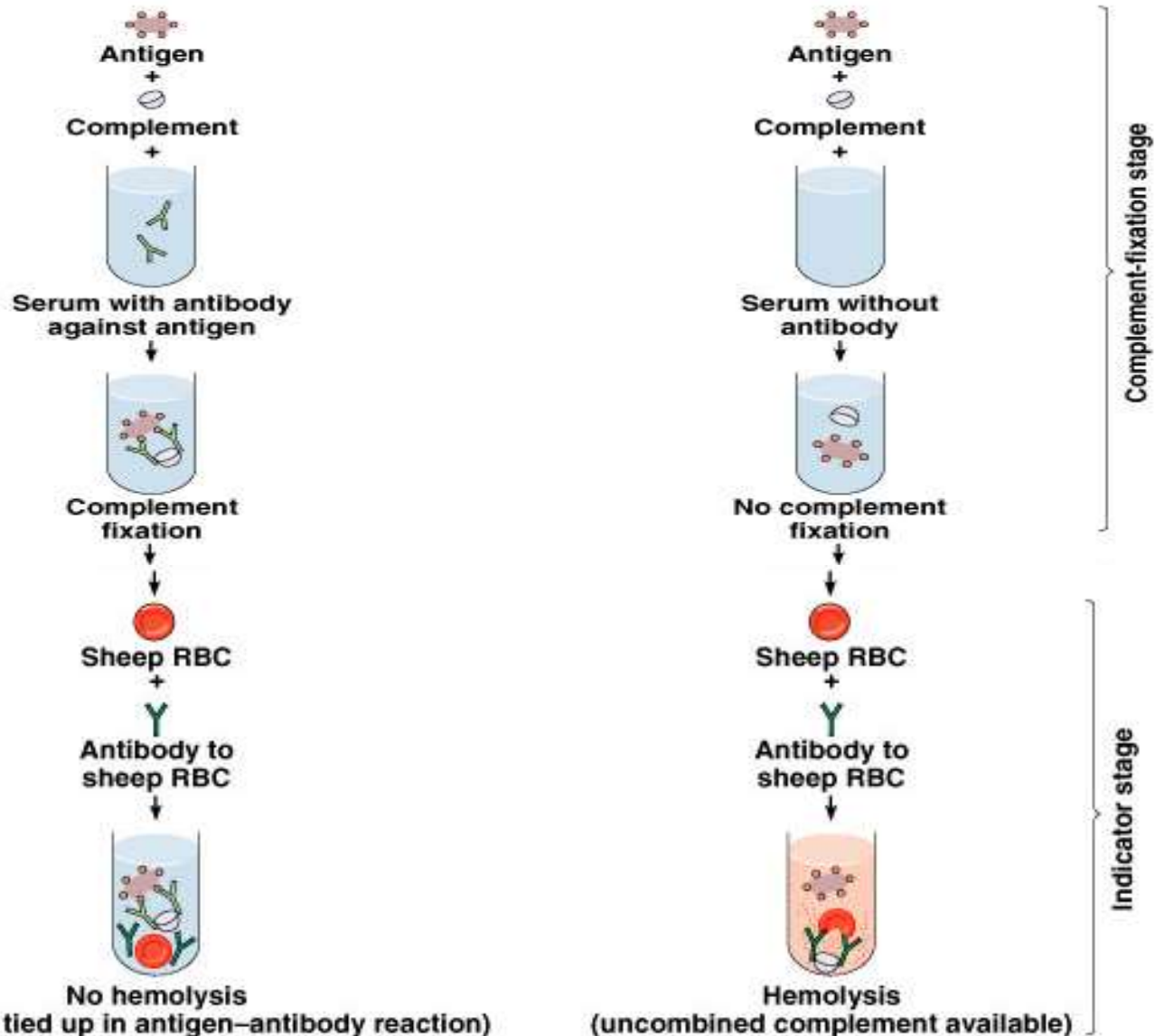
Performance, applications

- Advantages
 - Automated, inexpensive
 - Objective
 - Small quantities required
 - Class specific antibodies measurable
- Limitations
 - Expensive initial investment
 - Variable sensitivity / specificity of variable tests
 - Cross contamination
- Time taken - 1 day
- USE-commercial kit available to detect antibody for *mycoplasma, chlamydiae, borrelia*

Complement-Fixation Reactions

- Complement-fixation:
 - Complement (group of serum proteins) binds to antigen-antibody complex and is used up.
- Complement-fixation can be used to detect very small amounts of antibody.
 - Wasserman test for syphilis (in the past)
 - Certain viral & fungal diseases.

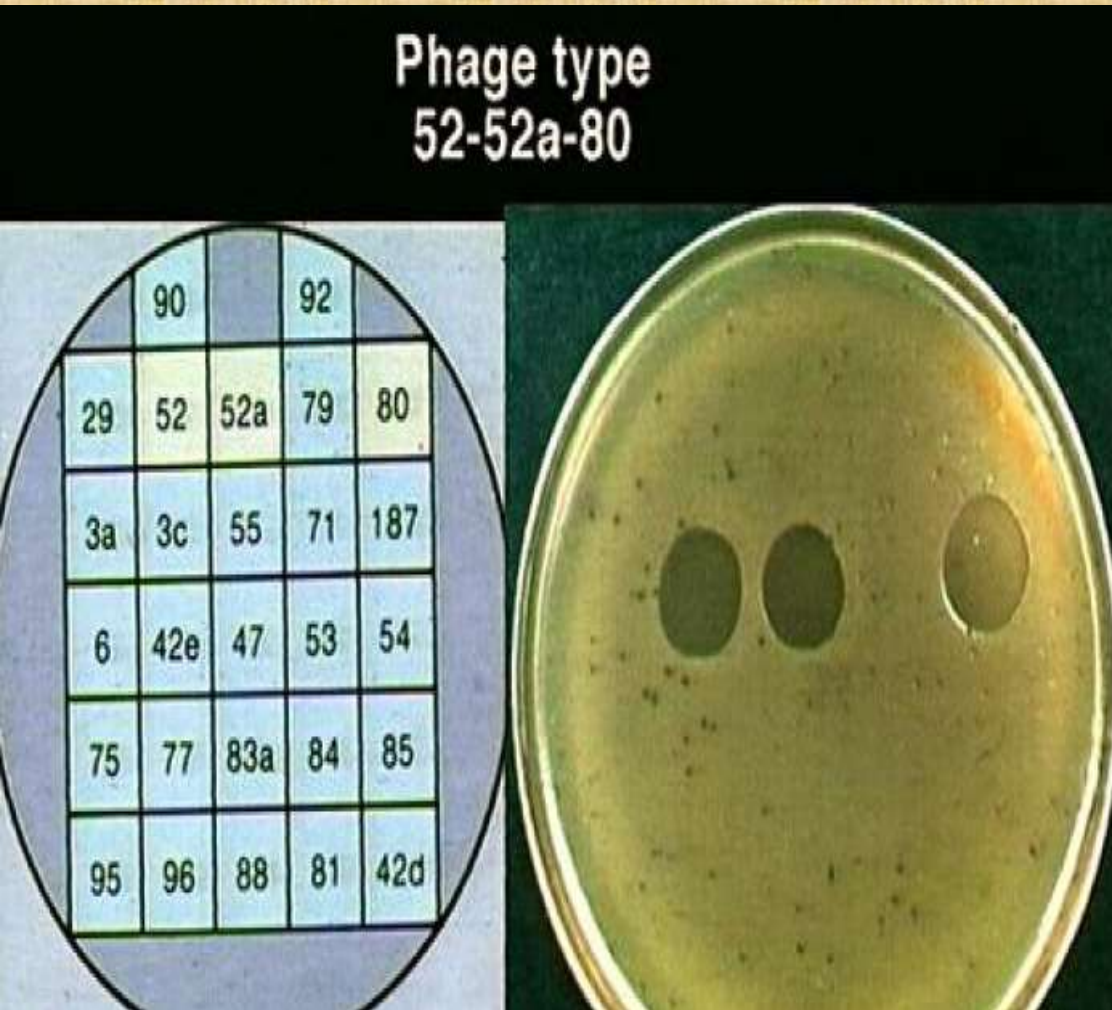
Complement Fixation



(a) Positive test. All available complement is fixed by the antigen-antibody reaction; no hemolysis occurs, so the test is positive for the presence of antibodies.

(b) Negative test. No antigen-antibody reaction occurs. The complement remains, and the red blood cells are lysed in the indicator stage, so the test is negative.

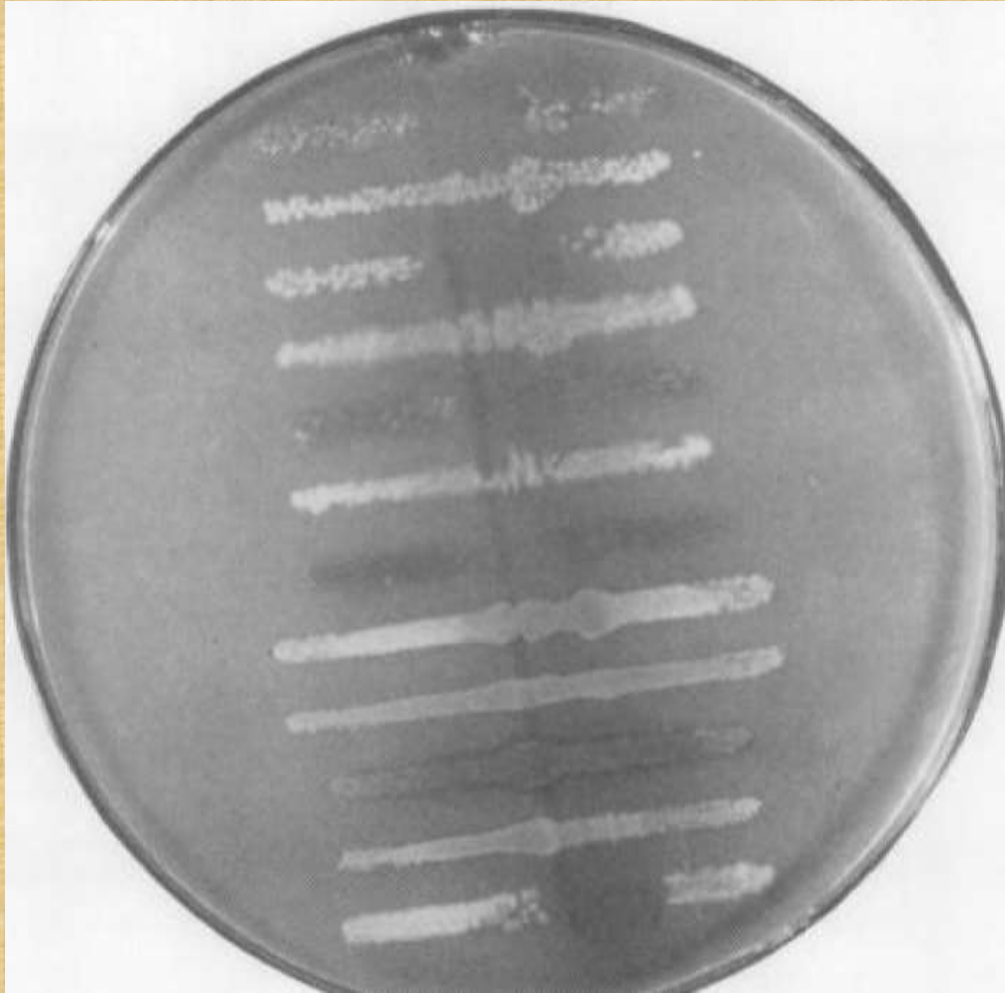
PHAGE TYPING



PHAGE TYPING

- **Classifies** bacterial organisms according to **susceptibility** of the bacteria to **lysis** by the **panel of bacteriophage**.
- Intraspecies differentiation of some bacteria.
- Phage typing has played useful in epidemiologic roles for *S.aureus* & *S.enterica* serotype Typhi.

BACTERIOCIN TYPING



- Bacteriocin is protein products produced by bacteria that inhibit growth of the strains of the same genus.
- **Classifies** bacteria according to their **susceptibility to bacteriocin**.
- Used in reference laboratories for typing *K.pneumoniae* *P. aeruginosa*.

BACTERIOCIN TYPING

Immunological Tests & Some of Their Uses

Table 17.3 Immunological Tests and Some of Their Uses

Test	Use
Immunodiffusion (precipitation)	Diagnosis of syphilis, pneumococcal pneumonia
Immuno-electrophoresis (precipitation)	Assay production of particular classes of antibodies
Agglutination	Blood typing; pregnancy testing; diagnosis of salmonellosis, brucellosis, gonorrhoea, rickettsial infection, mycoplasma infection, yeast infection, typhoid fever, meningitis caused by <i>Haemophilus</i>
Viral neutralization	Diagnosis of infections by specific strains of viruses
Viral hemagglutination inhibition	Diagnosis of viral infections including influenza, measles, mumps, rubella, mononucleosis
Complement fixation	Diagnosis of measles, influenza A, syphilis, rubella, rickettsial infections, scarlet fever, rheumatic fever, infections of respiratory syncytial virus and <i>Coxiella</i>
Direct fluorescent antibody	Diagnosis of rabies, infections of group A <i>Streptococcus</i>
Indirect fluorescent antibody	Diagnosis of syphilis, mononucleosis
ELISA	Pregnancy testing; presence of drugs in urine; diagnosis of hepatitis A, hepatitis B, rubella; initial diagnosis of HIV infection
Western blot	Verification of infection with HIV, diagnosis of Lyme disease

Problems with Serological Tests

1. Cross reacting antibody
2. Presence of Rheumatoid factors
3. Delay in Antibody response (Lyme disease - Legionnaire's Disease)
4. Competition for Antigen binding site of antibody.
 - IgM binds to the Antigen IgG site
 - IgG binds to the Antigen IgM site

Problems with Serology

Other Health conditions interfere

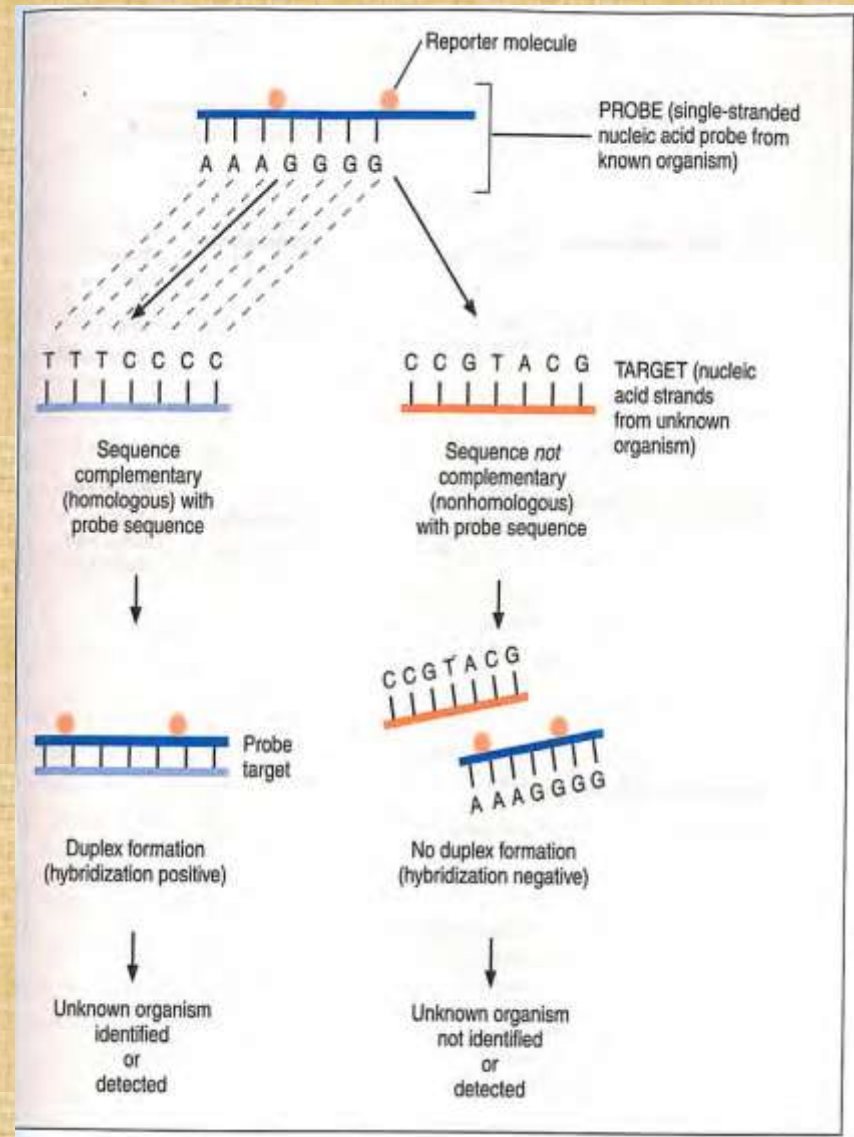
- Immunocompromised patients often give a reduced or absent humoral immune response.
- Patients with infectious mononucleosis and those with connective tissue diseases such as SLE may react non-specifically giving a false positive result.
- Patients receiving blood or blood products may give a false positive result due to the transfer of antibody

Genotypic methods

- The initiation of new molecular technologies in genomics is shifting traditional techniques for bacterial classification, identification, and characterization in the 21st century toward methods based on the elucidation of specific gene sequences or molecular components of a cell.
- Genotypic methods of microbe identification include the use of :
 - ✓ Nucleic acid probes
 - ✓ PCR
 - ✓ Nucleic acid sequence analysis
 - ✓ 16s rRNA analysis
 - ✓ RFLP
 - ✓ Plasmid fingerprinting.

Nucleic acid hybridization

- It is a technique involves using a labeled nucleic acid probe, which is a known DNA or RNA fragment, to bind with the target nucleic acids, which is usually a poorly understood, heterogeneous population of nucleic acids. A probe labeled with detectable tracer is the prerequisite for determining a specific DNA sequence or gene in a sample.
- Type:
 - i) Filter hybridization
 - ii) Southern hybridization
 - iii) Sandwich hybridization
 - iv) In situ hybridization

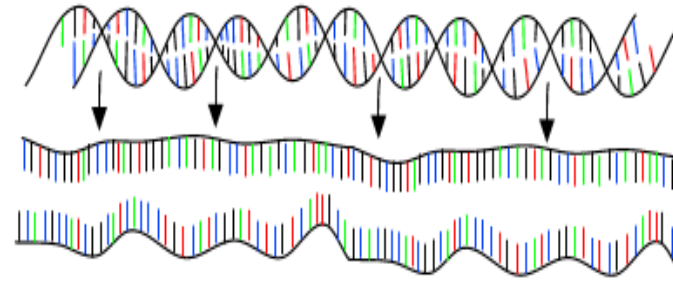


Polymerase Chain Reaction (PCR)

- PCR is widely used for the identification of microorganisms.
- Sequence **specific primers** are used in PCR for the amplification of DNA or RNA of specific pathogens.
- PCR allows for the detection even if **only a few cells are present** and can also be used on **viable nonculturables**.
- The presence of the **appropriate amplified PCR product** confirms the presence of the organisms.

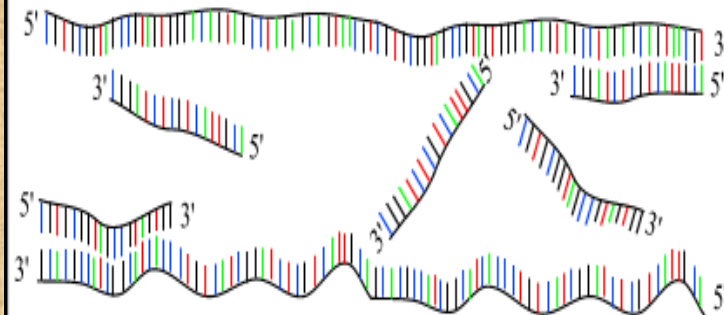
PCR : Polymerase Chain Reaction

30 - 40 cycles of 3 steps :



Step 1 : denaturation

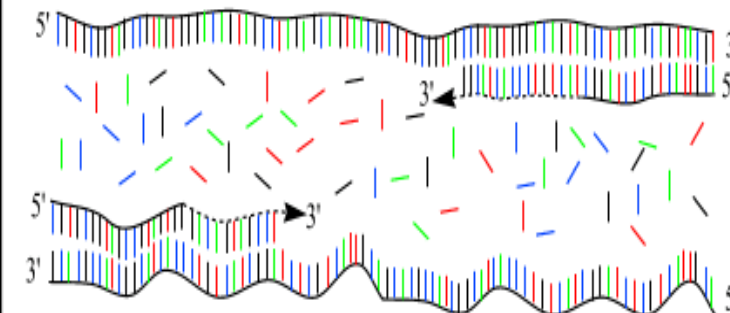
1 minut 94 °C



Step 2 : annealing

45 seconds 54 °C

forward and reverse primers !!!



Step 3 : extension

2 minutes 72 °C

only dNTP's

Variations of the PCR

- **Colony PCR**
- **Nested PCR**
- **Multiplex PCR**
- **AFLP PCR**
- **Hot Start PCR**
- **In Situ PCR**
- **Inverse PCR**
- **Asymmetric PCR**
- **Long PCR**
- **Long Accurate PCR**
- **Reverse Transcriptase PCR**
- **Allele specific PCR**
- **Real time PCR**

Table 8-3 Examples of Non-Polymerase Chain Reaction-Based Nucleic Amplification Tests

Amplification Method	Manufacturer/Name	Method Overview	Examples of Commercially Available Assays	Additional Comments
Nucleic acid sequence-based amplification (NASBA)	bioMérieux Inc. NucliSens technology: nucleic acid release, extraction, NASBA amplification, product detection.	(1) Isothermal amplification achieved through coordination of 3 enzymes (avian myxoblastosis, RNaseH, T7 RNA polymerase) in conjunction with 2 oligonucleotide primers specific for the target sequence. (2) Amplification based on primer extension and RNA transcription.	NucliSens HIV-1 QT NucliSens CMV pp67 NucliSens EasyQ HIV-1 NucliSens EasyQ enterovirus	(1) Can be adapted to real-time format using molecular beacons (2) Can develop in-house assays (3) Automated extraction available (NucliSens extractor) (4) Easy Q System = incubator, analyzer, and computer
Transcription-mediated amplification (TMA)	Gen-Probe Inc.: Sample processing, amplification, target detection by hybridization protection or dual kinetic assays for <i>Chlamydia trachomatis</i> and <i>Neisseria gonorrhoeae</i> . Also, ASRs for hepatitis C virus (HCV), Bayer Inc., Tarrytown, NY; Gen-Probe/Chiron Corp.: TMA for screening donated blood products for HIV-1 and HCV.	(1) Autocatalytic, isothermal amplification utilizing reverse transcriptase and T7 RNA polymerase and 2 primers complementary to the target. (2) Exponential extension of RNA (up to 10 billion amplicons within 10 minutes).	Gen-Probe: <i>Mycobacterium tuberculosis</i> Direct Test; APTIMA Combo 2 for dual detection of <i>C. trachomatis</i> and <i>N. gonorrhoeae</i> ; Bayer ASR reagents for HCV; Gen-Probe/Chiron: Procleix HIV-1/HCV	(1) Second-generation TMA assays of Gen-Probe better at removing interfering substances <ul style="list-style-type: none"> • Less labor-intensive • Uses target capture after sample lysis using an intermediate capture oligomer • TMA performed directly on captured target (2) Automated system for TMA-based assays: TIGRIS DTS system (Gen-Probe) <ul style="list-style-type: none"> • Instrument handles specimen processing through amplification and detection • 500 tests in 9 hours; 3.5 hours to first result (3) Real-time TMA-based assays using molecular beacons under development by Gen-Probe that will be compatible with a variety of thermal cyclers (e.g., iCycler, ABI Prism)
Standard displacement amplification (SDA)	BD ProbeTec ET System: SDA coupled with homogeneous real-time detection.	(1) Isothermal process in which single-stranded target is first generated. (2) Exponential amplification of target.	BDProbe Tec ET System for <i>C. trachomatis</i> and <i>N. gonorrhoeae</i> ; Panel assays for <i>Mycoplasma pneumoniae</i> , <i>Chlamydia philia pneumoniae</i> , and <i>L. pneumoniae</i> , <i>Chlamydiaceae</i> ; Assay that detects <i>C. trachomatis</i> , <i>C. pneumophila</i> , and <i>C. psittaci</i> ; BD ProbeTec <i>M. tuberculosis</i> Direct	(1) Reagents dried in separate disposable microwell strips (2) All assays have internal control to monitor for inhibition (3) Automated system for sample processing: BD Viper Sample Processor

Advantage of genotypic methods over phenotypic methods

- ❖ SPEED, ACCURACY, COST
- ❖ ability to detect nonviable organisms that are not retrievable by cultivation based method.
- ❖ identification of bacteria grown in culture
 - 1) Slow growing bacteria
 - 2) Common pathogen exhibit unusual phenotypic traits.
- ❖ detection of antimicrobial resistance.

Continue....

- ❖ characterization of bacteria beyond identification

- 1) For identifying virulence, resistance, strain relatedness of same species.

- ❖ ability to quantitative analysis of infectious agent burden directly in patients specimens.

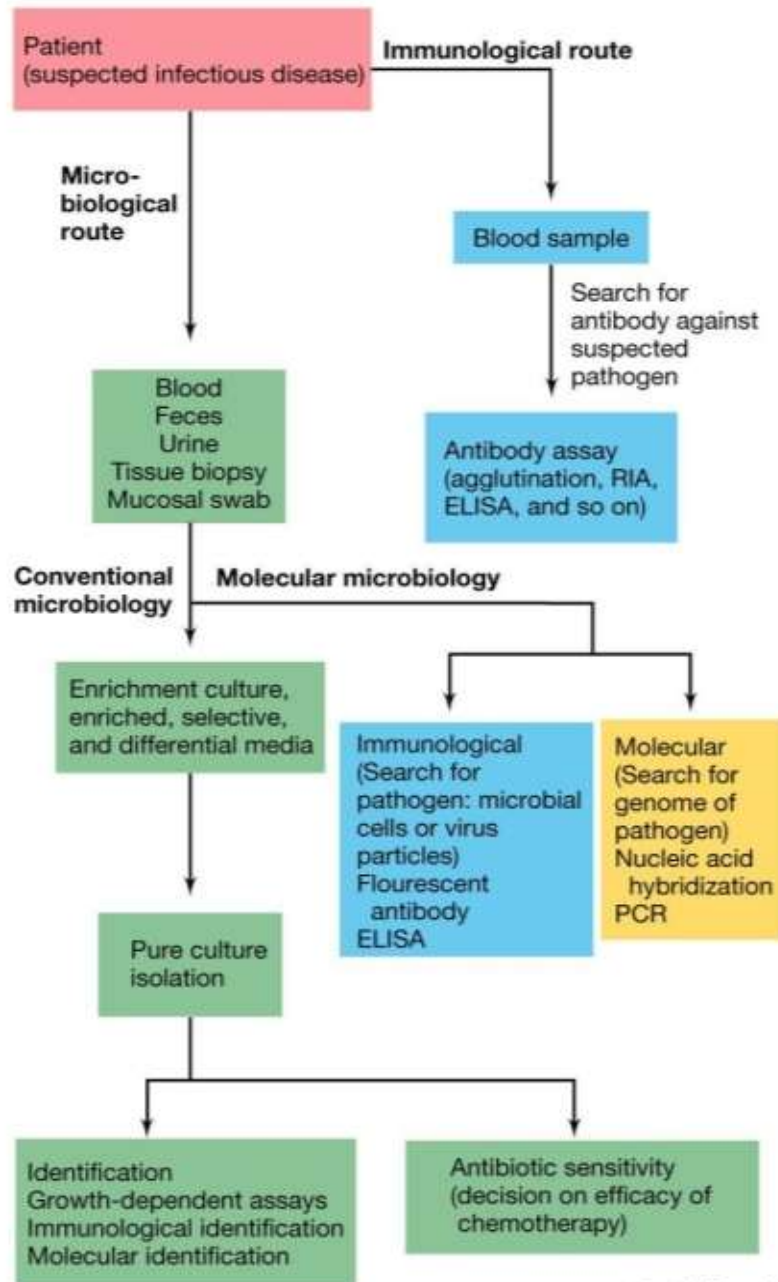
Continue....

❖ To recognizing and controlling disease outbreak inside or outside the hospital

- Ex-PFGE quickly identified nosocomial infection and outbreaks such as MRSA, VRE.



Figure 8-17 Although antimicrobial susceptibility profiles indicated that several methicillin-resistant *S. aureus* isolates were the same strain, restriction fragment length polymorphism analysis using pulsed-field gel electrophoresis (Lanes A through F) demonstrates that only isolates B and C were the same.



Microbe Identification

Thank You!

