University of Gondar

Institute of Biotechnology

Department of Medical Biotechnology

Course Title: Diagnostic technology

Course Code: Biot 3122

Credit Hour: 2

Instructor: Tadele Tamiru and Tekeba Sisay

Instructor's Contact Information:

- **Office Location:** College of Natural Science Building, Floor 3, Office No. 14
- **E-mail:** 21sisay@gmail.com or tadeletamiru13@gmail.com
- **Office Hours:** Wednesday 2:00 PM- 5:00 PM

Chapter one
Introduction about Diagnostic technology

Objectives of the chapter

❖ **At the end of the lesson you will be able to:**

- ➢ Define the term of diagnostics, diagnosis, prognosis, disease and health
- ➢ Describe what is infectious and non-infectious diseases
- ➢ Describe manifestations of infections and non infectious diseases
- ➢ Identify the diagnostic criteria and critical issues in disease diagnosis
- ➢ Describe types of diagnosis and components of general diagnosis
- ➢ Describe the historical highlights of diagnostic technology

Introduction

Definition of Terms

❖**Diagnostics:** is the identification of diseases by the examination of signs and symptoms and by other investigations an opinion or conclusion so reached .

❖**Diagnosis:**

➢The act or process of identifying or determining the nature and cause of a disease or injury through evaluation of patient history, examination, and review of laboratory data.

❖**Prognosis**:

- Is a medical term for predicting the likely or expected development of a disease, including whether the sign and symptoms will improve or remain stable over time.
- Expectations of quality of life, such as the ability to carry out daily activities; the potential for complications and associated health issues; and the likelihood of survival.
- A prognosis is made on the basis of the normal course of the diagnosed disease, the individual's physical and mental condition, the available treatments, and additional factors.
- A complete prognosis includes the expected duration, function, and description of the course of the disease, such as progressive decline, intermittent crisis, or sudden, unpredictable crisis.

❖**Health** is “a state of complete physical, mental and social well-being, not merely the absence of disease or infirmity”.

❖**Diseases:** is a disorder of structure or function in a human, animal, or plant, especially one that produces specific symptoms or that affects a specific location and is not simply a direct result of physical injury.

❖**Infection:** The colonization and/or invasion and multiplication of pathogenic microorganisms in the host with or without the manifestation of disease.

❖Classification of diagnostic tests

- ➢Immunopathology
- ➢Molecular Biology
- ➢ Clinical Chemistry
- ➢ Haematology
- ➢ Cytopathology
- ➢ Histopathology
- ➢Radiology
- ➢Electrophysiology
- ➢ Endoscopy

- **What is Diagnostic technology?**
- **What thing we diagnosis?**

Infectious & Non-Infectious Diseases

❖Infectious Diseases

- ➢Spread directly or indirectly, from one person to another. Caused by **pathogen**ic microorganisms
  - ✓Bacteria
  - ✓Viruses
  - ✓Parasites
  - ✓Fungi
- ➢Typically many infectious agents can be transmitted through different ways. Such as
  - ✓Respiratory/Droplets
    - Coughs or sneezes suspended in warm, moist droplets, may enter the body through the nose, mouth or eye surfaces.
    - Diseases that are commonly spread by coughing or sneezing include: Viral Meningitis, Chickenpox, Common cold, Influenza, Strep throat and Tuberculosis.

❖Fecal – Oral Transmission

- ➢ Food or water are contaminated (by people not washing their hands before preparing food, or untreated sewage being released into a drinking water supply) and the people who eat and drink them become infected.
- ➢ Examples: Cholera, Hepatitis A, Polio, Rotavirus and Salmonella

❖Sexual Transmission

- ➢This refers to any disease that can be caught during sexual activity with another person.
- ➢Examples: HIV/AIDS, Chlamydia, Genital warts, Gonorrhea, Hepatitis B, Syphilis and Herpes.

❖Direct Contact

- ➢Diseases that can be transmitted by direct contact are called contagious.
- ➢Examples: Athlete's foot, Impetigo and Warts.

❖Vector Transmission

- A *vector* is an organism that does not cause disease itself but that transmits infection by conveying pathogens from one host to another.
- Examples include mosquitoes, deer ticks, animal bites, etc.

- **The transmission cycle has different elements:**

✓The pathogen: the organism causing the infection

✓The host: the infected person or animal ‘carrying’ the pathogen

✓The exit: the sites in which the pathogen uses to leave the body of the host

✓Transmission: the pathogen is transferred from host to susceptible person or animal, which can include developmental stages in the environment, in intermediate hosts, or in vectors.

- ✓The environment: the environment in which transmission of the pathogen takes place.
- ✓The entry: the sites in which the pathogen uses to enter the body of the susceptible person or animal
- ✓The susceptible person or animal: the potential future host who is receptive to the pathogen.

❖Non – Infectious Disease (Non- communicable diseases)

- ➢Not caused by a pathogen and cannot be shared from one person to another.
- ➢The cause is may be due to different factors such as
  - ✓Environment, Nutritional deficiencies, Lifestyle choices or Genetic inheritance
- ✓Unlike infectious diseases, non-infectious diseases are not communicable or contagious, although some kinds can be passed down genetically to the children of a carrier.

❖Environmental Diseases

- Environmental disease are avoidable and unavoidable conditions caused by external factors, such as sunlight, food, pollution, and lifestyle choices.
  - ✓Many types of cardiovascular disease
  - ✓Chronic obstructive pulmonary disease caused by smoking tobacco
  - ✓Type 2 Diabetes
  - ✓Malnutrition caused by too little food, or eating the wrong kinds of food (e.g. scurvy from lack of Vitamin C)
  - ✓Skin cancer caused by radiation from the sun

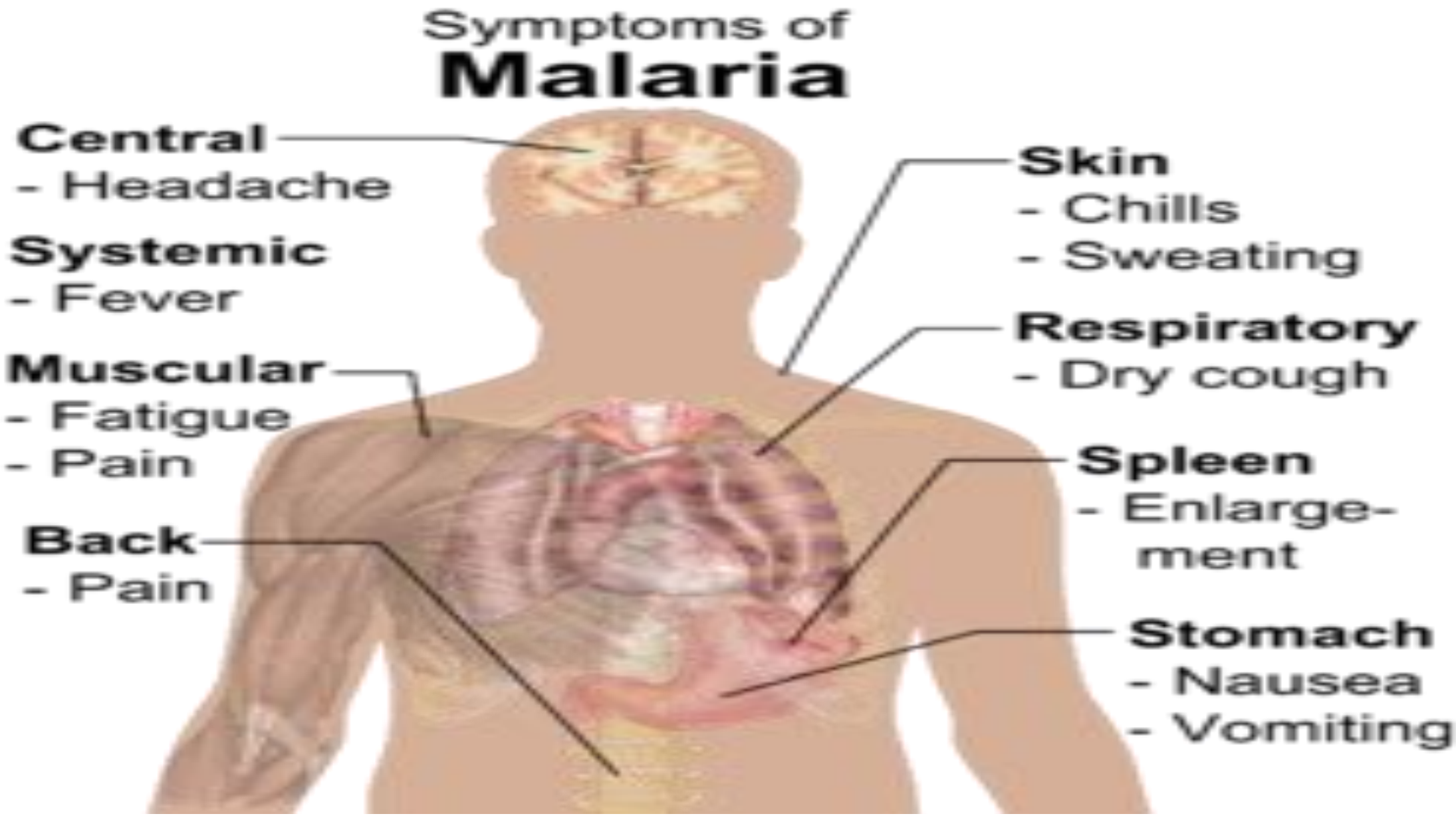
❖Cancer

- ➢Cancer is a non-infectious disease that affects millions of people.
- ➢The most common cancer type as nonmelanoma skin cancer, with about one million cases a year.
- ➢Lung cancer is the second-most common,
- ➢ Other common cancers include bladder, breast, leukemia, pancreatic, prostate, thyroid, colorectal, endometrial and kidney cancer.

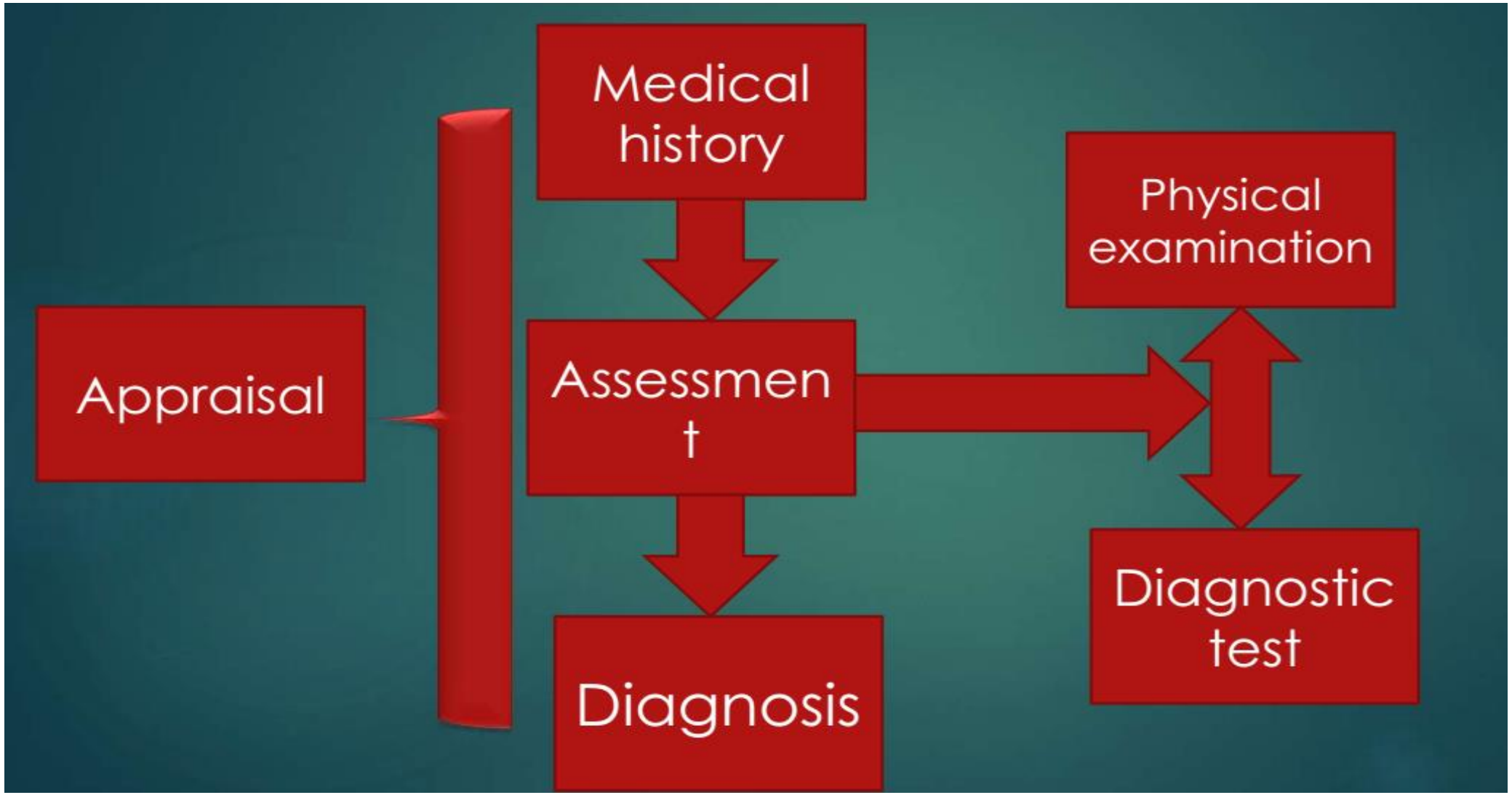
❖Other non-infectious diseases includes Heart Disease, Kidney Disease, Bone Disease, Mental Disease and etc…

Manifestations of infections

Symptoms	Sign
Are the expression of the disease caused by the manifestation of the physiological reaction of the body/ plant due to harmful activity of the pathogen.	Variety of structure produce by pathogen.
Symptoms are the changes that any patient experiences before or during the disease.	Signs refer to the changes in the normal functioning or structure of the body.
A doctor cannot see or observe it so the patient has to inform the doctor about the symptoms.	A physician/doctor can easily notice signs in the patient's body.
Symptoms include abdominal cramps, headache, fever, among numerous other symptoms.	Signs include rashes, inflammation, swelling, change in the colour of the skin, etc.
Two or more disease can have common symptoms.	
Therefore, symptoms of diseases cannot indicate the exact disease	


Symptoms of
Malaria
Central
- Headache
Systemic
- Fever
Muscular
- Fatigue
- Pain
Back
- Pain
Skin
- Chills
- Sweating
Respiratory
- Dry cough
Spleen
- Enlarge-
ment
Stomach
- Nausea
- Vomiting

Components of diagnosis



Types of Diagnostic Testing

- **CLINICAL diagnosis:** A diagnosis made on the basis of medical signs and patient reported symptoms, rather than diagnostic tests.
- **Laboratory diagnosis:** A diagnosis based significantly on laboratory reports or test results, rather than the physical examination of the patient.
    - For instance, a proper diagnosis of infectious diseases usually requires both an examination of signs and symptoms, as well as laboratory characteristics of the pathogen involved.
- **Radiology diagnosis:** A diagnosis based primarily on the results from medical imaging studies.
    - Magnetic resonating imaging (MRI) are common radiological diagnoses.

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❖**Reading assignment**

- **Principal diagnosis**
- **Admitting diagnosis**
- **Differential diagnosis**
- **Prenatal diagnosis:**
- **Dual diagnosis**
- **Remote diagnosis**
- **Nursing diagnosis**

A BRIEF HISTORY OF MEDICAL DIAGNOSIS

❖The sophistication of diagnostic techniques has come a long way and continues to develop at breakneck speed.

- ➢From tasting urine to microscopy to molecular testing,
- ➢The history of the diagnosis is the story of medicine's evolution from empirical to experimental techniques.

- Three distinct periods in the history of medicine are associated with three different places and, therefore,
  - ➢Middle ages to the 18th century different methods of determining diagnosis:
  - ➢1794 and 1848 bedside medicine was prevalent and came hospital medicine; and
  - ➢From these time forward, laboratory medicine has served as medicine's lodestar.

History con’t …

❖The laboratory’s/diagnostic technology contribution to modern medicine through the addition of another resource to medical science such as microscope, culture and other sophisticated instruments.

Microscopic method:

- Organisms must be present in sufficiently high concentration and
- Use of appropriate set stains and conditions make them visible

Cultivation method:

- Certain microbes may require special culture media and conditions and failure to consider these microbes may yield negative culture results.
- Cell culture can be used to detect some viruses and intracellular microbes but the cost, labor, and time required for this approach beg for better diagnostic method.

History con't …

❖Ancient diagnostic methods

➢300 BC Hippocrates

- ✓Promoted the use of the mind and senses as diagnostic tools
- ✓His doctrine is that all disease are associated to disorders of fluids of the body
- ✓He advocated a diagnostic protocol to obtain a clear picture of disease, that included
  - Tasting the patient's urine,
  - Listening to the lungs, and
  - Observing skin color and other outward appearances.

➢He related the appearance of bubbles on the surface of urine specimens to kidney disease and chronic illness.

➢He also related certain urine sediments, blood and pus in urine to disease.

History con’t …

- Rufus of Ephesus surfaced at around AD 50
  - The first description of hematuria, or the presence of blood in urine
  - Attributed to the failure of kidneys function properly in filtering the blood.
- Later (AD 180), Galen (AD 131–201),
  - Who is recognized as the founder of experimental physiology,
  - He described diabetes as “diarrhea of urine” and noted the normal relationship between fluid intake and urine volume.

History con't …

❖**Middle Ages between** 500 AD to 1500 AD

- In medieval Europe, early Christians believed that disease was either punishment for sin or the result of witchcraft or possession.
- Diagnosis was superfluous.
- The basic therapy was prayer, penitence, and invocation of saints.
- Lay medicine based diagnosis on symptoms, examination, pulse, palpitation, percussion, and inspection of excreta and sometimes semen.
- Diagnosis by "water casting" (uroscopy) was practiced, and the urine flask became the symbol of medieval medicine.
- By AD 900,
  - ✓Isaac Judaeus, a Jewish physician and philosopher, develop guidelines for the use of urine as a diagnostic aid;
  - ✓The first book detailing the color, density, quality and sediment found in urine was written around this time.

History con’t …

- AD 1267 R. Bacon experiments with optics, probably produces first microscope.
- By 1300
  - ✓ Uroscopy is widespread in Europe.
  - ✓Medieval medicine also included interpretation of dreams in its diagnostic repertoire.
  - ✓Repeated dreams of floods indicated “an excess of humors that required evacuation,” and dreams of flight signified “excessive evaporation of humors.”
- 1500 Physicians begin using urine color charts for visual urinalysis
- 1590 Hans Janssen invents the compound microscope.
- 1592 Galileo Galilei invents the thermometer.

History con’t …

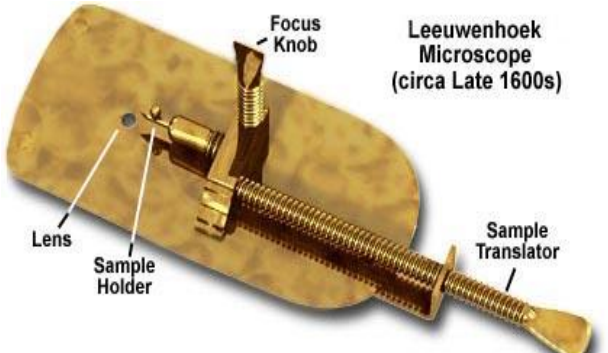
❖**Seventeenth century(17th century) between** 1601 to 1700

- Many of diagnostic techniques and therapeutic were discovered.
- Jesuit priest, Athanasius Kircher (1602–1680) of Fulda (Germany),
  - The first to use the microscope to investigate the causes of disease.
  - His experiments showed how maggots and other living creatures developed in decaying matter.
  - Kircher’s writings included an observation that the blood of patients with the plague contained “worms;”

History con't …

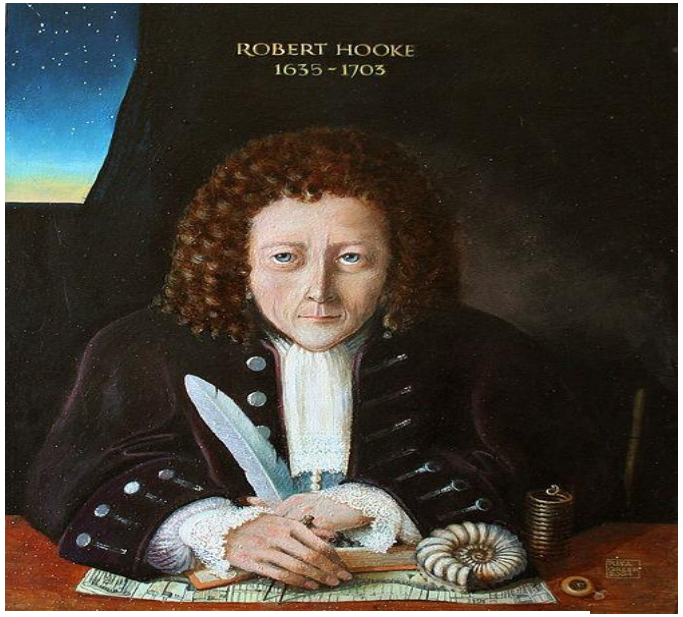
- Dutch microscopist,**Anton van Leeuwenhoek** (1632—1723)

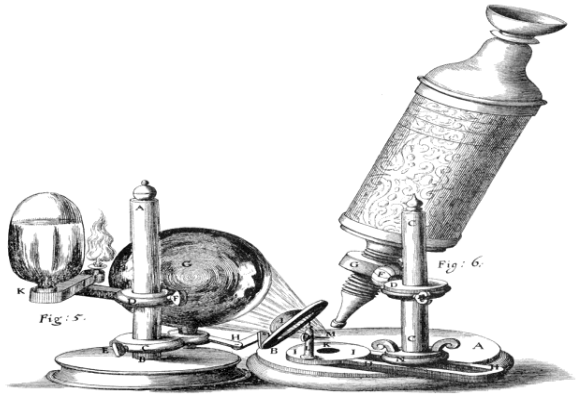
- improving the art of polishing lenses of short focal length (300x). Father of Microbiology.
- 1684 Anton van Leewenhoek publishes the first drawings of bacteria as seen under the microscope.
- He sketches
  - Small organisms(animalcules- protist) bacteria, yeasts
  - Sperm cells, movement of blood cells and the striated muscles

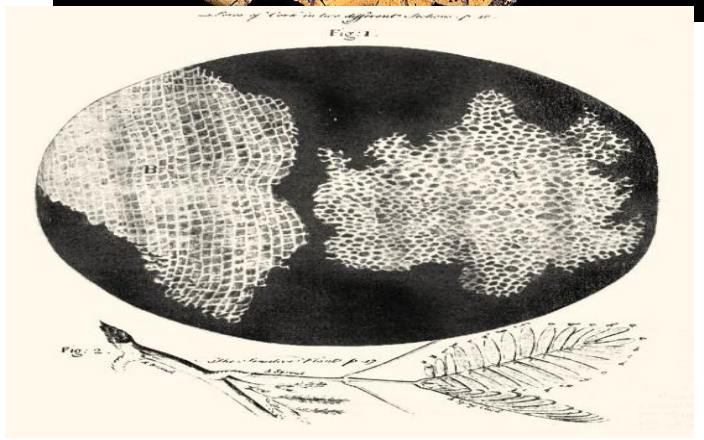


History con't …

- An English Scientist **Robert Hooke** (1635—1703)
  - Used microscope to look at thin slices of plant stems, wood, & pieces of cork coined term "cell".







History con’t …

❖Marcello Malpighi (1628–1694) Italian microscopist,

- ➢Who is the founder of histology
- ➢Famous for his investigations of the embryology of the chick and the histology and physiology of the glands and viscera.
- ➢His work in embryology describes the aortic arches, the head fold, the neural groove, and the cerebral and optic vesicles.

❖Thomas Willis (1621–1675), an English physician

- ➢The best qualitative analysis of urine at the time
- ➢The first to notice the characteristic sweet taste of diabetic urine,
- ➢Established the principle for the differential diagnosis of diabetes mellitus and diabetes insipidus.

History con’t …

❖Richard Lower (1631–1691).

- ➢The first to perform direct transfusion of blood from one animal to another.

❖William Harvey (1657)

- ➢Discovery of the circulation of blood
- ➢The beginning of a period of mechanical explanations for a variety of functions and processes, including digestion, metabolism, respiration and pregnancy.

❖Other medical innovations of the time included the intravenous injection of drugs, transfusion of blood, and the first attempts to use pulse rate and temperature as indicators of health status

History con't …

❖Eighteenth century (1700 to 1800)

- "Golden Age" of the successful practitioner.
- The advancement of medicine during this time was more theoretical than practical.
- Internal medicine was improved by new textbooks that described many new forms of disease and the introduction of new drugs.
- 1714 Gabriel Fahrenheit develops the mercury thermometer and the Fahrenheit temperature scale.
- William Hewson (1739–1774)
  - ✓The discovery of the cause of blood coagulation and coagulation tests such as measurement of prothrombin time, plasma thromboplastin time and other coagulation tests

History con't …

- Coagulation was the formation in the plasma of a substance, he called "coagulable lymph," which is now known as fibrinogen.
- A later discovery that fibrinogen is a plasma protein and that in coagulation it is converted into fibrin.

❖In 1707, Sir John Floyer (1649–1734) of Staffordshire, England, introduced the concept of measuring pulse rate by timing pulse beats with a watch.

❖Stephen Hales (1677–1761), an English clergyman

- Measuring blood pressure

❖1770 John Hill introduces a method of obtaining specimens for microscopic study.

❖1789 de Fourcroy discovers cholesterol

History con't …

- James Currie (1756–1805), a Scot,
  - The first to use cold baths in treatment of typhoid fever; and
  - Monitoring the patient's temperature using a thermometer, he was able to adjust the temperature and frequency of the baths to treat individual patients.
- 1816 R. T. H. Laennec invents the stethoscope.
- 1828 British hospitals are organized into clinical wards.
- 1830 Gerardus Mulder performs the first elemental chemical analysis of proteins;
- 1836 James Marsh develops a standard test for arsenic.
- **1841** P. S. Denis describes the separation of blood proteins into albumins and globulins by salt precipitation.
- **1847** The American Medical Association is founded.

❖Nineteenth century (1800-1900)

- The emergence of sophisticated diagnostic techniques and
- The laboratories happen together roughly with the worldwide political, medical, industrial and philosophical revolutions of the 19th century.
- An era of public health in which
  - ✓New discoveries in bacteriology allowed for water treatment and pasteurization of milk
  - ✓Advent of antiseptic surgery
  - ✓Medical practitioners relied, for a time, more on increased hygiene and less on drugs.
- At the beginning of the century, physicians depended on:
  - ✓ Primarily on patients' accounts of symptoms, superficial observation to make diagnoses and manual examination.

History con't …

- By the 1850s, a series of new instruments, including the stethoscope, ophthalmoscope and laryngoscope, began to expand the physician's sensory powers in clinical examination.
- Diagnostic tools: including the microscope and the X-ray, chemical and bacteriological tests, and machines that generated data on patients' physiological conditions, such as the spirometer and the electrocardiograph produced data independent of the physician's and patient's subjective judgment.
- **1849** A. E. Berthold demonstrates endocrine function.
- **1852** K. Vierordt develops a method for performing accurate blood counts (hemocytometry) and G. G. Stokes discovers fluorescence.

History con't …

- **1856** William Perkin prepares the first synthetic dye.
- **1859** E. Becquerel invents the fluorescent lamp.
- Louis Pasteur (1822–1895) discovered the anaerobic character of the bacteria of butyric fermentation and introduced the concepts of aerobic and anaerobic bacteria around the year 1861.
  - ✓He discovered *Mycoderma aceti* in woine.
  - ✓In 1867 partial heat sterilization (Pasteurization) at a temperature of 55–60°C without any alteration of the taste
- **1866** Voit establishes the first hygienic laboratory in Munich.
- **1869** Luer invents the glass hypodermic syringe.

History con't …

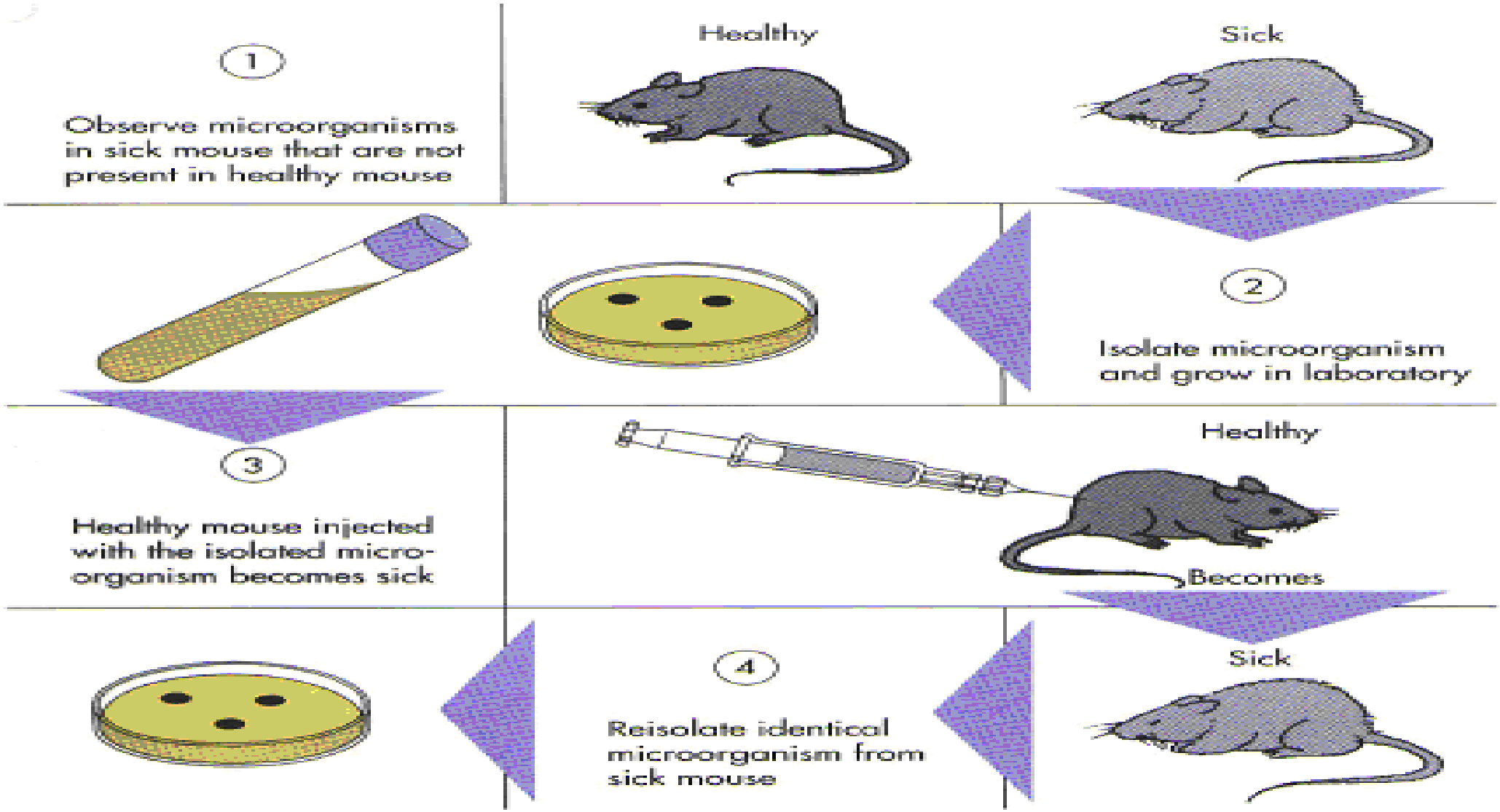
- **1872** Oscar Brefeld develops the use of gelatin medium for isolation of fungi in pure culture.
- **1875** Corfield establishes the first public health laboratory in England; Osaka, Japan, establishes the Imperial Hygienic Laboratory.
- In 1877, Koch published his methods of fixing and drying bacterial films on coverslips, of staining them with Weigert's aniline dyes, of staining flagella, and of photographing bacteria for identification and comparison.
  - ✓In 1881, he developed a method of obtaining pure cultures of organisms by spreading liquid gelatin with meat infusion on glass plates, forming a solid coagulum.
  - ✓Koch also played a role in perfecting the method of steam sterilization.

History con't …

✓The year after, he discovered the tubercle bacillus by other special culture and staining methods and formulated a rule for determining the specificity of disease-causing organisms called Koch's postulates or Koch's law.

❖Koch's postulates or Koch's law, four criteria that were established by Robert Koch to identify the causative agent of a particular disease, these include:

- ➢The pathogen must be **present in all cases of the disease**
- ➢ The pathogen can be isolated from the diseased host and **grown in pure culture**
- ➢ The pathogen from the pure culture must **cause the disease when inoculated into a healthy, susceptible laboratory animal**
- ➢The pathogen must be **re-isolated** from the new host and **shown to be the same** as the originally inoculated pathogen.


1
Observe microorganisms in sick mouse that are not present in healthy mouse
Healthy
Sick
2
Isolate microorganism and grow in laboratory
3
Healthy mouse injected with the isolated micro-organism becomes sick
Healthy
Becomes
Sick
4
Reisolate identical microorganism from sick mouse

History con't …

- In 1881, Pasteur produced a vaccine against anthrax and lowered the mortality rate to 1% in sheep and to 0.34% in cattle.
- In 1883, Koch discovered *Cholera vibrio* and recognized its routes of transmission as drinking water, food and clothing.
- In 1885, the Pasteur Institute was opened.
- In 1893, Koch wrote an important paper on waterborne epidemics showing how they could be prevented by proper filtration.
  - ✓In the 1890s and proved to be effective in preventing typhoid.
  - ✓Finally, in 1905, Koch received the Nobel Prize.

❖Major contribution of Louis Pasteur.

- Microbial theory of fermentation
- Principles and practices of sterilization and pasteurization
- Control of disease of silk worm
- Development of vaccines against anthrax and rabies
- Discovery of streptococci.
- Germ theory of diseases, which states that a specific disease is caused by a specific type of microorganism.
- Disprove Theory of spontaneous generation.

History con’t …

- **Major achievements of Robert Koch**

1. Discovery and use of solid medium in bacteriology
2. Discovery of causative agents of tuberculosis and cholera
3. Koch’s postulate

History con't …

- **1892** Charles and William Mayo found the first public diagnostic bacteriology lab in the U.S.

- **1893**
  - J. Elster and H. F. Geitel invent the photoelectric cell;
  - T. W. Richards invents the nephelometer;

- **1895**
  - Franz Ziehl and Friedrich Neelsen introduce their modification of the acid-fast stain for tuberculosis;

- **1896**
  - S. Riva-Rocci invents the sphygmomanometer;
  - C. W. Purdy publishes Practical Urinalysis and Urinary Diagnosis;
  - Ferdinand Widal develops the agglutination test for identification of the typhoid bacillus; in Great Britain, clinical laboratories existed in Edinburgh.

- **1897**

- The first commercial clinical laboratory established in England, The Clinical Research Association, receives specimens by mail.

The History and Evolution of Molecular Diagnostics

- Molecular Diagnostics is a branch of the biotechnology industry, and involves the detection of genetic patterns in DNA and RNA, as well as patterns in protein generation and usage.
- These detected patterns (both genomic and proteomic) are used clinicians for aiding with diagnosis, prognosis and therapeutic monitoring.

History con't …

❖Uses of Molecular Diagnostics are diverse, including

- ✓Classification of organism based on genetic relatedness (genotyping)
- ✓Identification and confirmation of isolate obtained from culture
- ✓Early detection of pathogens in clinical specimen
- ✓Rapid detection of antibiotic resistance
- ✓Clinical pathology,
- ✓Forensics testing,
- ✓Epigenetics,
- ✓Immunotherapy and immunosuppression,
- ✓ Metagenomics,
- ✓Molecular endocrinology, Molecular oncology, Toxicology, and Personalized medicine and more.

History con't …

- ❖Before the rise of Molecular Diagnostics in the 1980s, clinicians were limited to a generalized approach to diagnosing health problems and for creating solutions for those problems.
- ➢The theoretical gestation of genetics spanned well over 2,000 years, these is what they did to access the human history.
- ➢From classical Greek thinkers like Hippocrates and Aristotle, all the way to the advent of cellular biology in the 19th Century and through to modern times.
- ❖Fast-forward to the mid-1800s with Charles Darwin the controls of evolutionary biology, promoting the idea that evolution was "descent with modification".
- ❖Then in 1866 Gregor Johann Mendel, whose pioneering work on the pea plant clearly showed documentable inheritance patterns.

History con't …

- On the clinical side in 1902 , Oxford physician Sir Archibald Edward Garrod
  - Collected historical information about the family of one of his patents, and concluded that his patent's **alkaptonuria** was a recessive disorder passed down through inheritance.
- In 1869 a Swiss physician named Friedrich Miescher analyzed the pus that was in the discarded bandages of one of his patents, and discovered that the discharge contained microscopic material called **'nuclein'.**
- Then in 1878, Albrecht Kossel was the first scientist to isolate nucleic acid, and went on to isolate the five primary nucleobases later in his career.

History con't …

❖'Thymus nucleic acid' which is deoxyribonucleic Acid (DNA) and 'yeast nucleic acid' which is ribonucleic Acid (RNA) were formally named in the first part of the 20th Century.

❖**Phoebus Levene**

- ➢Identified the nucleotide unit of yeast nucleic acid in 1909, carefully noting the base, sugar and phosphate components.
- ➢In 1929, studying thymus nucleic acid, he identified deoxyribose sugar.
- ➢Leven's 'tetranucleotide hypothesis' thymus nucleic acid was composed of four nucleotide units linked by the phosphate groups – although he thought that the ordering of the nucleotide units was the same in every cell.

History con’t …

- Nikolai Koltsov, in 1927, proposed his idea that hereditary traits were carried by two-stranded or “mirrored” molecules, with each strand acting as a genetic template.
- The double-helix model of DNA was provided by Francis Crick and James Watson in 1953.

History con’t …

- Genetic coding mechanism finally cracked in 1965.
- The Human Genome Project (HGP) was begun by the US government in1987.
  - ✓The project would make extensive use of technologies that were in development at that time, which included PCR and capillary sequencing, restriction fragment-length polymorphisms (RFLP) and pulsed field gel electrophoresis
- The Human Genome Project was completed in 2003

Conclusion

- The advancement of diagnostic technology is now coming to the aid, through
  - Reducing the time it takes to diagnosis the cause of infection, via the detection of the specific microbial agent.
  - New systems are allowing a patent’s sample to be loaded into a cartridge and placed into a process that will test for hundreds of different pathogens at once.
  - Testing for antibiotic resistance markers.

Thank You

Chapter-2
Sample Collection, handling and shipping to the laboratory

❖ **After completion of this chapter, you will be able to:**

- Define different types of specimen
- Identify different types of specimen
- Know how to collect them
- Know sample rejection and accepting criteria
- Labeling, transporting and storing of different types of specimens

Definition

❖**Definition of specimen**

- o Specimen is a part taken to determine the character of the whole

❖ **Safety during collection, processing & transportation**

- o Masks (e.g. sputum)
- o Gloves
- o Protective eye ware (goggles)
- o Protective clothing (gown)

❖**Specimen collection**

- o Correct treatment (Rx) depend on accurate result
- o Accurate result depend on quality specimen

➢Several different kinds of specimens are used in the biotechnology laboratory.

- These are: - serum, whole blood, cerebrospinal fluid (CSF), urine, genital swabs, respiratory swabs, stool, different tissue biopsies, sputum, hair, saliva, nail, autopsy, soil, water, plant materials,

Specimen collection con’t…

- ❖ Quality of specimen depend on proper:
  - ✓ Collection
  - ✓ Transportation
  - ✓ Processing of specimens
- ❖ The purpose of quality specimen is to**:**
  - ✓ Provide high yield positive result
  - ✓Provide that a negative result indicates the absence of the pathogen

❖ **Importance of Quality Specimen**

- ➢ Key to accurate laboratory diagnosis
- ➢ Directly affects outcome of your conclusion
- ➢ Influences therapeutic decisions
- ➢ Impacts on length of stay and exposed for additional costs
- ➢ Influences laboratory/your research center efficiency

Basic Concept in Specimen Collection

1. Specimen site selection

a. If the sample is from animal/human

- Should locate right anatomic site & select appropriate tests & specimens based on:
  - Physical examination (sign & symptoms) by trained personnel
  - Should collect specimens from right site
  - Use aseptic technique and Sterile container
    - To prevent contamination of specimen &
    - To protect animals/patients from infection

2. **Volume/amount of specimens**

- Collecting & processing too little specimen lower sensitivity
- Collecting adequate volume:
  - enhance recovery of the pathogen
  - enable to perform all procedures required or to permit complete examination
- For example;
  - Sputum: 5 -10 ml for mycobacterium examination
  - Blood:
    - ✓ Serology: minimum 2 - 3 ml
    - ✓Clinical chemistry: 3ml
    - ✓ Culture: 10 – 20 ml (adult) & 1-5ml (infant)
    - ✓Molecular techniques: 2ml
  - CSF: 5 – 10 ml

3. Time of collection

- Provide best chance of recovery of the causative agent
  - **Sputum & urine**
    - ✓ early in the morning soon after the patient awaken
  - **Blood**
    - ✓ when the patients temperature begins to rise

4. Collect specimens before the administration of antimicrobial

- Because antimicrobials limits recovery of pathogens

5. Age of specimens

- Age of the specimen directly influences the recovery of protozoan organism
- Is preferable to use fresh sample for molecular diagnosis however sample age is not as a great burden for DNA analysis.

6. Stage of the disease at which the specimen is collected

- Enteric pathogens are present in great numbers during the acute or diarrheal stage of intestinal infection

7. Labeling

- Make sure that you are collecting/drawing the right person first.
- Then label with:
  - Patient/sample name
  - Unique identification number
  - Patient demographic information
  - Specimen collection date
  - Specimen collection location
  - Diagnostic test results
- **During Labeling:**
  - Make sure that container label & the requisition match
  - Label should be on the container not on the lid, since the lid can be mistakenly placed on a different container
  - Ensure the labels on the containers are adherent under refrigerated conditions

General Sample Rejection Criteria

1. Unlabelled Specimens

- Common specimen like blood, urine, swabs, sputum, stool, soil, water, plant material can be easily recollected.
- Less common specimens like CSF, fluids, tissues, etc. are more difficult to recollect
- Call the person who collected it for the identification of the specimen.

2. Incorrectly labeled (mislabeled) specimens

- Use same criteria as for Unlabelled Specimens

3. Incorrect container or Preservative

- Specimens received in an incorrect container, or without appropriate preservative, will require recollection
- So the patient will be contacted to arrange for recollection of the specimen

4. Insufficient specimen for procedure

- If insufficient, recollect (urine, stool, sputum, blood, etc.)
- If the specimen is not recollectable (CSF, fluids, etc.), the physician will be contacted to establish a priority order of tests

5. Unsuitable Specimen for Procedures

- Specimens which are unsuitable for the procedure requested saliva for sputum test or specimen too long for a valid result

Specimen Transportation

❖**Required when:**

- ➢ Specimens are to be sent to referral laboratory
- ➢ For teaching purpose
- ➢ For quality assurance
- ➢ Unavailability of trained personnel
- ➢ Specimens are collected in the field
- ➢ Lack of time to examine within the recommended time due to laboratory workload

❖This transportation is made by using different preservation methods

- Physical or chemical
- chemical method of preservation is most common

- Reading assignment
  - Physical or chemical method of specimen preservation

- **Purpose of preservation :**
  - o Maintain protozoan morphology
  - o Maintain viability of microorganisms
  - o Prevent overgrowth of normal flora
  - o Prevent instability of solutes & degeneration of sediments
- **Specimen packaging**
  - o Screw container tops on firmly
  - o Wrap in absorbent wadding to absorb any fluid leakage
  - o Place in a self-sealing plastic bag

- Place the request form into the secondary pocket of the specimen bag
- Hazard labels with internationally accepted biohazard label (HIGH RISK)
- Pack specimens in strong cardboard box or a grooved polystyrene box
- Seal with self-adhesive tape.

❑**Specimen Storage (Prior to Dispatch)**

- Blood samples should be kept at +4 ^{o}c for short time or -21 ^{o}c for long time for DNA analysis.
- Serum & plasma can be stored either frozen or at +4^{o}c
- Any sample for DNA analysis can store -21 ^{o}c

❑ **Transport**

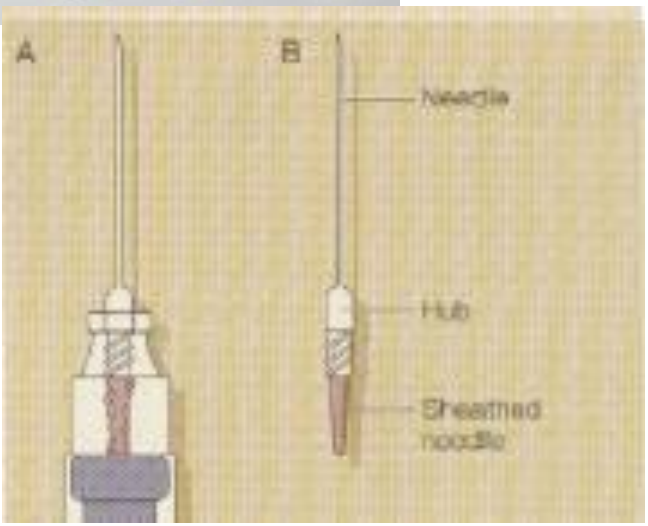
- o Urgent requests within normal laboratory hours
- o Transport with labelled, tightly fitted, leak proof container
- o Wet ice or ice pack should be used.

Guideline in blood collection

❖ Collected by trained personnel only.

Equipment and Supplies

- Gloves
- Needle
- Holder
- Tubes
- Gauze
- Alcohol Pad
- Band Aid
- Sharps Container
- Tourniquet



Collection of sputum specimen

Definition

- Saliva: - is secreted by the salivary glands and is limited in the oral region.
- Sputum: - is the material coughed up from the throat and lungs.
- Sputum is usually examined to determine the presence of disease of the lungs or of the upper respiratory tract e.g. diagnose pulmonary tuberculosis.

sputum specimen con’t…

❖Collection Method

- Provide the patient with a sterile wide- mouthed glass bottle of about 50 ml capacities with a screw top.
- The lab technician or the nurse should be present when the sample is taken.
- First, the patient should be standing, if possible.
- Then, he/she should take a very deep breath, filling his/her lungs.
- She/he should empty his/her lungs in one breath, coughing as hand and deeply as she/he can.
- She/he should spit what he brings up into the jar

After Collection

- Check that a sufficient amount of sputum has been produced.
- The sputum of an infected person usually contains:
    - thick mucus with air bubbles
    - threads of fibrin
    - patches of pus
    - occasional brownish streaks of blood
- **N.B.** The first sputum coughed out in the early morning is the most desirable specimen for the laboratory investigation.
    - ➢ Liquid frothy saliva and secretions from the nose and pharynx are not acceptable expectations. Have the patient produce another specimen.

sputum specimen con't…

- Dispatch of sputum: - Fluid transport medium
  - A wide-mouthed, screw-top bottle containing: 25ml of a solution of 0.6% cetylpyridinium bromide in distilled water
  - The patient should expectorate directly into the liquid in the bottle
  - Screw on the top and dispatch

Guideline in soil sample collection

- Sampling sites should not be previously sprayed with microbial insecticides.
- The locations are best as diverse as possible.
- Soil samples should be collected by scraping off surface material with a sterile spatula
- Obtaining approximately 100-g samples from 2-5 cm below the surface.
- Use sterilized container
- Labeled appropriately such as site, soil type, date of collection etc.
- Samples should to be stored at 4 °C until use.

Guideline in food , water, milk and beverages sample collection

- This is important to identify sources of contamination and types of microorganisms through microbiological examination of foods, water and beverages.

❖For food sample

➢Representative sample

➢Mix well (blender, Stomacher)

❖ For water sampling

➢Samples of water for bacteriological testing must be collected in sterile bottles and care *must* be taken to prevent accidental contamination of the water during its collection.

➢Glass bottles used for water sampling should have a capacity of at least 200 ml.

➢They should be fitted with ground glass stoppers or screw caps.

water sampling con’t..

- Sampling bottles should be sterilized.
- Sampling bottles stopper or cap and neck of the bottle should be protected from contamination by a suitable cover such as thin aluminum foil.
- After being sterilized the bottle should not be opened before the sample is collected.

water sampling con't..

❖***Neutralizing chlorine in water samples***

- When the water to be examined is likely to contain, sufficient sodium thiosulphate ($Na_2S_2O_3.5H_2O$) should be added to neutralize chlorine or chloramine to each bottle as follows:
- Add 100–200 µl (0.1–0.2 ml) sodium thiosulphate 30 g/l (3% w/v) to each bottle of 200 ml capacity before it is sterilized.
- This will give a concentration of approximately 18 mg/liter of water.

- **Note**: Sodium thiosulphate at a concentration of approx. 18 mg/litre has no significant effect on the coliform or *E. coli*.

Information to be supplied with water samples

❖Each water sample should be given a code number and the following information should accompany the sample (preferably using a standardized form):

- Reasons for examination, e.g. whether a routine sample or otherwise.
- Source and the exact place from where the water has been collected
- State whether the water has been filtered, chlorinated, or treated in some other way.

Sample from tap

- Sample from a house tap:

Mention

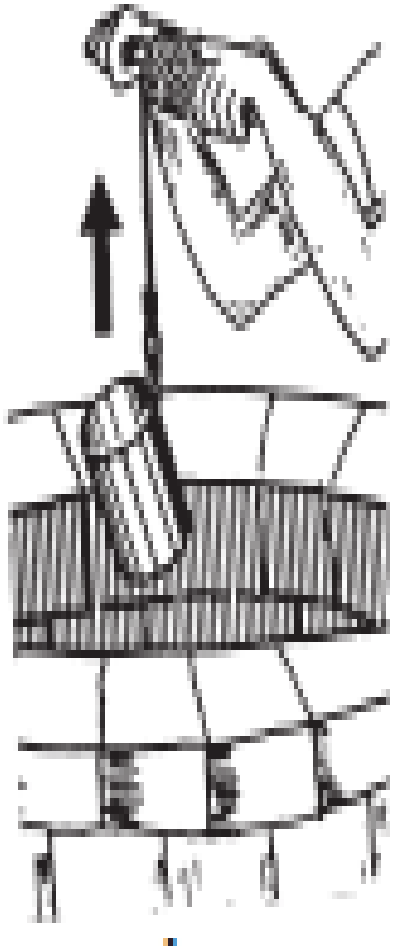
- Whether the water was drawn from a cistern or direct from the main.
- Remove any external fittings from the tap, such as an antisplash nozzle or rubber tube.
- Clean carefully the outside nozzle of the tap, especially any grease which has collected.
- Turn the tap on full, and allow the water to run to waste for 1 minute.
- This allows time for the nozzle of the tap to be flushed and any stagnant water in the service pipe to be discharged.

Sample from well

Sample from a well:

Give details of :

- The well's depth
  - Covered or uncovered
  - Recently constructed or altered

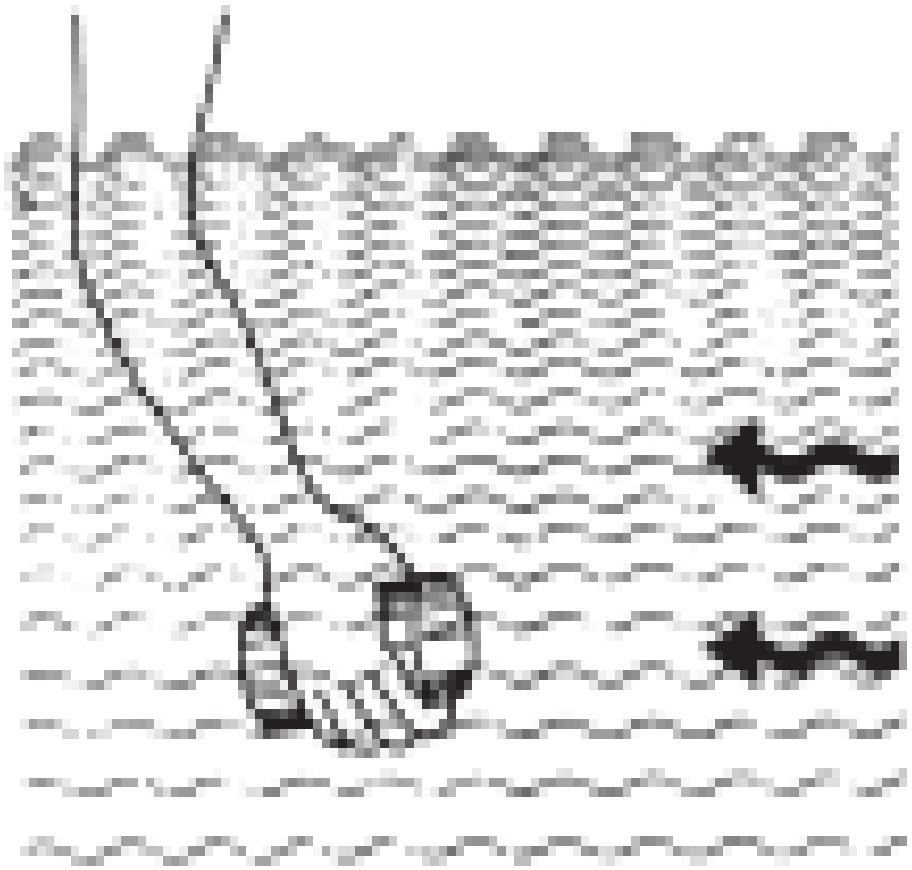


Sample from a river

- **Sample from a river or stream:**

Mention the depth at which the sample will collected

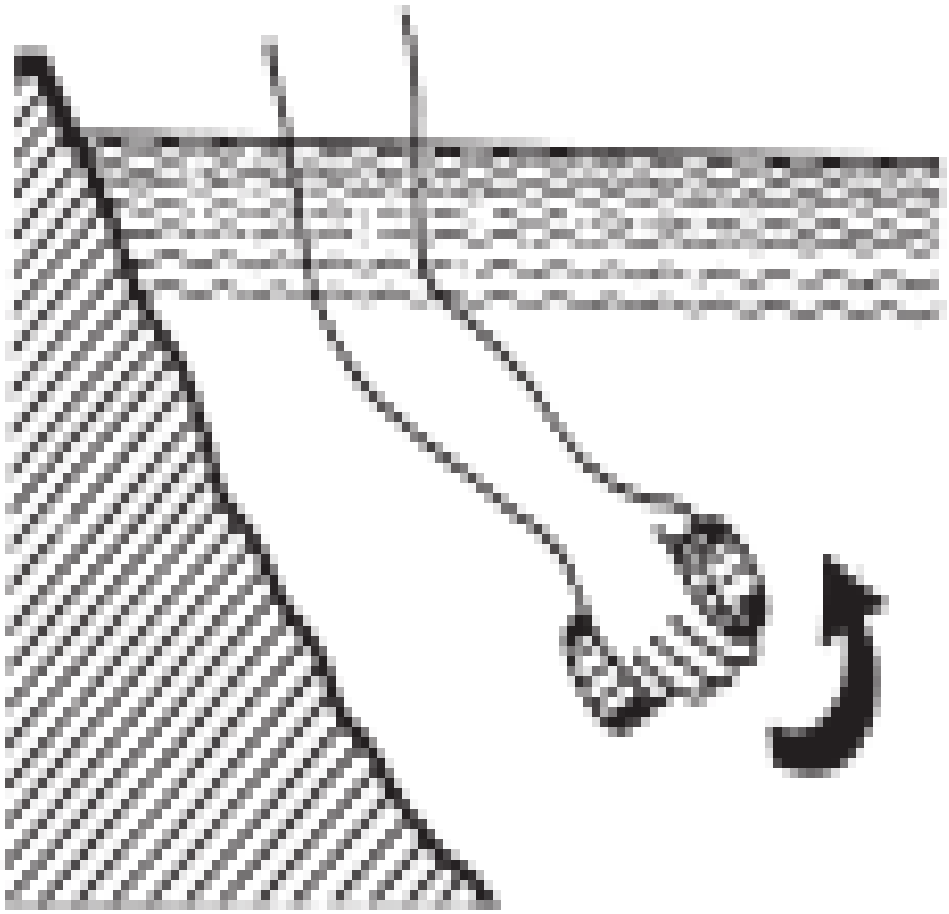
- Whether from the side or the middle of the stream
- Whether the water level was above or below average
- Whether there had been heavy rainfall or flooding.



Sample from a lake or reservoir :

Give the detail of :

- Exact position and the depth
- Temperature of the source
- Possible sources of pollution in the area
- Their approximate distance from the sampling point.
- Date and time when the sample was taken (and dispatched if sent to a testing laboratory).



water sampling con’t..

- Hold the sterile bottle by the base in one hand. Use the other hand to remove the stopper and cover together.
- The stopper and cover should be retained in the hand while the bottle is filled and then they should be replaced together.
- To prevent contamination, the person collecting the water must not touch, or allow any surface to touch, the screw thread of the bottle neck or the inside of the cap.
- If the bottle becomes contaminated, it must not be used.

Transporting water samples to a water testing laboratory

- Immediately after collection, samples should be placed in an insulated cold box for transport to a water testing laboratory.
- Water samples should be examined as soon as possible on arrival and always within 6 hours of collection.
- Whenever possible, process water samples in the field.

Collection, Preparation And Preservation Of Specimens For Serologic tests

- Specimens that are used for serologic test include: serum, plasma and cerebrospinal fluid.
- Collected by the laboratory personnel. physician or trained nurse.

❖**Serum or plasma sample collection procedure:**

- ➢Collect 2-3ml of venous blood from a patient using a sterile syringe and needle.
- ➢If serum is required, allow the whole blood to clot at room temperature for at least one hour,
- ➢Centrifuge the clotted blood for 10 minutes at 2000 rpm.
- ➢Transfer the serum to a labeled tube with a paster pipette and rubber bulb.
- ❖ if there is a need to obtain plasma samples:
- ➢treating fresh blood with anticoagulant,
- ➢Centrifuge and separate the supernatant.

Specimens For Serologic tests con’t…

- The specimen should be free from hemolyzed blood.
- Finally, seal the specimen containing tube; the tube should be labeled with full patient's identification (age, sex, code number, etc).
- The test should be performed within hours after sample collection, if this could not be done preserve it at -20^{o}c.

❖Before shipment, the following things should be considered.

- Don't ship whole blood unless the test to be performed required whole blood.
- Remove cells from plasma and clot from serum before shipment.

Specimens For Serologic tests con’t…

- Don't inactivate serum or plasma before mailing.
- Keep the specimen and packing container in the refrigerator until time of shipment.
- Shipment is requires several days preserve by refrigeration in transit. First, freeze the specimen; then pack and ship in a well-insulated container with dry ice.

Conclusion

- **Pre-Analytical Errors**
  - Insufficient sample
  - Sample condition
  - Sample handling
  - Incorrect Identification
  - Incorrect sample

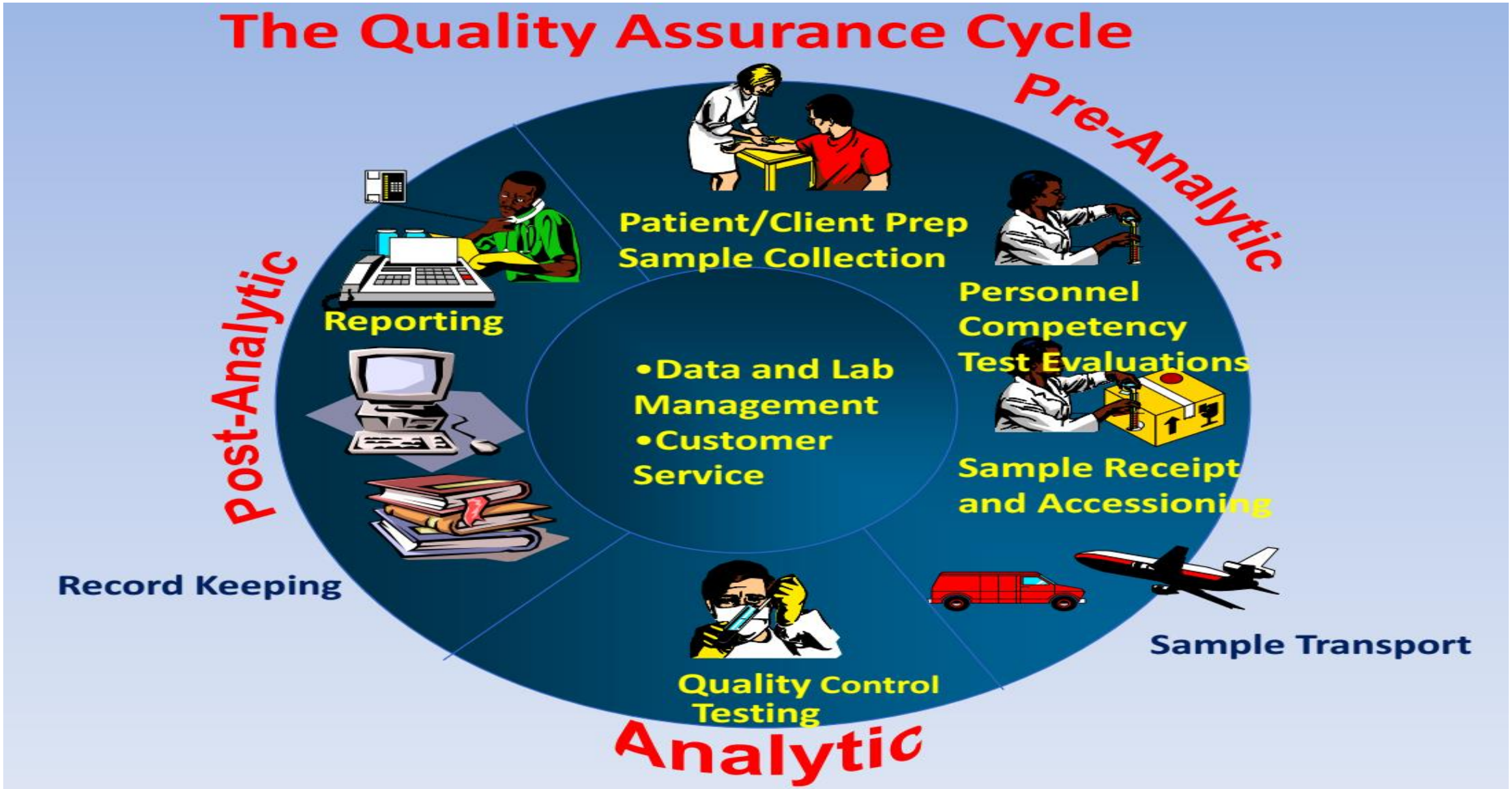
Analytical Errors

- Test System Not Calibrated
- Improper measurements of specimens and/or reagents
- Reagents prepared incorrectly
- Reagents stored inappropriately or used after expiration date
- Instrument maintenance is not accurate
- Dilution and pipetting errors

❖Post-Analytical Error

- Transcription errors in reporting
- Report sent to the wrong location
- Report illegible
- Report not sent

The Quality Assurance Cycle



Thank You

Unit Three:
Microbe Identification Methods

Objectives of the chapter

- **At the end of this lesson, the student will be able:**

- Characterize microorganisms based the:
  - Phenotypic Methods through manually observation on the culture media and microscopic (staining) characteristics.
  - Culturing microorganisms using different culture media such as enrichment, selective or differential media.
  - Identify the species of bacteria using different biochemical tests.
  - Perform drug susceptibility tests and categorize organism as susceptible, intermediate and resistance.
  - Identify Immunological techniques and Molecular (genotypic) techniques.

Phenotypic Characteristics Useful in Classification and Identification of microbes

- **Morphologic Characteristics**

  - Both wet-mounted and properly stained bacterial cell suspensions can yield a great deal of information.
  - These simple tests can indicate the Gram reaction of the organism; whether it is acid-fast; its motility; the arrangement of its flagella; the presence of spores, capsules, and inclusion bodies; and its shape.
  - Colony characteristics and pigmentation are also quite helpful. For example, colonies of several *Porphyromonas* species auto fluoresce under long-wavelength ultraviolet light, and *Proteus* species swarm on appropriate media.

- **Growth Characteristics**

➢ A primary distinguishing characteristic is whether an organism grows aerobically, anaerobically, facultatively (i.e., in either the presence or absence of oxygen), or microaerobically (i.e., in the presence of a less than atmospheric partial pressure of oxygen).

➢ The proper atmospheric conditions are essential for isolating and identifying bacteria.

➢ Other important growth assessments include the incubation temperature, pH, nutrients required, and resistance to antibiotics.

- **Antigens and Phage Susceptibility**

➢ Cell wall (O), flagellar (H), and capsular (K) antigens are used to aid in classifying certain organisms at the species level, to serotype strains of medically important species for epidemiologic purposes, or to identify serotypes of public health importance.

➢ Serotyping is also sometimes used to distinguish strains of exceptional virulence or public health importance, for example with *V cholerae* (O1 is the pandemic strain) and *E coli* (enterotoxigenic, enteroinvasive, enterohemorrhagic, and enteropathogenic serotypes).

- **Biochemical Characteristics**

➢ Most bacteria are identified and classified largely on the basis of their reactions in a series of biochemical tests.

➢ Some tests are used routinely for many groups of bacteria (oxidase, nitrate reduction, amino acid degrading enzymes, fermentation or utilization of carbohydrates); others are restricted to a single family, genus, or species (coagulase test for staphylococci, pyrrolidonyl arylamidase test for Gram-positive cocci).

CLASSIFICATION OF BACTERIA

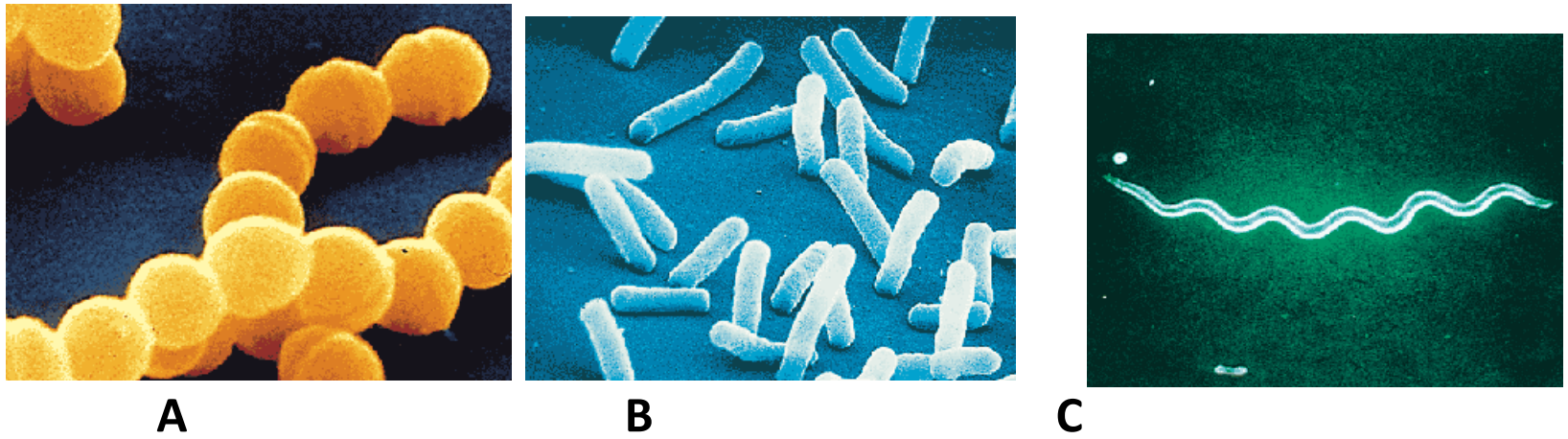
- Bacteria are classified in to 19 different categories in Bergey's manual of determinative bacteriology, 8th (1974), and the classification is based on

1. Morphology
2. Staining
3. Growth
4. Nutritional requirement
5. Bio chemical and metabolic activity
6. Pathogenecity
7. Amino acid sequencing of proteins
8. Genetic composition

Morphology: - Bacteria vary widely in size, ranging from 0.2 um to 10um long

-There are there basic shapes

1. Spherical or coccoid/cocci- (singular –coccus)
2. Rods or bacilli (singular - bacillus)
3. Spirals or spirilla (Singular - Spirillum)



A B C

Fig . Different bacterial morphologies A. Cocci, B. Bacilli, C. Spiral shape

- The cells of cocci may be found in various arrangements depending on the species and the way they divide

e.g – Cocci occurring single:- Micrococcus

- Pairs of cocci- Diplococci
- Cocci in chain - Strepto cocci
- Cocci in cluster- Staphylococci
- Four cocci as in box - Tetrads
- Eight cocci as in box – Octads

✓Bacilli (rods) may be short or long, thick or thin, pointed or with blunt ends,

✓ Some rods resemble cocci and are often called coccobalilli because they are very short small bacilli.

✓Some bacilli stack up next to each other eg. Diphteroids, Some are coma shaped e.g. V.*cholera*

✓Spiralls usually occur singly.

-The different species of spirilla varies in size, length, rigidity, number and amplitude of their coils.

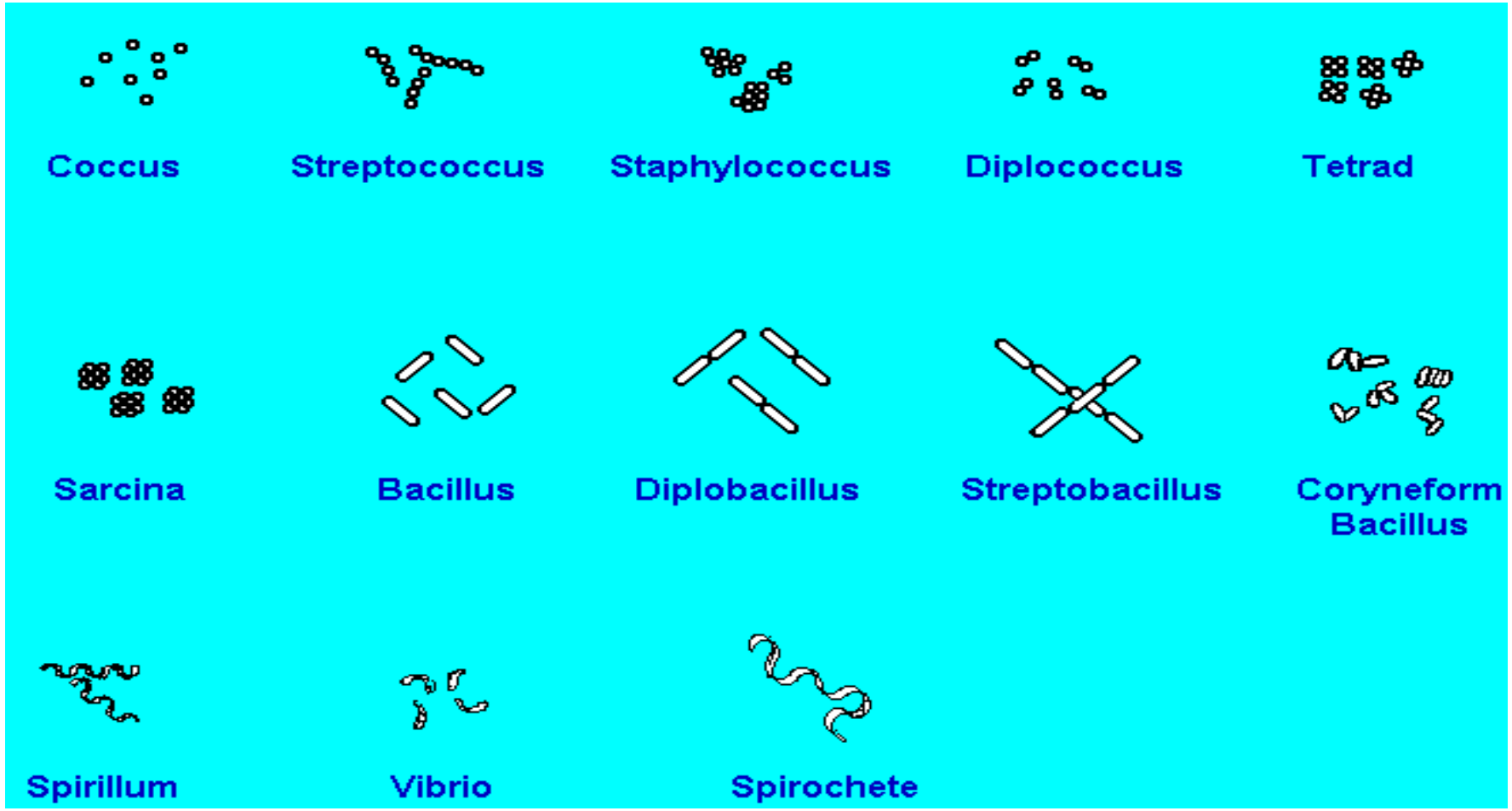


Fig. different bacterial arrangements

BACTERIAL STAINING

Learning objectives

At the end of this chapter, the student will be able to:

1.Define stains and bacterial staining.

2.Describe the uses of staining solutions.

3.List the types of staining methods that are used to stain bacteria.

4.List the different types of bacterial staining techniques.

5.Discuss the principles of Gram’s stain and Ziehl - Nelseen stain.

6.Describe the factors that contribute for false Gram’s stain and Zihel -Nelseen stain results.

7.Prepare the Gram’s stain and Zihel -Nelseen stain reagents.

Bacterial staining con't…

Definition

- Stains (Dyes) are coloured chemical compounds that are used to selectively give (imprat) colour to the colourless structures of bacteria or other cells.
- Bacterial staining is the process of imparting colour to the colourless structures (cell wall, spore, etc) of the bacteria in order to make it visible under the microscope.

Uses:

1. To observe the morphology, size and arrangement of bacteria

2. To differentiate one group of bacteria from the other group.

Staining reactions are made possible because of the Physical phenomena of capillary osmosis, solubility, adsorption, and absorption of stains or dyes by cells of micro-organisms.

Bacterial staining con't…

- Individual variation in the cell wall constituents among different groups of bacteria will consequently produce variations in colours during microscopic examination.
- Nucleus is acidic in character and hence, it has greater affinity for basic dyes. Whereas, cytoplasm is basic in character and has greater affinity for acidic dyes.
- Why are stains not taken up by every micro-organism?

❖**Factors controlling selectivity of microbial cells are:**

1. Number and affinity of binding sites
2. Rate of reagent uptake
3. Rate of reaction
4. Rate of reagent loss (differentiation or regressive staining)

Bacterial staining con't…

Why dyes colour microbial cells?

Because dyes absorb radiation energy in visible region of electromagnetic spectrum i.e., "light"(wave length 400-650). And absorption is anything outside this range it is colourless.

e.g., acid fuschin absorbs blue green and transmit red.

Type of stain in Microbiology

There are three kinds of stains

1. Basic stains
2. Acidic stains
3. Neutral stains

NB: This classification is not based on the p^H of stains

Bacterial staining con't…

A. Basic Stains

- Are stains in which the colouring substance is contained in the base part of the stain and the acidic part is colourless. The bacterial cells can easily stain with basic dyes. E.g. Methylene blue stain, Safranin, Genetian violet, Carbolfuchsin etc

B. Acidic Stains

Are stains in which the colouring substance is contained in the acidic part of the stain and the base part is colourless. Acidic dyes are mainly used in histology laboratory. It is not commonly used in microbiology laboratory ; e.g. eosin.

C. Neutral Stains

- Are stains in which the acidic and basic components of stains are coloured.
- Neutral dyes stain both nucleic acid and cytoplasm. these stains are commonly used in hematology laboratory. e.g Giemsa's stain, Wright's stain.

Bacterial staining con’t…

Type of staining methods

- Simple staining method
- Differential staining method
- Special staining method

Bacterial staining con’t…

1. Simple staining method

- It is a type of staining method in which only a single dye is used.
- There are two kinds of simple staining methods

A. Positive staining

B. Negative staining

A. Positive staining: The bacteria or its parts are stained by the dye. e.g. Methylene blue stain, Crystal violent stain

Bacterial staining con’t…

Procedure

- Make a smear and label it
- Allow the smear to dry in air
- Fix the smear over a flame
- Apply a few drops of positive simple stain
- Wash off the stain with water
- Air -dry and examine under microscope
- Result-all the bacterial surface is stained.

Bacterial staining con't…

B. Negative staining-

The dye stains the background and the bacteria component remain unstained. e.g. Indian ink stain.

2. Differential staining method

A method in which multiple stains (dye) are used to distinguish different group of bacteria. e.g. Gram's stain, Ziehl-Neelson stain.

Gram's stain

This method was developed by the Danish bacteriologist Christian Gram in 1984.

Basic concepts:

- Most bacteria are differentiated by their gram reaction due to differences in their cell wall structure.
- The surface of bacterial cell has got a negative charge due to the presence of polysaccharides and lipids (PG) this has made the surface of the bacteria to have affinity to cationic or basic dyes (when the colouring part is contained in the basic part.)

Bacterial staining con't…

Principle

- The principle of Gram's stain is that cells are first fixed to slide by heat or alcohol and stained with a basic dye (e.g. crystal violate), which is taken up in similar amounts by all bacteria. The slides are then treated with an Gram's iodine (iodine KI mixture) to fix (mordant) the crystal violet stain on Gram positive bacteria, decolorized with acetone or alcohol, and finally counter stained with Safranin.

Required reagents:

- Crystal violet
- Gram's Iodine
- Acetone-Alcohol
- Safranin

Gram's stain reagents preparation

1.Crystal violet stain

- Crystal violet ……. 20g
- Ammonium oxalate …… 9g
- Ethanol or methanol absolute …….. 95ml
- Distilled water …….. to 1 litre

2. Gram's iodine

- Potassium iodide …….. 20 g
- Iodine …… 10 g
- Distilled water …… to 1 litre

Should be stored in a brown bottle

Bacterial staining con't…

3. Acetone -alcohol

➢To make 1 litre

✓Acetone(500ml), Ethanol or methanol absolute (475 ml) and Distilled water (25ml)

4. Safranin

1.Prepare a stock solution

➢Safranine O (2.5g) and Ethanol (95%) (100ml)

➢Mix until all the safranine is dissolved. Transfer the solution to a glass – stoppered bottle.

➢Label the bottle (Safranine stock solution) and write the date.

2. Prepare a working solution in a glass stoppered bottle

- Stock solution ----------10ml
- Distilled water ----------90ml
- Label the bottle (Safranine working solution) and write the date

Bacterial staining con't…

Procedure

1. Prepare smear from specimen or culture.
2. Allow the smear to air-dry.
3. Rapidly pass slide three times through flame.
4. Cover fixed smear with crystal violet for one minute and wash with tap water.
5. Tip off the water and cover the smear with Gram's iodine for one minute.
6. Wash off iodine solution with tap water.
7. Decolorize with acetone-alcohol for 30 seconds.
8. Wash off the acetone-alcohol with clean water.
9. Cover the smear with safranin for one minute.
10. Wash off the stain and wipe the back of the slide. Let the smear air-dry.
11. Examine the stained smear with oil immersion objective to look for bacteria.

Bacterial staining con't…

Results

- Gram positive bacteria …….. purple
- Yeast cells …….. Dark purple
- Gram negative bacteria …….. Pale to red
- Nuclei of pus cell …….. Red
- Epithelia cells …….. Pale red

Bacterial staining con't…

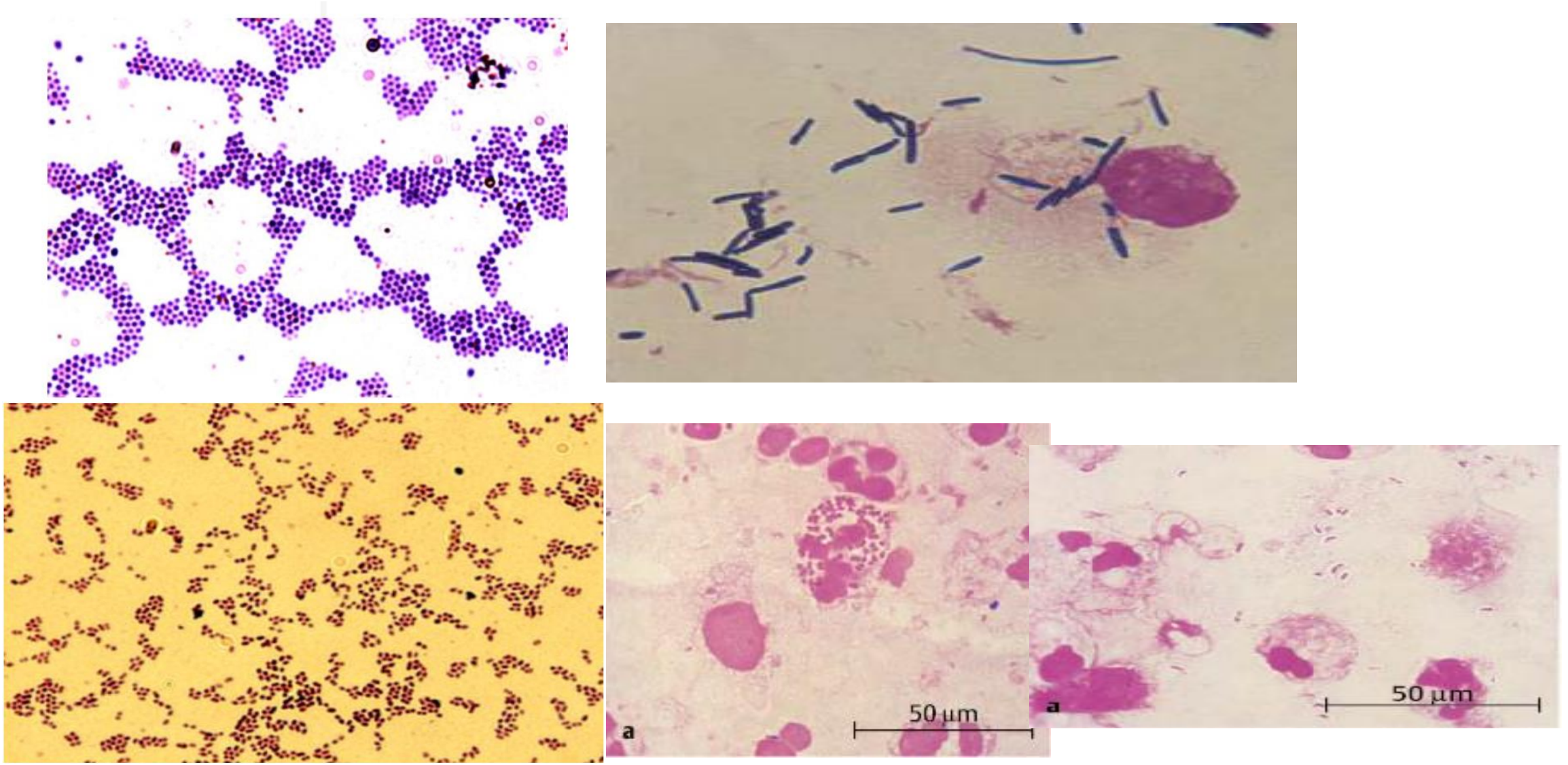


Figure---. **Gram positive and Gram negative bacteria**

Bacterial staining con't…

Reporting of Gram's stained smear

The report should include the following:

1.Number of bacteria present whether many, moderate, few or scanty

2. Gram reaction of the bacteria whether Gram positive or Gram negative

3. Morphology and arrangement of the bacteria whether cocci**,** diplococci,streptococci, rods, or coccobacili, also whether the organisms are intracellular.

4. Presence and number of pus cells.

5. Presence of yeast cells and epithelia cells.

Factors which contribute to false Gram’s staining result

I. False Gram positive reaction

- Preparation of too tick smear – the stain will not be fully washed.
- Application of crystal violet -presence of sediment in crystal violet -this can be avoided by filtering the stain before use.
- Fixing before drying brings alteration of cell wall and morphology.
- Decolourizing time - when insufficient decolourization time is used or (when not fully decolorized).

II. False Gram Negative reactions

- Making smear from old culture.
- Cell wall damage due to antibiotic.
- Excessive heat fixation of smear.
- Over decolourization of smear (decolourization for longer time).
- Use of an iodine solution which is too old (i.e. yellow in stead of brown in colour) (its mordant effect will be decreased).

B. Ziehl-Neelson (Acid fast) staining method

- Developed by Paul Ehrlich in1882, and modified by Ziehl and Neelson
- Ziehl-Neelson stain is used for staining mycobacteria which are hardly stained by Gram‘s staining method.
- Once the Mycobacteria is stained with primary stain it can not be decolorized with acid, so named as acid-fast bacteria.

Bacterial staining con't…

Principle

- Sputum smear is heat –fixed, flooded with a solution of carbilfusin (a mixture of basic fuschin and phenol) and heated until steam rises. The heating allows melting of cell wall of mycobacteria which facilitate entrance of the primary stain into the bacterium.
- After washing with water, the slide is covered with 3% HCL (decolourizer). Then washed with water and flooded with methylene blue (*Mycobacterium tuberculosis)* and malachite green (*Mycobacterium leprae*).

Required reagents

- Carbol-fuchsin
- Acid-Alcohol
- Methylene blue/Malachite green

Bacterial staining con’t…

Ziehl Neelson stain reagents preparation

A. Carbol fuscin

Solution A (saturated solution of basic fuchsin)

Basic fuchsin (3gm) and Ehanol (100ml)

Solution B (phenol aquous solution, 50g/L (5%)

Phenol (10gm) and Distilled water (200ml), Mix 10 ml of solution A with 90ml of solutin

B.Transfer resulting mixture to a glass stopperd bottle and label.

B. Acid –alcohol

Hydrocloric acid (conc)(3ml) and Ehanol (97ml)

Label the bottle.

C. Methylene Blue Stain (Loeffler's)

Solution A

- Methylene blue (90% dye content)----------0.3g
- Ethanol (95%) ----------30ml

Solution B

- Diluted potassium hydroxide (0.01%) ----------100ml

Mix solutions A and B and store in a brown bottle.

D. Malachite green

Malachite green …… 1gm

Distilled water …… 100ml

Using a pestle and mortar, grind the malachite green crystal to a powder. Dissolve the grind powder I 100ml distilled water and store in a dark brown bottle.

Bacterial staining con’t…

Procedure

1. Prepare the smear from the primary specimen and fix it by passing through the flame and label clearly

2. Place fixed slide on a staining rack and cover each slide with concentrated carbol fuchsin solution.

3. Heat the slide from underneath with sprit lamp until vapor rises (do not boil it) and wait for 3-5 minutes.

4. Wash off the stain with clean water.

5. Cover the smear with 3% acid-alcohol solution until all color is removed (two minutes).

6. Wash off the stain and cover the slide with 1% methylene

Blue for one minute.

7. Wash off the stain with clean water and let it air-dry.

8. Examine the smear under the oil immersion objective to look for acid fast bacilli

Bacterial staining con’t…

Results

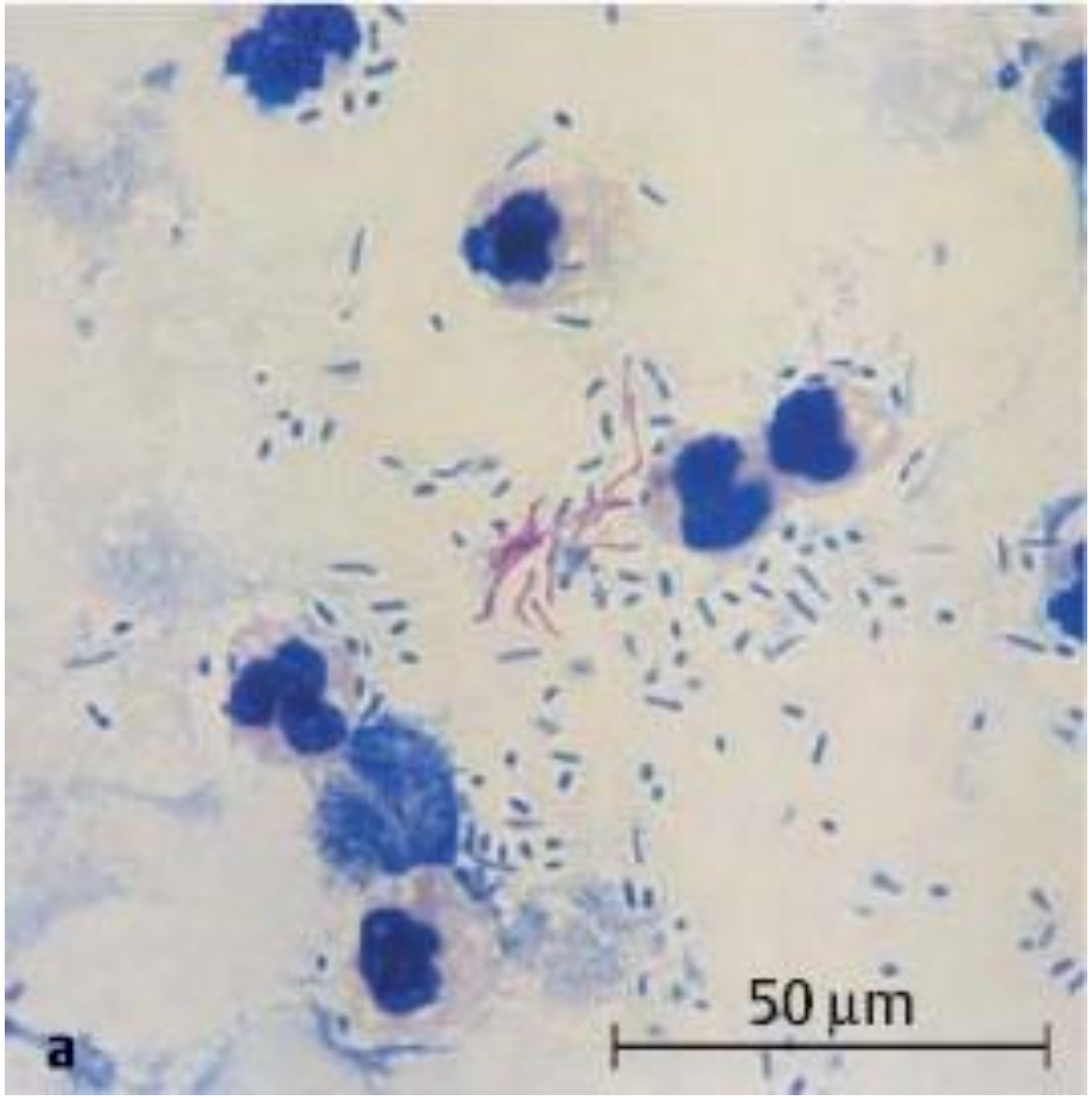
- AFB..... Red, straight or slightly curved rods, occurring single or in a small groups
- Cells..... Blue
- Background Material ……. Blue

Bacterial staining con't…

Reporting system

- 0 AFB/100 field ……No AFB seen
- 1-2 AFB/ 300 field……..(±) or scanty
- 1-9 AFB/100 field……1+
- 1-9AFB/10 field……2+
- 1- 9AFB/field…… ……...3+
- >9 AFB/field…… ……4+

N.B: AFB means number of acid fast bacilli seen.



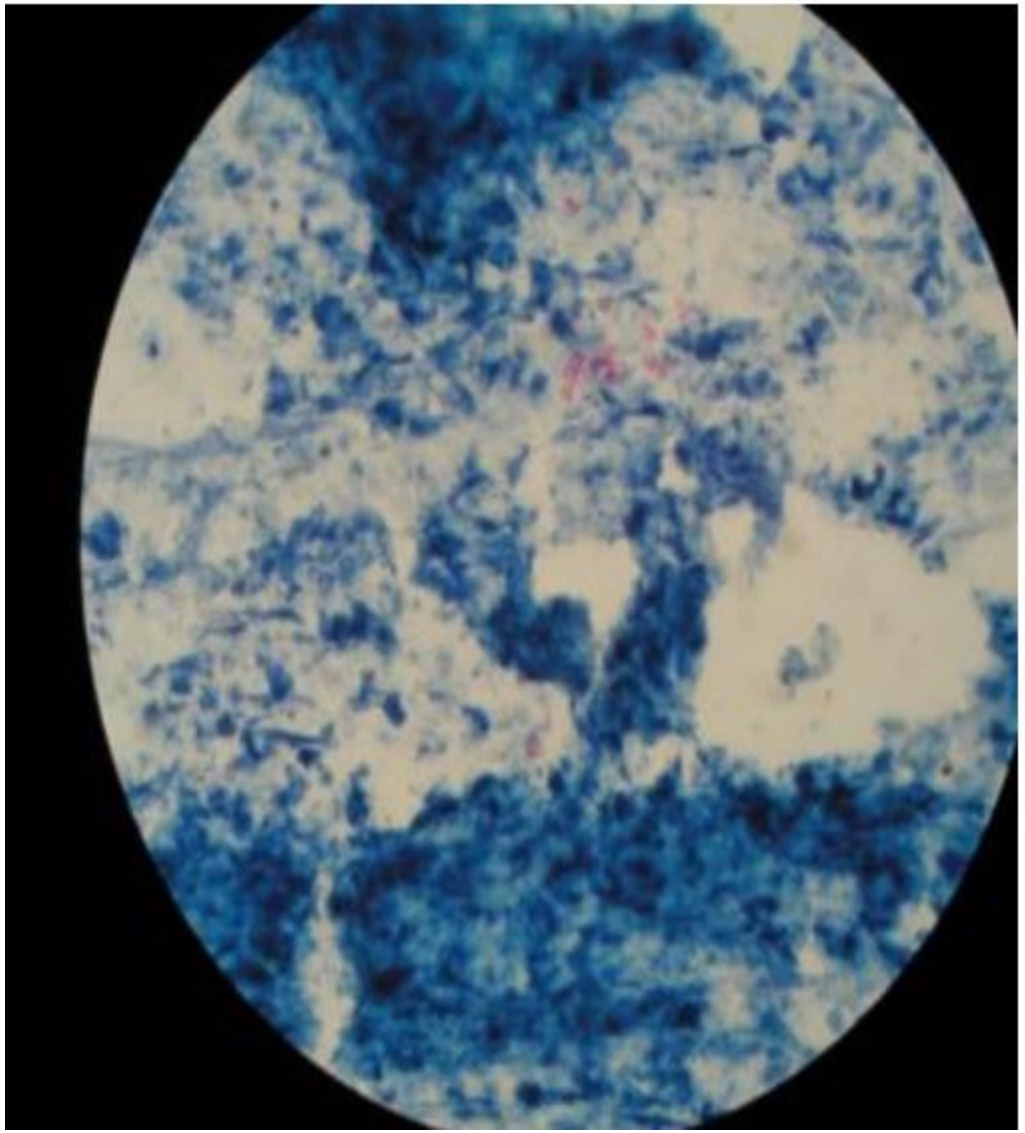


Figure----. AFB under the microscope

Bacterial staining con’t…

Factors which bring false positive results

- Using scratched slides on which deposits of stain may look like bacilli.
- Using unfiltered carbol fuchsin which may contain crystals .
- Carelessness in heating the carbol fuchsine, allowing it to dry and crystallize on the smear.
- Inadequate decolorizing of the smear.

Bacterial staining con’t…

3. Special Staining method

- These are stains, which are used to stain capsules and spores.

There are two types of special staining methods

A. Capsule Staining method

B. Spore staining method

Bacterial staining con't…

A. Capsule staining method

- This technique is used for showing the presence of capsules around bacteria

Required reagents

- Crystal Violet ……..10g/l (1% w/v) stain
- Copper sulphate……200g/l (20%w/v)

Bacterial staining con’t…

Procedure

1. Fix the direct smear using alcohol

2. Cover the smear with crystal violet stain and heat gently until the steam begins to raise and leave to stain for one minute.

3. Wash off the stain with the copper sulphate solution (don’t use water).

4.Wipe the back of the slide and place it in a draining rack to air dry.

5. Examine the smear microscopically using oil immersion objective to look for capsulated bacteria.

Result

- Bacterial cell ---------- dark purple
- Capsule outline ---------- Pale blue

B. Spore staining method

- The primary dye malachite green is a relatively weakly binding dye to the cell wall and spore wall. In fact, if washed well with water, the dye comes right out of the cell wall. That is why there does not need a decolourizer in this stain.
- It is based on the binding of the malachite green and the permeability of the spore vs. cell wall.
- The steaming helps the malachite green to permeate the spore wall.

Bacterial staining con’t…

Procedure

1. Prepare smear of the spore-forming bacteria and fix in flame.

2. Cover the smear with 5% malachite green solution and heat

over steaming water bath for 2-3 minutes.

3. Wash with clean water.

4. Add1% safranine for 30 seconds.

5. Wash with clean water.

6. Dry and examine under the oil immersion objective.

Bacterial staining con't…

The cold method spore stain

- Without heat you have to really rough up the spore wall to get in the dye.
- Heat fix the smear by running the slide through the flame about 20 times, and leave malachite green on for 20 minutes during the stain process.

Interpretation

- Spores ……light green
- Vegetative cell walls will pick up the counter stain safranin…… ……Red

PREPARATION OF CULTURE MEDIA, INOCULATION AND IDENTIFICATION OF BACTERIAL GROWTH CHARACTERISTICS

At the end of this lesson, the student will be able:

1.List the common ingredients of culture media.

2.List the different types of culture media.

3.Discuss the different steps while preparing culture media.

4.Describe the storage methods for culture media.

5.Discuss the inoculation and aseptic techniques during inoculation of culture media.

6.List the different inoculation techniques.

7.Discuss the culturing of anaerobes.

8.Perform quality control on culture media.

Culture con't…

❖Culture media are artificially prepared media containing the required nutrients used for propagation of micro-organisms or living tissue cells.

❖**Uses**

- Isolation and identification of micro-organisms
- Performing anti-microbial sensitivity tests

❖**Common ingredients of culture media**

➢H2O
➢Peptone
➢Meat extract
➢Yeast extract
➢Mineral salts
➢Carbohydrates
➢Solidifying agents
➢Accessory growth factors

Types of culture media

The main types of culture media are:

1. Basic media

2. Enriched or enrichment media

3. Selective media

4. Indicator (differential) media

5. Transport media

1. Basic Media

These are simple media that will support the growth of micro-organisms that do not have special nutritional requirements.

Example - Nutrient Agar
- Nutrient broth

Purposes of basic media

1. Are used in preparation of enriched media.

2. Are used to maintain stock culture.

2. Enriched

- These media are required for growth of organism with extra nutritional requirements such as *H. influenza*, *Neisseria spp*, *and some streptococcus* species.
- An enriched medium increases the number of a pathogen by containing all the necessary ingredients to promote its growth.
- The media can be enriched with whole blood, lyzed blood, serum, vitamins, and other growth factors.

Example: - Blood Agar (contain whole blood)

- Chocolate agar (contain lyzed blood)

3. Enrichment media

- Fluid medium that increases the number of a pathogen by containing enrichments and/ or substances that discourage multiplication of unwanted bacteria.

Example:

- Selenite Faecal (F) broth is used as an enrichment medium for Salmonellae in faeces or urine prior to subculture.
- Tryptosoya broth supports the growth fastidious organisms such as Neisseriae and Pnemococci.

4. Selective media

- These are media which contain substances that prevent or slow down growth of micro-organisms other than pathogen for which the media are intended.
- The medium is made selective by incorporation of certain substances like bile salt, crystal violet, antibiotics, etc.
- Selective medium is used when culturing a specimen form a site having normal microbial flora to prevent unwanted contaminants overgrowing a pathogen.

Example:.

1.Thiosulphate citrate bile salt sucrose agar(TCBS) - is alkaline medium and selective for V. cholera.

2. Xylose Lysine Deoxy Cholate (XLD) agar - selective for Salmonella and Shigella.

3. Modified New York City (MNYC) medium - Selective for *Neisseria gonorrhoeae.*

4. Butzler medium - Selective for Campylobacter species.

5. Salmonella and Shigella (SS) agar - Selective for Salmonella and Shigella species.

5. Differential (Indicator) media

- These are media to which dyes or other substances are added to differentiate micro-organisms.
- Many differential media distinguish between bacteria by incorporating an indicator which changes colour when acid is produced following fermentation of a specific carbohydrate.

Example :

- ➢Mac Ckonkey agar - contain neutral red as an indicator and lactose as carbohydrate.
- ➢Lactose fermenting bacteria will become pink and other bacteria become colourless.

6. Transport media

- These are mostly semisolid media that contain ingredients to prevent the overgrowth of commensals and ensure the survival of aerobic and anaerobic pathogens when specimens cannot be cultured immediately after collection.
- Their use is particularly important when transporting microbiological specimens form health centres to the district microbiological laboratory or regional public health laboratory.

Example

1.Cary-Blair medium :– is used for preserving and transporting enteric pathogens.

2.Amies transport medium:- is used for transportation of gonococci.

Culture con’t…

Culture media can be classified by their consistency (form) as:

- **Solid media-** Are solidified by agar, mainly in Petri dishes as plate cultures, in bottles or tubes as stab (deeps) or slope cultures.
- **Semi-solid media-** small amount of agar (0.4 - 0.5% W/V) to a fluid medium, mainly as transport media, and for motility and biochemical tests.
- **Fluid media-** Is used for biochemical testing as enrichment media and blood culture. e.g. Peptone water.

Preparation of culture media

Steps for the preparation of culture media

1. Weighing and dissolving of culture media
2. Sterilization and sterility testing
3. Addition of heat sensitive ingredients
4. p^H testing of culture media
5. Dispensing of the culture media

6. Quality assurance of culture media
7. Storage of culture media

Culturing of bacteria

Materials for Culturing Bacteria

Basic materials used for culturing bacteria.

- Culture media
- Petri dishes
- Test tubes
- Inoculating loops, straight wire loops
- Bunsen burner
- Incubator

Culture con’t…

❖Depending on the characteristics and nutritional requirement, bacteria can be inoculated on different culture media hence the choice of culture media for inoculation of samples depends on:

1.The major pathogens to be isolated, their growth requirements, and the features by which they are recognized.

2. Whether the specimens being cultured are from sterile sites or from having normal microbial flora.

➢Although a selective medium is usually more expensive than a non selective one, it often avoids sub culturing , isolates a pathogen more quickly, and makes it easier to differentiate and interpret bacterial growth.

➢Cost, availability, and stability of different media in tropical countries.

➢Training and experience of laboratory staff in preparing, using, and quality controlling culture media.

Inoculation of culture media

- Inoculation: - is artificial seeding or introduction of micro organisms on/in to culture media or animal body.
- When inoculating culture media, an aseptic technique must be used to prevent contamination of specimens and culture media, and laboratory worker and the environment.
- Immediately before inoculating a culture medium, check the medium for visual contamination or any change in its appearance medium for visual contamination or any change in its appearance which may indicate deterioration of the medium.

Example. Darkening of the medium and any growth of micro organisms.

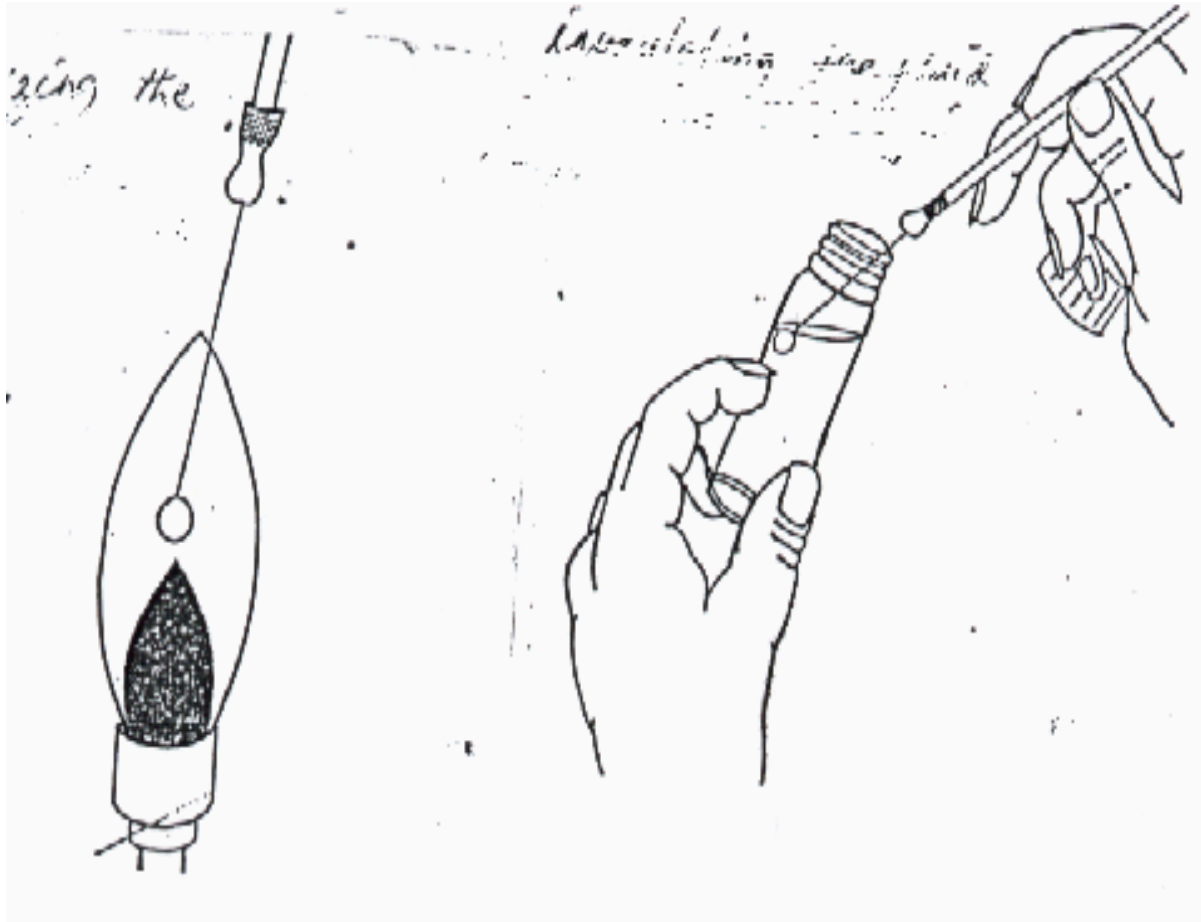
❖The inoculation of media in Petri dishes is referred to as ‘**plating out**’ or ‘**looping out**’.

Aseptic technique during inoculation of culture media

- Decontaminate the workbench before and after the work of the day.
- Use facemask and gloves during handling highly infectious specimens.
- Flame sterilize wire loops, straight wires, and metal forceps before and after use.
- Flame the neck of specimen and culture bottles, and tubes after removing and before replacing caps and plugs.

Culture con’t…

Sterilizing the inoculating loop with flame



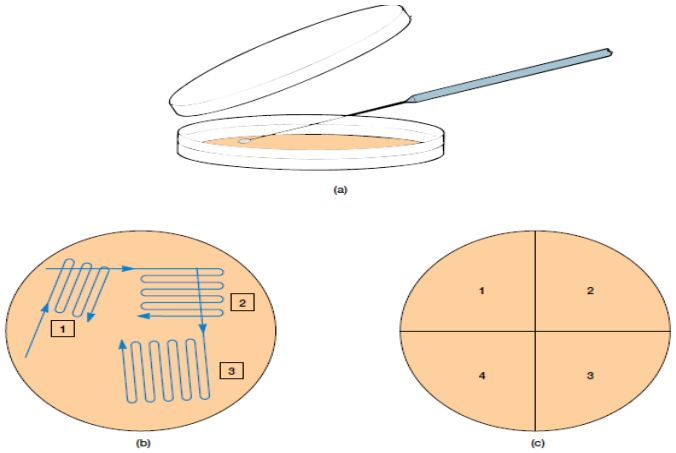
Inoculating the fluid media with sterilized loop

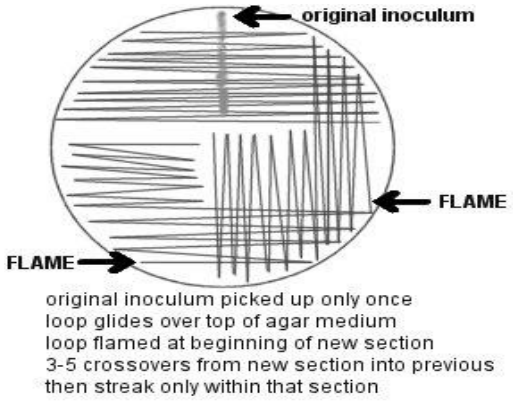
Figure. ----- **Aseptic inoculation technique**

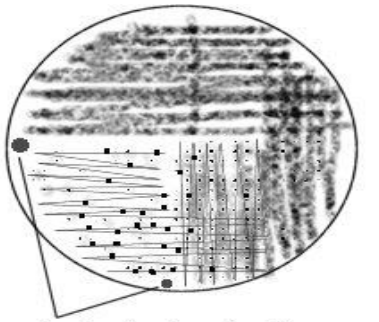
- **Inoculating Techniques**

1.Using a sterile loop or swab of the specimen, apply the inoculation to a small area of the plate.

2. Flame sterilize the loop, when cool or using a second sterile loop, spread the inoculation systematically. This will ensure single colony growth







Inoculation of Slopes

To inoculate slopes (such as Dorset egg medium or TSI) use a sterile straight wire to streak the inoculation down the center of the slope and then spread the inoculation in a zigzag pattern as shown in the figure.

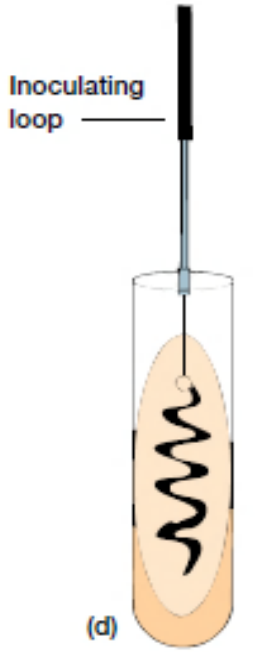


Figure. … Inoculation of Slopes

Inoculation of Stab Media (deeps)

Use a sterile straight wire to inoculate a stab medium. Stab through the centre of the medium taking care to with draw the wire along the line of inoculation with out making further stab lines.

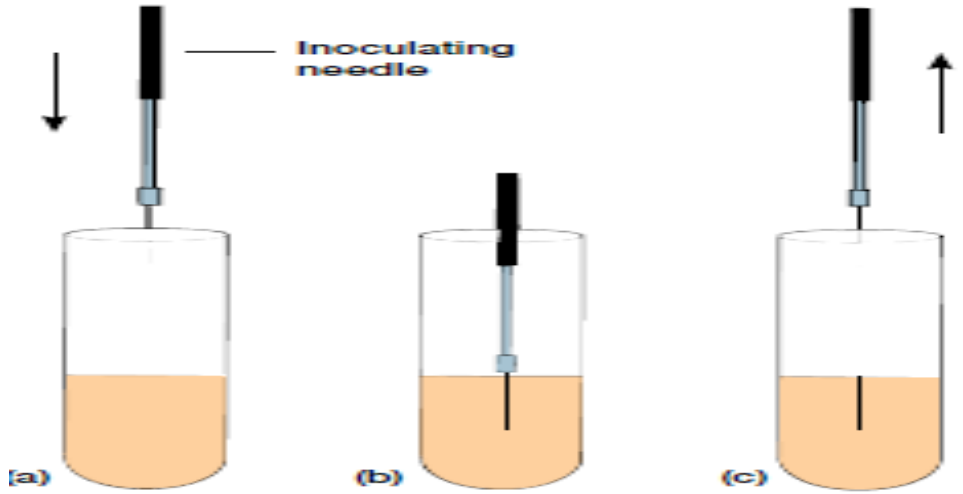


Figure. **… Inoculation of a deep (stab)**

Inoculation of fluid media

- Broths and other fluid media are inoculated using a sterile wire loop, straight wire, or pasture pipette depending on whether the inoculums is a colony, a fluid culture or a specimen.
- When using a wire loop, hold the bottle or tube at an angel and rub the loop against the side of the container below the level of the fluid.

Incubation of inoculated media

- Inoculated media should be incubated as soon as possible. A delay in incubation can affect the viability of pathogens.
- Micro organisms require incubation at the temperature, humidity & gaseous atmosphere most suited for their metabolism.

Appearance of Bacterial colonies on solid Media

Bacterial colonies should be examined in a good light and a low power magnifying lens can help to see morphological details.

1. When viewed form above: colonies may appear round, irregular crenated, or branching.

They may be transparent or opaque and their surface may be smooth or rough, dull or shiny. Eg. The colonies of pneumococci have a ringed appearance.

Culture con’t…

2. When viewed form the side: Colonies may appear flat or raised in varying degrees some times with beveled edges or with a central elevation or depression.

3. When touched with wire loop: Some colonies are soft and easily emulsified such as *S.aureus.* Where as others are difficult to break up such as *S. pyogens.*

4. The colour of colonies: this also helps to identify bacteria, especially when using differential media containing indicators.

Example- *V. cholera* in TCBS agar appear Yellow

- *Corny bacterium diphteriae* in Tellurite agar appear black.

Reporting culture results

The manner of reporting culture depends on whether the specimen is either from a sterile site or from site with a normal microbial flora.

Sites normally Sterile: Identify and report all bacteria isolated up to their genera. And if helpful identify the actual species using bio chemical tests. Sterile sites include blood, bone marrow, CSF, pleural and peritoneal fluids.

Sites Having a Microbial Flora:

- Interpretation requires patients' clinical data to judge whether an isolate is a pathogen which causes the patients illness.
- The laboratory report should indicate those organisms for which isolation technique has been performed. For example when

the pathogen have been isolated from feacal specimen cultured on selective medium like SSA (salmonella shigela agar). The report should state as 'no salmonella or shigella organism

isolated'.

Reporting culture results

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Culture con’t…

Sterility testing

1. Contamination by micro organism capable of over night growth will be shown by turbidity in a fluid medium and growth on or in a solid medium.
2. The simplest way to test for contamination is to incubate the prepared sample media at 35-37 ^{O}C for 24 hours. Turbidity in fluid media and microbial growth in solid media confirm contamination.

BIOCHEMICAL TESTS

At the end of this chapter the students will be able to:

- List the types of biochemical tests
- Describe the principles of biochemical tests
- List the required materials for the biochemical tests
- Describe the procedures of biochemical tests
- Identify test result and positive and negative control bacteria for the specific biochemical tests
- Discuss the principle, material required, procedures and interpretation of API-20E test strips used to identify the enteric gram negative rods

Introduction

- Biochemical tests are used to differentiate different organisms based on their genus and species characteristics.
- Biochemical tests are performed on pure culture.
- The following are some of the common biochemical tests used for differentiation of different bacteria.

1. Catalase test

This test is used to differentiate those bacteria that produce the enzyme catalase such as staphylococci from non catalase producing bacteria such as streptococci.

Principle:

- Catalase acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water.
- An organism is tested for catalase production by bringing it in to contact with hydrogen peroxide.
- Bubbles of oxygen are released if the organism is a catalase producer. The culture should not be more that 24 hour old.

Material Required

- Hydrogen peroxide,
- 3% H_2O_2 (10 volume solution)
- Test tubes

Biochemical tests con't…

Method

- Pour 2-3 ml of the hydrogen peroxide solution into a test tube.
- Using a sterile wooden stick or a glass rod (not a nichrome wire loop), remove several colonies of the test organism and immerse in the hydrogen peroxide solution.
- Look for immediate bubbling.
    - *Important:* Care must be taken when testing an organism cultured on a medium containing blood because catalase is present in red cells. If any of the blood agar is removed with the organism, a false positive reaction may occur.

Results

- Active bubbling ---------- Positive test- Catalase produced
- No release of bubbles ---------- Negative test- No catalase produced

Biochemical tests con't…

Note:

- if the organism has been cultured on an agar slope, pour about 1ml of the hydrogen peroxide solution over a good growth of the organism, and look for the release of bubbles.
- When the rapid slide technique is used, the hydrogen peroxide solutions should be added to the organism suspension after placing the slide in a petridish. The dish should then be covered immediately, and the preparation observed for bubbling through the lid.
- **Control**
  - Positive catalase control – staphylococcus species
  - Negative catalase control – streptococcus species

2. Coagulase Test

This test is used to differentiate the bacteria which produces the enzyme coagulase such as Staphylococcus aureus , from S.epidermidis and S.saprophyticus which do not produce coagulase.

Principle

- Coagulase causes plasma to clot by converting fibrinogen to fibrin.
- Two types of coagulase are produced by most strains of S.aureus.

❖**Free Coagulase** which converts fibrinogen to fibrin by activating a coagulase-reacting factor present in plasma.

- Free coagulase is detected by the appearance of a fibrin clot in the tube test.

Biochemical tests con't…

❖**Bound coagulase** (clumping factor) which converts fibrinogen directly to fibrin without requiring a coagulase reacting factor.

- It can be detected by the clumping of bacterial cells in the rapid slide test.
- It is usually recommended that a tube test should be performed on all negative slide tests.
- A tube test must always be performed if the result of the slide test is not clear, or when the slide test is negative and the Staphylococcus has been isolated from a serious infection.
- Before performing a coagulase test, examine a Gram stained smear to confirm that the organism is a Gram positive coccus.

Material Required

- EDTA anticogulated human plasma.
    - The plasma should be allowed to warm to room temperature before being used.
- Oxalate or heparin plasma can also be used.
- Do not use citrated plasma because citrate-utilizing bacteria e.g. Enterococci, *Pseudomonas* and *Serratia* may cause clotting of the plasma (in tube test).
- Occasionally, human plasma may contain inhibition substances which can interfere with coagulase testing. It is therefore essential to test the plasma using a known coagulase positive S. aureus.
- The plasma can be stored frozen in amounts ready for use.

Slide test method (detects bound coagulase)

- Place a drop of distilled water on each end of a slide or on two separate slides
- Emulsify a colony of the test organism (previously checked by Gram staining) in each of the drops to make two thick suspensions.

• **Note:** Colonies from a mannitol salt agar culture are not suitable for coagulase testing. The organism must first be cultured on nutrient agar or blood agar.

- Add a loopful (not more) of plasma to one of the suspensions, and mix gently.
- Look for clumping of the organisms within 10 seconds.

• **Note:** plasma is added to the second suspension. This is used to differentiate any granular appearance of the organism from true coagulase clumping

Biochemical tests con't…

Results

- Clumping within 10 seconds……S. aureus
- No clumping within 10 seconds … ……No bound coagulase

Control

- Positive coagulase control ……*Staphylococcus aureus*
- Negative coagulase control …… *E.coli* or *Staph epidermides*

3. Oxidase test/Cytochrome oxidase test

- The oxidase test is used to detect bacteria that produce the enzyme cytochrome oxidase which catalyze oxidation of reduced cytochrome by oxygen molecule
- It assist in the identification of *Pseudomonas*, *Neisseria*, *Vibrio*, *Brucella*, *and pasteurella species*, which are oxidase positive.

Principle

- A piece of filer paper is soaked with a few drops of oxidase reagent.
- A colony of the test organism is then smeared on the filter paper.
  - Alternatively an oxidase regent strip can be used.
- When the organism is oxidase-producing, the phenylenediamine in the reagent will be oxidized to a deep purple colour.
- Occasionally the test is performed by flooding the culture plate with oxidase reagent but this technique is not recommended for routine use because the reagent rapidly kills bacteria. It can however be useful when attempting to isolate *N.gonorrhoeae* colonies from mixed cultures in the absence of a selective medium.
- The oxidase positive colonies must be removed and subcultured within 30 seconds of flooding the plate.

Biochemical tests con't…

Material Required

- Fresh Oxidase reagent (Tetramethyle-p-phenylenediamine dihydrochloride, 1%)
- Filter paper or oxidase regent strip

Note:

- Fresh oxidase reagent is easily oxidized. When oxidized it appears blue and must not be used.

Method

- Place a piece of filter paper in a clean petri dish
- add 2 or 3 drops of freshly prepared oxidase reagent,
- Using a piece of stick or glass rod (not an oxidized wire loop), remove a colony of the test organism and smear it on the filter paper.
- Look for the development of a blue-purple colour within a few seconds as shown in colour plate 3.

Biochemical tests con't…

Result

- Blue – purple color ……..positive Oxidase test (With in 10 seconds)
- No blue – Purple color …Negative Oxidase test (With in 10 seconds)

Note: Ignore any blue – purple color that develops after 10 seconds.

Method using an oxidase regent strip

- Moisten the strip with a drop of sterile water.
- Using a piece of stick or glass rod (not an oxidized wire loop) remove a colony of the test organism and rub it on the strip.
- Look for a red-purple colour within 20 seconds.
- Red-purple colour……positive oxidase test.

Controls

- Positive oxidase control: Pseudomonas aeruginosa
- Negative oxidase control: Escherichia coli

4. Urease test

- This test is used to detect the enzyme urease, which breaks down urea into ammonia.
- Testing for urease enzyme activity is important in differentiating enterobacteria.
- Proteus strains are strong urease producers.
- Y. enterocolitica also shows urease activity (weakly at 35-37^{0}C).

Principle

- The test organism is cultured in a medium which contains urea and the indicator phenol red.
- When the strain is urease-producing, the enzyme will break down the urea (by hydrolysis) to give ammonia and carbon dioxide.
- With the release of ammonia, the medium becomes alkaline as shown by a change in colour of the indicator to pink-red.
- *Ways of performing a urease test*
    - Using modified christensen's urea broth.
    - Using a Rosco urease identification tablet.

Method

A. Urease test using Christensen's (modified) urea broth

- Inoculate heavily the test organism in a bijou bottle containing 3 ml sterile Christensen's modified urea broth
- Incubate at 35-37^{0}C for 3-12 h (preferably in a water bath for a quicker result).
- Look for a pink colour in the medium.

Results

- Pink colour……..Positive urease test
- No pink colour…… Negative urease test

4. Urease test…Cont'd

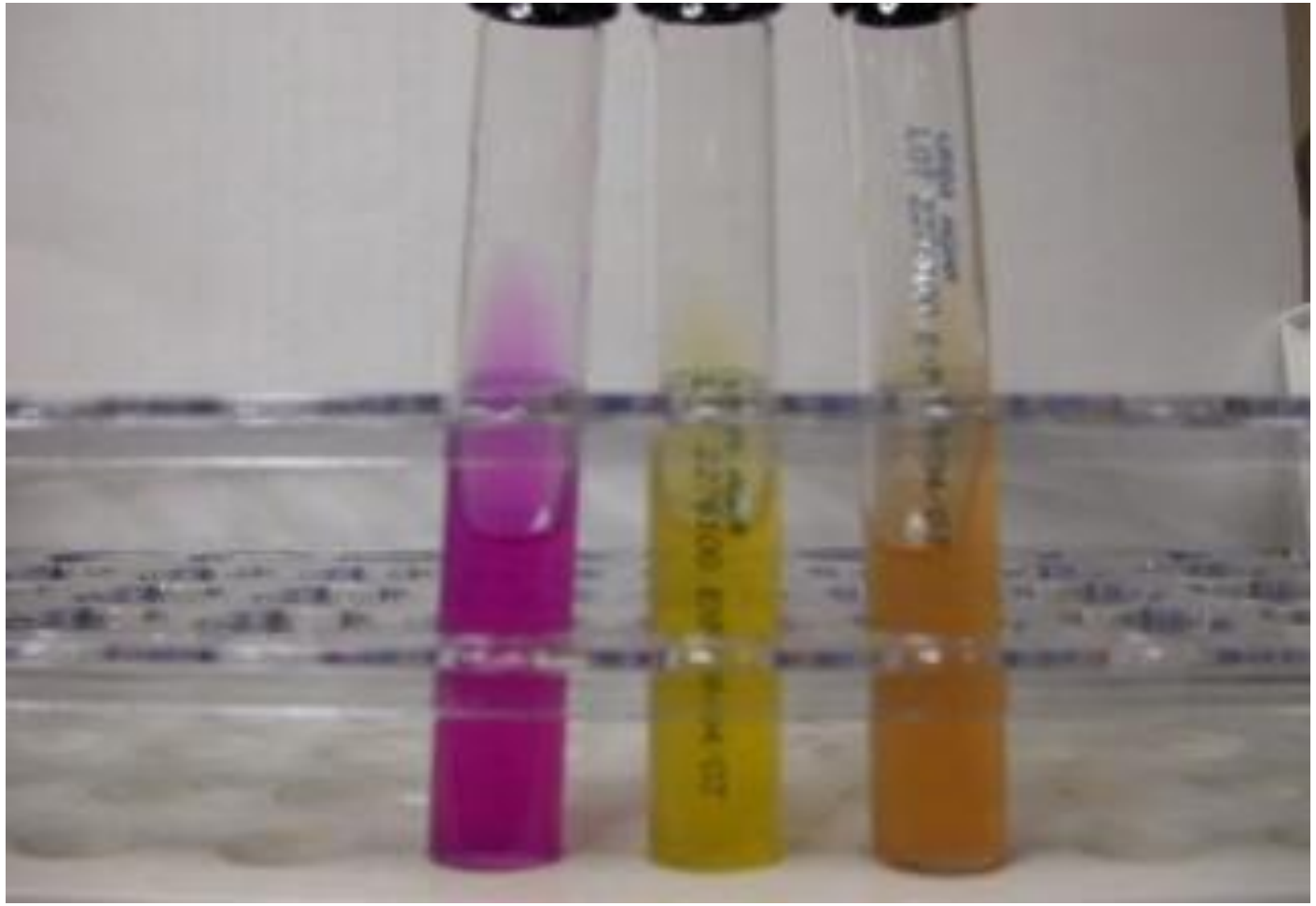


Fig. Urease test: *The tube on the left is a positive reaction; the tube in the middle is a negative reaction and the tube on the right is an un-inoculated control.*

4. Urease test…Cont'd

B. Urease test using a Rosco urease tablet

- Urease identification tablets are available commercially
- Prepare a dense 'milky' suspension of the test organism in 0.25 ml physiological saline in a small tube.
- Add a urease tablet, close the tube and incubate at 35-37^{0}C (preferably in a water bath for a quicker result) for up to 4 hours or overnight.
- Proteus and M. morganii organism give a positive reaction within 4 hours.

Results

- Red/purple colour……positive urease test
- Yellow/orange…….. Negative urease test

Control

- Positive urease control: Proteus spp,
- Negative urease control: Salmonellae

5. Indole test

- The test detect the ability of an organism to produce indole from Tryptophan present in the medium.
- Testing for indole production is important in the identification of enterobacteria.
- Most strains of E. coli, P. vulgaris, P. rettgeri, M. morganii, and Providencia species are indole positive organisms.

Principle

- The test organism is cultured in a medium which contains tryptophan.
- Indole production is detected by Kovac's or Ehrlich's reagent which contains 4(p)-dimethylamino-benzaldehyde.
- This reacts with the indole to produce a red coloured compound.
  - Kovac's reagent is recommended in preference to Ehrlich's reagent for the detection of indole from enterobacteria.

Biochemical tests con't…

Material required

- Kovac's or Ehrlich's reagent
- bijou bottle/test tube

Method

An indole test can be performed:

- As a single test using tryptone water and kovac's reagent.
- As a combined beta-glucuronidase-indole test using a Rosco PGUA/indole identification tablet and kovac's reagent. This is useful when identifying E. coli.
- As a combined lysine decarboxylase-indole test using a Rosco LDC/indole identification tablet. This is useful in helping to identify salmonellae and shigellae

Biochemical tests con't…

Indole test using tryptone water and kovac's reagent.

- Inoculate the test organism in a bijou bottle containing 3 ml of sterile tryptone water.
- Incubate at 35 – 37^{o}C for up to 48 hr
- Test for indole by adding 0.5ml of Kovac's reagent.
- Shake gently.
- Examine for a red color in the surface layer with in 10 minutes.

Results

- Red surface layer……Positive indole test
- No re surface layer……. Negative indole test

Biochemical tests con't…

Control

- Positive control ….. Escherichia coli
- Negative control…. Klebsiella pneumoniae

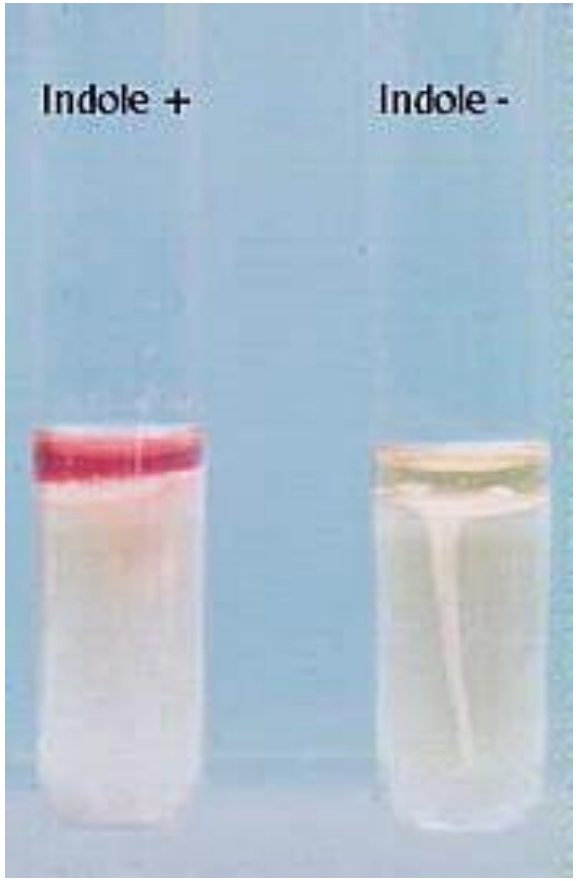


Fig. Indole test: The tube on the left with the red ring is positive for indole production while the tube on the right shows a negative result.

6. Citrate utilization test

The test detect the ability of an organism to use citrate as its only source of carbon. This test is one of several techniques used occasionally to assist in the identification of enteric bacteria.

Principle

- Some bacteria can obtain energy in a manner other than by the fermentation of carbohydrate by using citrate as source of carbon.
- The utilization of citrate by a test bacterium is detected in citrate medium by the production of alkaline by-products.
- The medium includes sodium citrate as the sole source of carbon and ammonium phosphate as the sole source of nitrogen.
- Bacteria that can use citrate can also extract nitrogen from the ammonium salt, with the production of ammonia (NH^+), leading to alkalinization of the medium.
- In the presence of the indicator Bromthymol blue the medium will be converted from green (at pH 6.0) to blue (at a pH above 7.6).

Biochemical tests con't…

Material required

- Simmon's citrate medium/agar
- Inoculating loop

Method

- Prepare slopes of the medium in bijou bottles as recommended by the manufacturer (store at 2-8 C)
- Using a sterile straight wire, first streak the slope with a saline suspension of the test organism and then stab the butt.
- Incubate at 35 ^{0}C for 48 hours
- Look for a bright blue colour in the medium

Results

- Bright blue----------Positive citrate test
- No change in colour of medium ----------Negative citrate test

Controls

- Positive control ---------- *klebsiella pneumoniae*
- Negative control---------- *Escherichia coli*

Biochemical tests con’t…

Fig. Citrate utilization test: Left tube is a negative result. Right tube is a positive result.

7. DNase test

- This test is used to identify S. aureus which produces deoxyribonuclease (DNase) enzymes.
- The DNase test is particularly useful when plasma is not available to preform a coagulase test are difficult to interpret.

Principle

- Deoxyribonuclease hydrolyzes deoxyribonucleic acid (DNA).
- The test organism is cultured on a medium which contains DNA.
- After overnight incubation, the colonies are tested for DNase production by flooding the plate with a weak hydrochloric acid solution.
- The acid precipitates unhydrolyzed DNA.
- DNase-producing colonies are therefore surrounded by clear areas due to DNA hydrolysis.

Biochemical tests con't…

Material Required

- DNase agare plate
  - Up to six organisms may be tested on the same plate.
- Hydrochloric acid, 1 mol/L (1 N)

Method

- Divide a DNase plate into the required number of strips by marking the underside of the plate.
- Using a sterile loop or swab, spot-inoculate the test and control organisms.
- Make sure each test area is labelled clearly.
- Incubate the plate at 35-37^{0}C overnight.
- Cover the surface of the plate with 1 mol/L hydrochloric acid solution.
- Tip off the excess acid.
- Look for clearing around the colonies within 5 minutes of adding the acid.

Biochemical tests con't…

Results

- Clearing around the colonies…….DNase positive strain
- No clearing around the colonies…… DNase negative strain

Note:

- Some methicillin resistant S.aureus (MRSA) strains give a negative DNase test.
- Some coagulase negative staphylocci are weakly positive.
- Rarely, S. pyogenes, Moraxella and Serratia species may give a positive DNase test.

Controls

- Controls Postive Dnase control:…….. Staphylococcus aureus
- Negative DNase control: ……Staphylococcus epidermidis

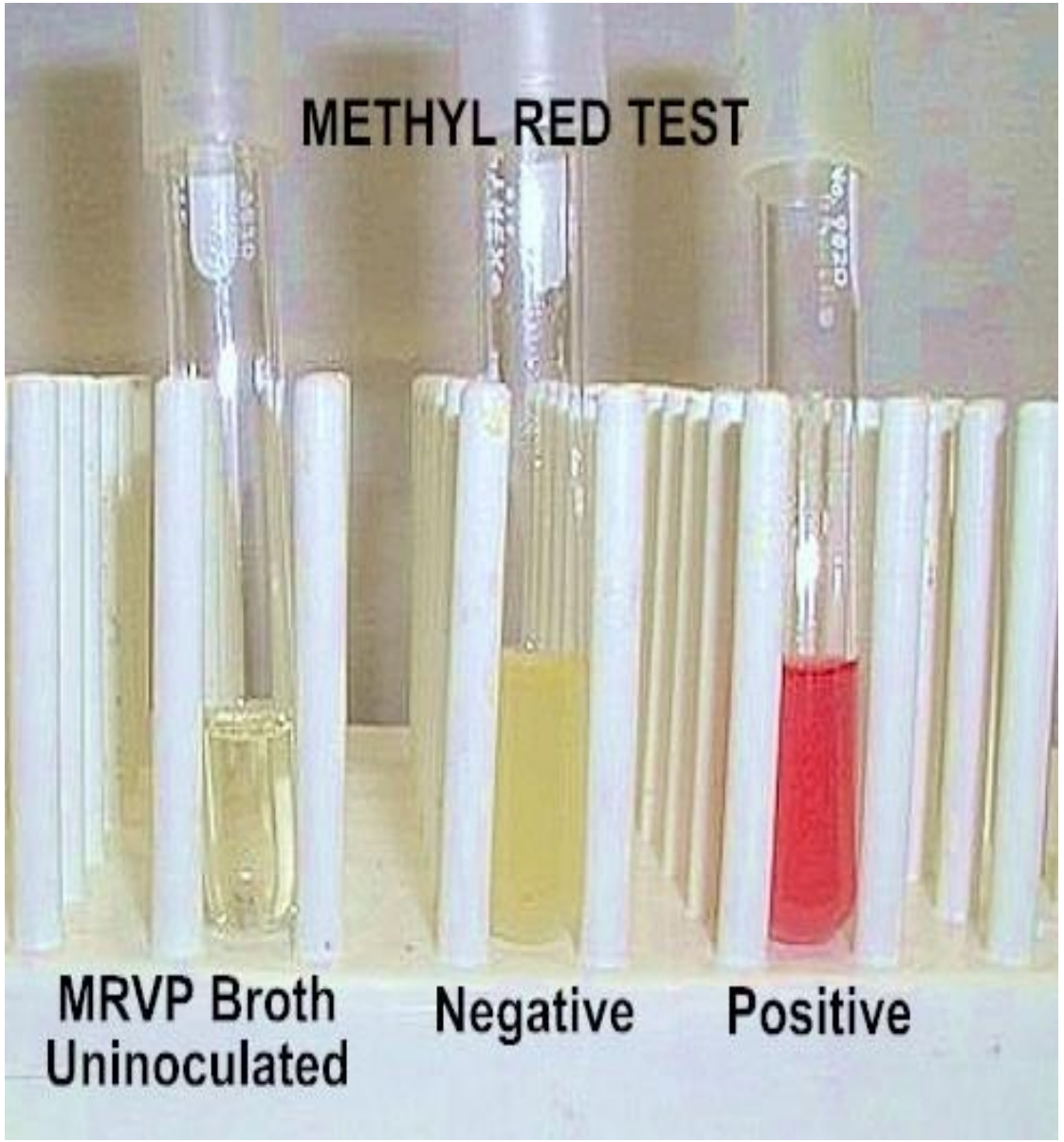
8. MRVP (methyl red-Vogues Proskauer) test

- This test is used to determine two things.
  - The MR portion (methyl red) is used to determine if glucose can be converted to acidic products like lactate, acetate, and formate.
  - The VP portion is used to determine if glucose can be converted to acetoin.
- These tests are performed by inoculating a **single tube of MRVP** media with a transfer loop and then allowing the culture to grow for 3-5 days.
- After the culture is grown, about half of the culture is transferred to a clean tube.
- One tube of culture will be used to conduct the MR test, the second tube serves as the VP test.

Biochemical tests con’t…

A. Methyl red (MR) test:

- Methyl red is added to the MR tube.
- A red color indicates a positive result
  - glucose can be converted into acidic end products such as lactate, acetate, and formate.
- A yellow color indicates a negative result,
  - glucose is converted into neutral end products.



B. **VP (Vogues Proskauer) test:**

- First alpha-napthol (also called Barritt’s reagent A) and then potassium hydroxide (also called Barritt’s reagent B) are added to the VP tube.
- The culture should be allowed to sit for about 15 minutes for color development to occur.
- If acetoin is produced then the culture turns to red color (positive result);
- if acetoin is not produced then the culture appears yellowish in color (a negative result).

Biochemical tests con't…

- **VP (Vogues Proskauer) test positive bacteria include**
  - *Klebsilla* spp, *Enterobacter*, spp and *Serratia* spp, Eltor vibrio, Staphylococci
- **Methyl Red positive bacteria include**
  - *Escherchia* spp., *Citobacter* spp., *Salmonella* spp, *Proteus* spp., *Yersinia* spp., Staphylococci Etc
- **MR-VP test positive bacteria include**
  - Staphylococci

9. Triple sugar Iron (TSI) & Hydrogen sulfide production (H_2S):

- Looks at fermentation of glucose, lactose, and sucrose and checks if hydrogen sulfide and gas is produced in the process.
- Basically a pH indicator will change the color of the media in response to fermentation.
- The color change that occurs in the tube will indicate what sugar or sugars were fermented.
- The presence of a black color indicates that H_2S was produced.
- In this media, H_2S reacts with the ferrous sulfate in the media to make ferrous sulfide, which is black in colour.
- To inoculate, use a needle to stab agar and then use a loop to streak the top slanted region.
- In addition to TSI media, KIA media can be used to determine if H_2S production.

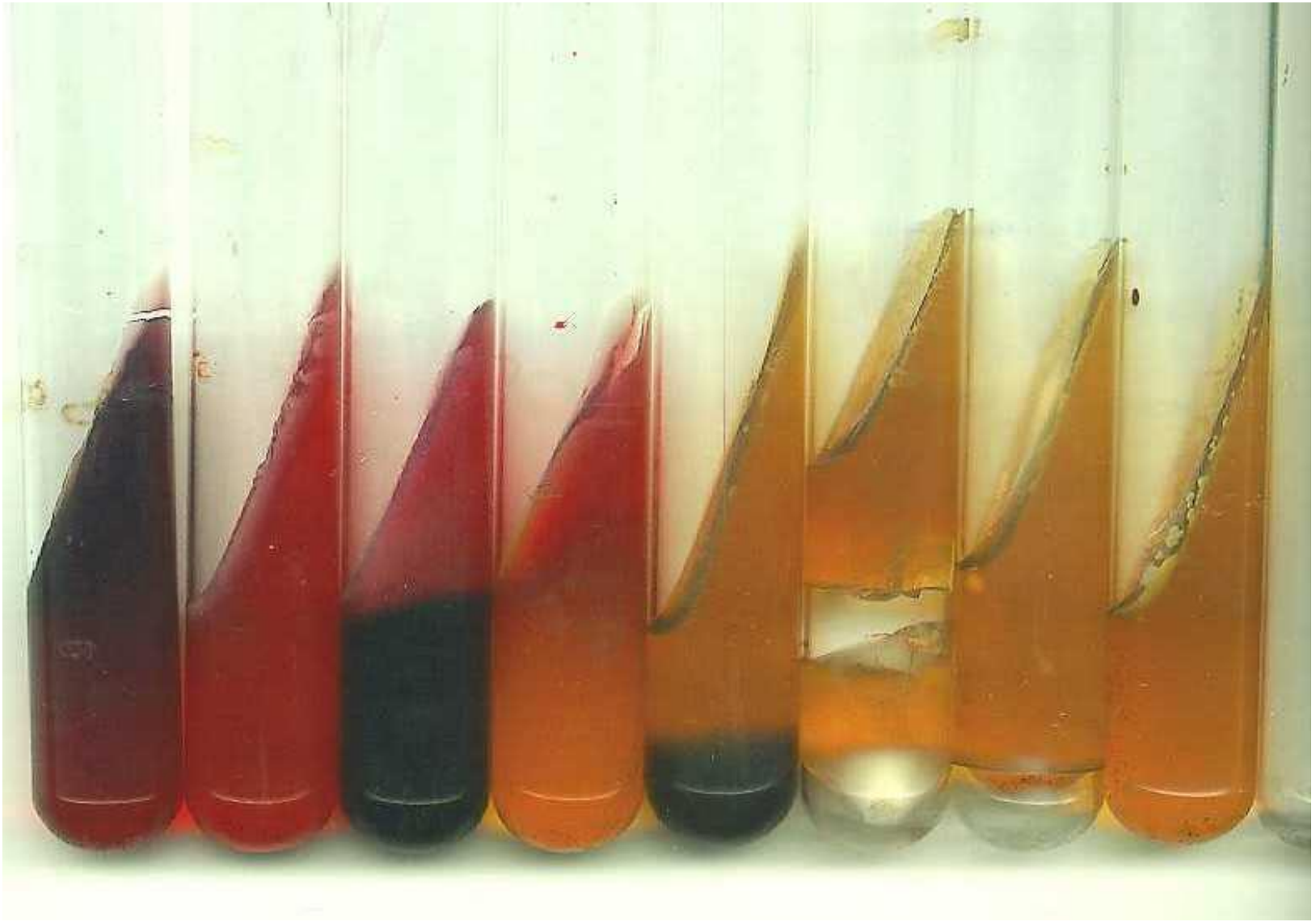
Biochemical tests con't…

Result

- Slant colour **red** ……. **does not ferment** either lactose or sucrose
- Slant colour **yellow**……**Ferments** lactose and/or sucrose
- Butt colour **red**…….**no fermentation** of glucose
- Butt colour **yellow**……. **some fermentation** of glucose has occurred and acid has been produced
- **Cracks seen** in the agar, ……**Gas formed**

  bubbles occured, or the entire slant pushed out of the tube.
- **Blackening** in the Butt**…. H_2S has been produced**

Biochemical tests con’t…



A B C D E F G

Fig. TSI test

Biochemical tests con't…

From left to right of the previous picture:

A. Uninoculated control

B. **Red slant and red butt, no black color**= no fermentation of glucose, sucrose or lactose. No Hydrogen sulfide produced

C. **Red slant and black butt**= no lactose or sucrose fermentation, H_2S has been produced

D. **Red slant with yellow butt**= no lactose or sucrose fermentation, Glucose is fermented, no H_2S has been produced

E. **Yellow slant, yellow butt and black coloration**= Lactose, sucrose and glucose fermented, and H_2S has been produced

F. **Yellow slant, yellow butt and lifting and/or cracking of media, no black coloration**= Lactose, sucrose and glucose fermented, H_2S has not been produced but gas has been produced

G. **Yellow slant, yellow butt and no lifting and/or cracking of media, no black coloration**= Lactose, sucrose and glucose fermented, H2S has not been produced nor has gas been produced

Biochemical tests con't…

10. Kligler Iron Agar (KIA)

- KIA is a composit medium containing glucose, lactose, phenole red and ferric citrate.
- A yellow base indicates glucose fermentation
- A yellow base and slope indicates both glucose and lactose fermentation.
- Bubble in the medium indicate gas production from glucose
- Blackening of the medium indicate H_2S production

11. Nitrate reduction test

- Nitrate broth is used for the test

Principle

- The test detects the ability of the organism to produce the enzyme nitrate reductase which reduses nitrate to nitrite

Method

- Growing the bacteria for 5 days at 37^0C in a broth containing 1% KNO_3
- Add 0.1 ml of the test reagent to the culture
- The test reagent consists of a mixture of equal volume of solutions of Sulphonilic acid and α-naphthylamine in 5 N acetic acid. Mixed just before use

Result

- Positive ---------red colour developing within a few minutes
- Negative--------No colour change

12. Additional non biochemical test

Motility test

- The motility test is not a biochemical test since we are not looking at metabolic properties of the bacteria.
- Rather, this test can be used to check for the ability of bacteria to migrate away from a line of inoculation.
- To perform this test, the bacterial sample is inoculated into motility media using inoculating wire.
- Simply stab the media in as straight a line as possible and withdraw the needle very carefully to avoid destroying the straight line.

Biochemical tests con't…

- After incubating the sample for 24-48 hours, observations can be made.
- Check to see if the bacteria have migrated away from the original line of inoculation.
- If migration away from the line of inoculation is evident then you can conclude that the test organism is motile (positive test).
- Lack of migration away from the line of inoculation indicates a lack of motility (negative test result).

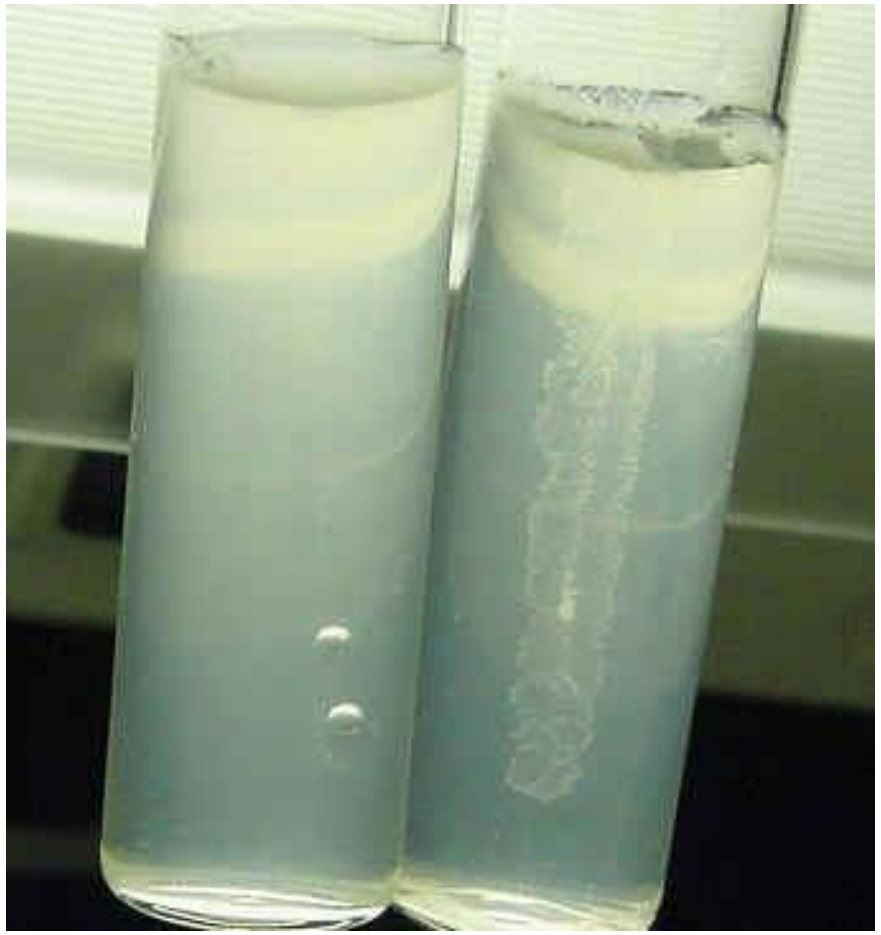


***Fig. Motility test**: Left tube is the result for a non-motile bacterium. Right tube is the result for a motile organism.*

13. Motility-Indole-Urea (MIU)

- MIU is a composie medium containing tryptone, phenol red, urea and a paper strip moistened in kovac's reagent.
- It is inoculated by straight wire through the center of the medium
- Non-motile organism grow only in the line of the inoculum, but motile organism grow through out the medium which become turbid
- Urease positive organism turn the medium red
- Indole positive organism turn the Kovac's strips red

14. STARCH HYDROLYSIS

- Starch is a polysaccharide that appears as a branched polymer of the simple sugar glucose. This means that starch is really a series of glucose molecules hooked together to form a long chain. Additional glucose molecules then branch off of this chain as shown below.

GLU

|

(---GLU-GLU-GLU-GLU-GLU-GLU-GLU---)n

- Some bacteria are capable of using starch as a source of carbohydrate but in order to do this they must first **hydrolyze or break down** the starch so it may enter the cell. The bacterium secretes an exoenzyme that hydrolyzes the starch by breaking the bonds between the glucose molecules. This enzyme is called a **diastase**.

(---GLU GLU GLU GLU GLU GLU GLU---)n

- The glucose can then enter the bacterium and be used for metabolism.

Biochemical tests con't…

- **MEDIUM**

  Starch agar (one plate)
- **ORGANISMS**

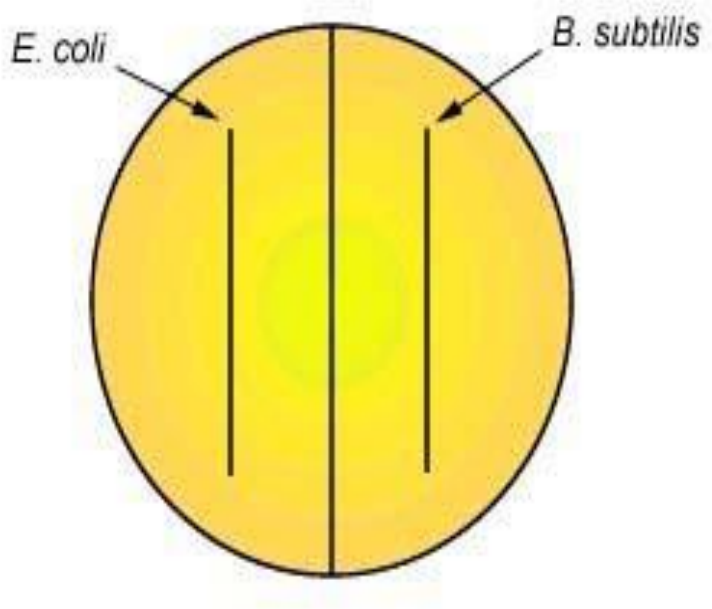
  Trypticase Soy broth cultures of *Bacillus subtilis* and *Escherichia coli*.
- **PROCEDURE**

  1. Using a wax marker, draw a line on the bottom of a Starch agar plate so as to divide the plate in half. Label one half *B. subtilis* and the other half *E. coli*.

  2. Make a single streak line with the appropriate organism on the corresponding half of the plate as shown below.
- 3. Incubate at 37^{o}C over night
- 4. Iodine will be added to see if the starch remains in the
- agar or has been hydrolyzed by the exoenzyme diastase.
- Iodine reacts with starch to produce a dark brown or
- blue/black color.

Biochemical tests con’t…

If starch has been hydrolyzed there will be a clear zone around the bacterial growth because the starch is no longer in the agar to react with the iodine. **If starch has not been hydrolyzed, the agar will be a dark brown or blue/black color**.



14. PROTEIN HYDROLYSIS

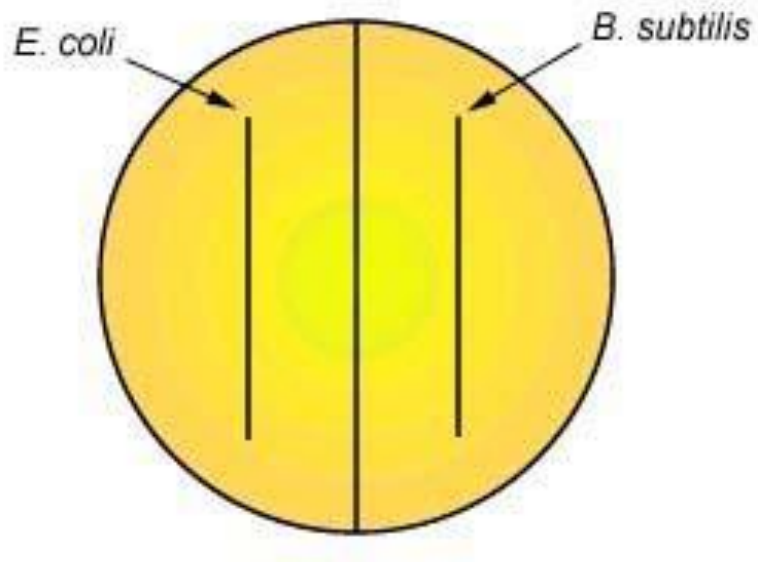
- Many bacteria can hydrolyze a variety of proteins into peptides (short chains of amino acids) and eventually into individual amino acids. They can then use these amino acids to synthesize their own proteins and other cellular molecules or to obtain energy.
- The hydrolysis of protein is termed **proteolysis** and the enzyme involved is called a **protease**. In this exercise we will test for bacterial hydrolysis of the protein **casein,** the protein that gives milk its **white, opaque appearance**
- **MEDIUM**

  Skim Milk agar (one plate)
- **ORGANISMS**

  Trypticase Soy broth cultures of *Bacillus subtilis* and *Escherichia coli*.
- **PROCEDURE**

  1. Divide the Skim Milk agar plate in half and inoculate one half with *Bacillus subtilis* and the other half with *Escherichia coli* as done above with the above starch agar plate.

Biochemical tests con’t…



2. Incubate at 37^{o}C until the next lab period. If casein is hydrolyzed, there will be a clear zone around the bacterial growth (see Fig.). If casein is not hydrolyzed, the agar will remain white and opaque.

15. FERMENTATION OF CARBOHYDRATES

- Carbohydrates are complex chemical substrates that serve as energy sources when broken down by bacteria and other cells.
- Facultative anaerobic and anaerobic bacteria are capable of fermentation, an anaerobic process during which carbohydrates are broken down for energy production.

Biochemical tests con’t…

- We can detect whether a specific carbohydrate has been fermented by looking for common end products of fermentation. When carbohydrates are fermented as a result of bacterial enzymes, the following fermentation end products may be produced:

1. acid end products, or
2. acid and gas end products.

- In order to test for these fermentation products, inoculate and incubate tubes of media containing a single carbohydrate (such as lactose or maltose), a pH indicator (such as phenol red) and a Durham tube (a small inverted tube to detect gas production).
- If the particular carbohydrate is fermented by the bacterium, acid end products will be produced which lowers the pH, causing the pH indicator to change color (phenol red turns yellow).

Biochemical tests con't…

- If gas is produced along with the acid, it collects in the Durham tube as a gas bubble (Fig.). If the carbohydrate is not fermented, no acid or gas will be produced and the phenol red will remain red.

MEDIA

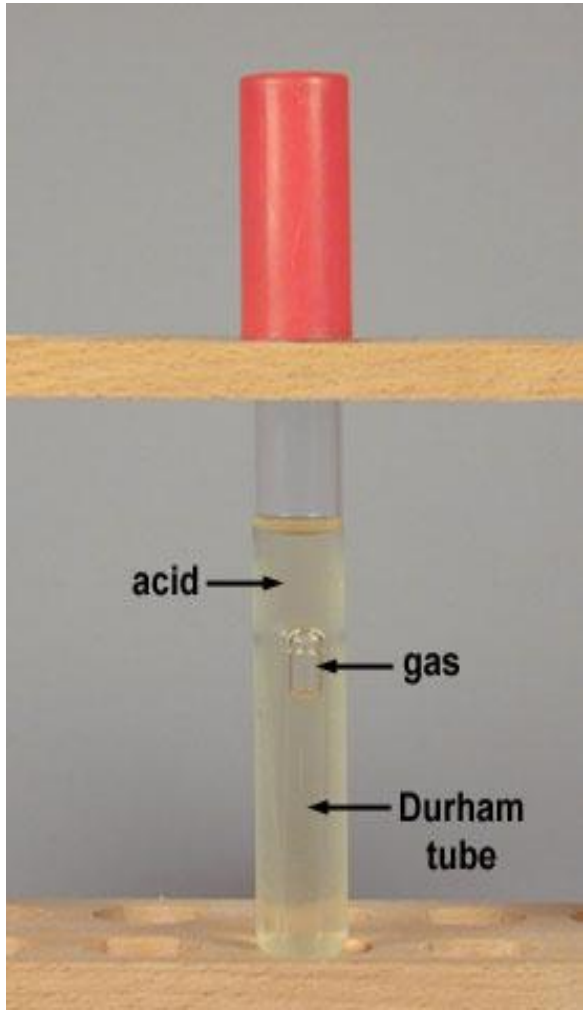
3 tubes of Phenol Red Lactose broth and 3 tubes of Phenol Red Maltose broth.

ORGANISMS

Trypticase Soy agar cultures of *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus*.

PROCEDURE

1. Label each tube with the name of the sugar in the tube and the name of the bacterium you are growing.
2. Inoculate one Phenol Red Lactose broth tube and one Phenol Red Maltose broth tube with *Bacillus subtilis.*
3. Inoculate a second Phenol Red Lactose broth tube and a second Phenol Red Maltose broth tube with *Escherichia coli*.
4. Inoculate a third Phenol Red Lactose broth tube and a third Phenol Red Maltose broth tube with *Staphylococcus aureus*.
5. Incubate all tubes at 37°C overnight.



Biochemical tests con't…

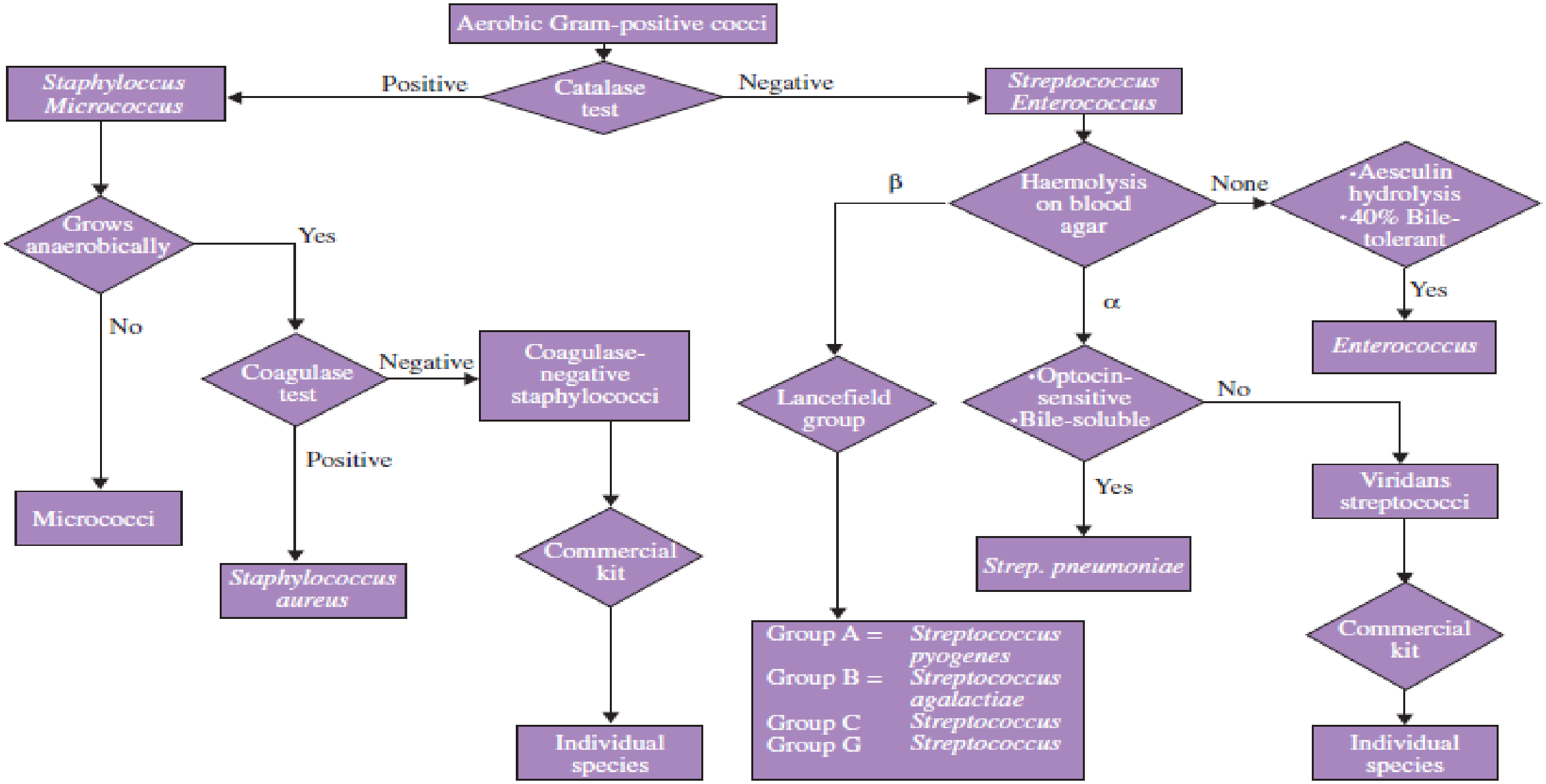


Figure: Identification of aerobic Gram-positive cocci of medical importance

Biochemical tests con't…

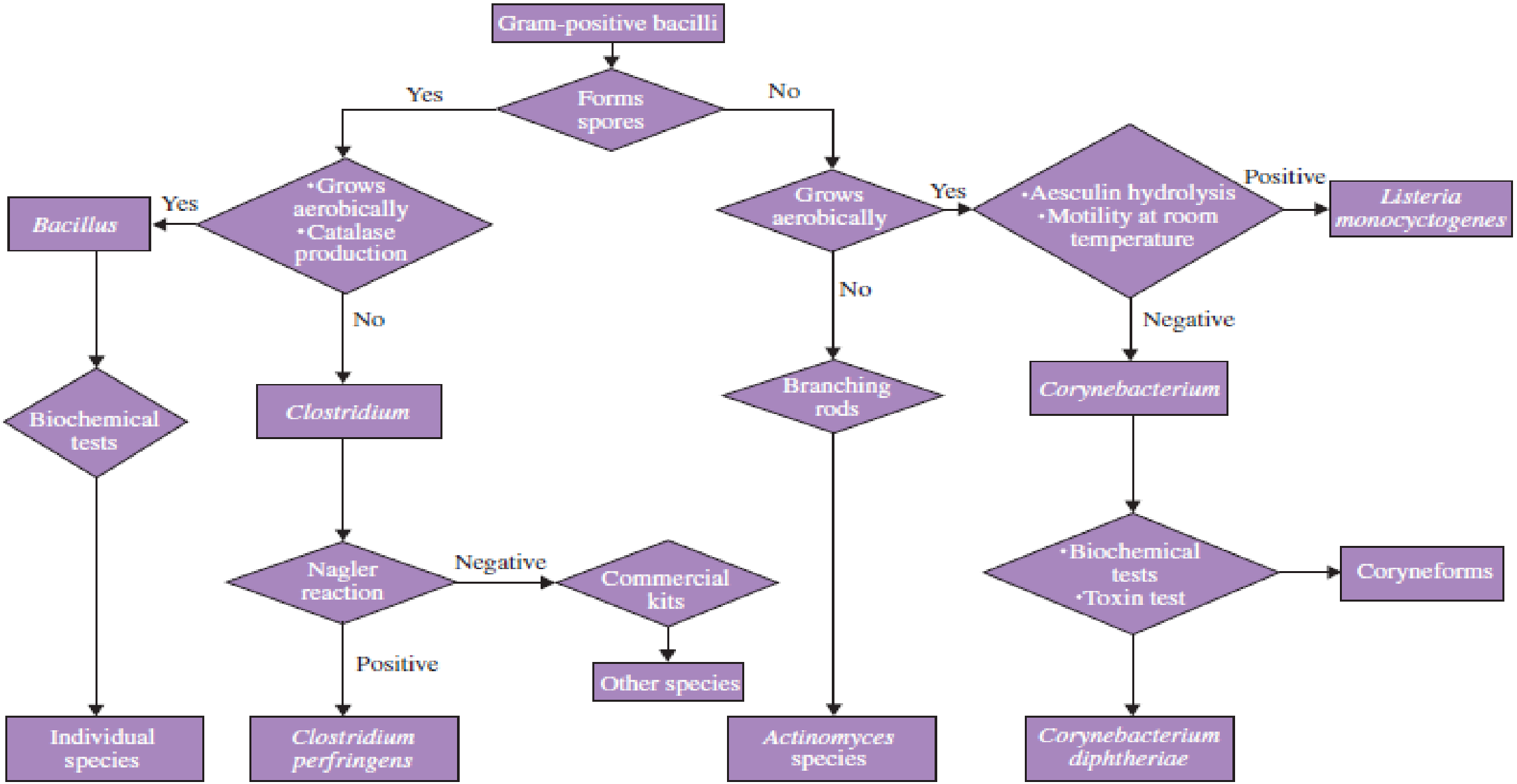


Figure: Preliminary identification of Gram-positive bacilli of medical importance

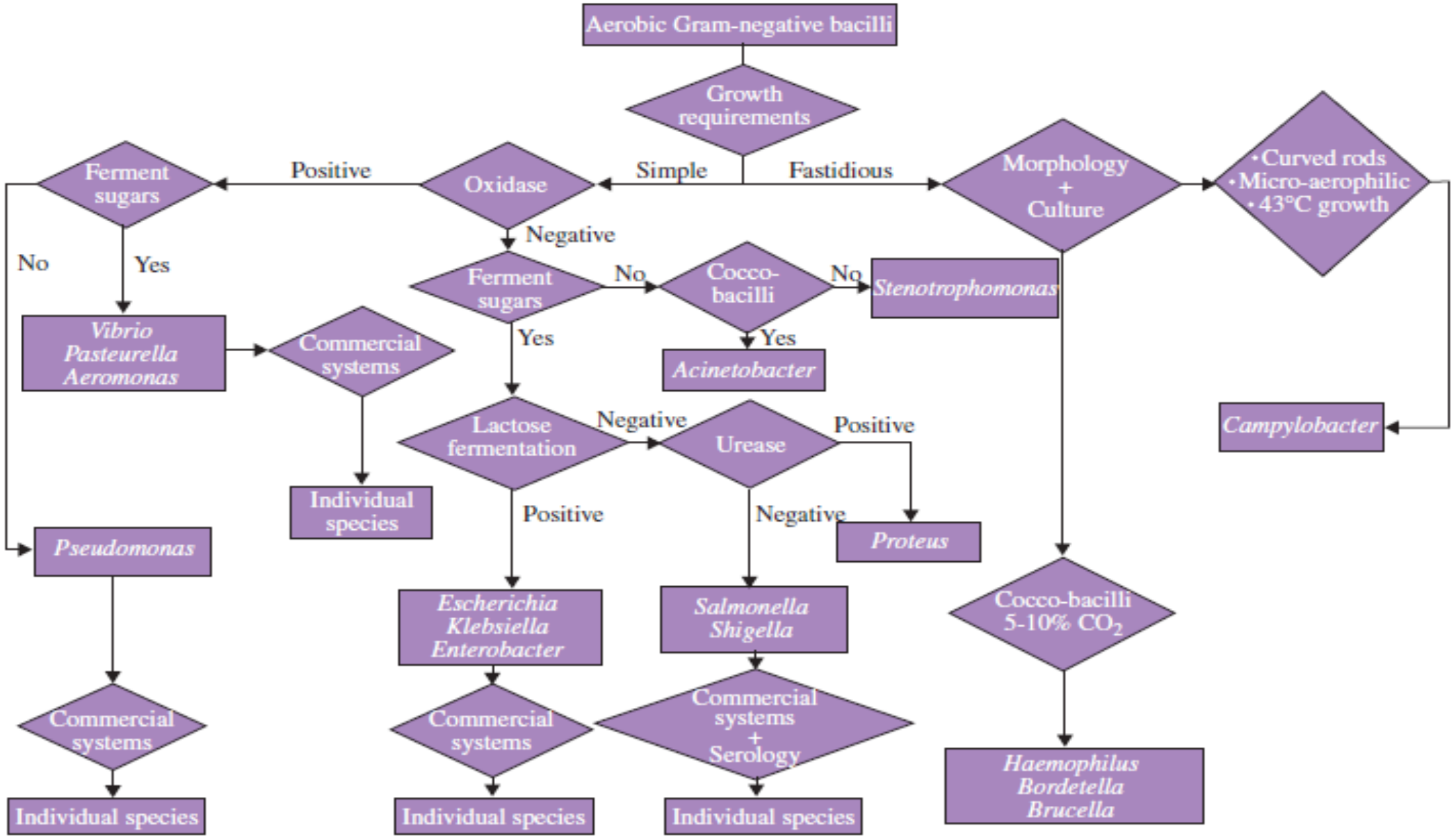


Figure: Preliminary identification of aerobic Gram-negative bacilli of medical importance

ANTIMICROBIAL AGENTS & SUSCEPTIBILITY TESTING

Definition of Antimicrobial agent

- **Antimicrobial agent:** A general term for drugs, chemicals, or other substances that either kill or slow the growth of microbes. Among the antimicrobial agents are antibacterial drugs, antiviral agents, antifungal agents, and antiparisitic drugs.
  - Most microbiologist distinguish two groups of antimicrobial agents used in the treatment of infectious disease:
- **Antibiotics**, which are natural substances produced by certain groups of microorganisms, and
- **Chemotherapeutic agents**, which are chemically synthesized.

- A hybrid substance is a **semi synthetic antibiotic**, wherein a molecular version produced by the microbe is subsequently modified by the chemist to achieve desired properties.
- Furthermore, some antimicrobial compounds, originally discovered as products of microorganisms, can be synthesized entirely by chemical means. They might be referred to as **synthetic antibiotics** to distinguish them from the chemotherapeutic agents.

Susceptibility testing con't…

Characteristics of Antibiotics

- Antibiotics are low-molecular weight substances that are produced as secondary metabolites by certain groups of microorganisms, especially *Streptomyces*, *Bacillus*, and a few molds (*Penicillium* and *Cephalosporium*) that are inhabitants of soils.
- Antibiotics may have a **cidal (killing) effect** or a **static (inhibitory) effect** on a range of microbes.
- The range of bacteria or other microorganisms that are affected by a certain antibiotic is expressed as its **spectrum of action**.
- Antibiotics effective against prokaryotes which kill or inhibit a wide range of Gram-positive and Gram-negative bacteria are said to be **broad spectrum**.
- If effective mainly against Gram-positive or Gram-negative bacteria, they are called **narrow spectrum**.
- If effective against a single organism or disease, they are referred to as **limited spectrum**.

Susceptibility testing con't…

- A clinically-useful antibiotic should have as many of these characteristics as possible.
  - ✓It should have a wide spectrum of activity with the ability to destroy or inhibit many different species of pathogenic organisms.
  - ✓It should be nontoxic to the host and without undesirable side effects.
  - ✓It should not eliminate the normal flora of the host.
  - ✓It should be able to reach the part of the human body where the infection is occurring.
  - ✓It should be inexpensive and easy to produce.
  - ✓It should be chemically-stable (have a long shelf-life).
  - ✓Microbial resistance is uncommon and unlikely to develop.

Susceptibility testing con't…

Antimicrobial Modes of action

- The following are antimicrobial agents based on their mode of action in bacterial cells.

Cell wall synthesis inhibitors

- Cell wall synthesis inhibitors generally inhibit some step in the synthesis of bacterial peptidoglycan. Generally they exert their selective toxicity against bacteria because human cells lack cell walls.
- **Beta lactam antibiotics**. They are the products of two groups of fungi, *Penicillium* and *Cephalosporium* molds, and are correspondingly represented by the **penicillins** and **cephalosporins**.
- **Semi synthetic -Amoxycillin** and **Ampicillin** have broadened spectra against Gram-negatives and are effective orally; **Methicillin** is penicillinase-resistant.

Susceptibility testing con't…

E.g.

- Penicillin's
  - Benzyl penicillin (penicillin G)
  - Ampicillin
  - Amoxicillin
  - Cloxacillin
  - Carbenicillin
  - Ticarcillin
  - Azlocillin
  - Pipecacillin etc
- Cephalosporins
  - Cepharadine
  - Cefuroxime
  - Ceftazidime etc.
- Glycopeptides
  - Vancomycine
  - Teicoplanin

Susceptibility testing con't…

Cell membrane inhibitors

- These antibiotics disorganize the structure or inhibit the function of bacterial membranes. The integrity of the cytoplasmic and outer membranes is vital to bacteria, and compounds that disorganize the membranes rapidly kill the cells. Eg. **polymyxin**, produced by *Bacillus polymyxis*

Protein synthesis inhibitors

- Many therapeutically useful antibiotics owe their action to inhibition of some step in the complex process of protein synthesis. The most important antibiotics with this mode of action are the **tetracyclines**, **chloramphenicol**, the **macrolides** (e.g. erythromycin) and the **aminoglycosides** (e.g. streptomycin).

Susceptibility testing con’t…

- Amino glycosides
  - Gentamicin
  - Kanamycin
  - Streptomycin etc
- Macrolides
  -Erythromycin
- Lincosamides
  - Clindamycin
- Chloramphenicol
- Tetracyclines
  - Tetracycline
  - Doxycycline

Drugs which act on Nucleic Acids

- Some antibiotics and chemotherapeutic agents affect the synthesis of DNA or RNA, or can bind to DNA or RNA so that their messages cannot be read. Either case, of course, can block the growth of cells.
- Two nucleic acid synthesis inhibitors which have selective activity against procaryotes and some medical utility are the **quinolones** and **rifamycins**.

❖Inhibitors of bacterial Nucleic acid synthesis include

- Quinolones
  - ✓Naldic acid, Ciprofloxacin,Norfloxiacin etc.
- Sulphonamides and Trimethoprim
  - ✓ Co – trimoxazole,Trimethoprim,Sulphadoxine etc.
- Metronidazole

Competitive Inhibitors: Many of the synthetic chemotherapeutic agents are **competitive inhibitors** of essential metabolites or growth factors that are needed in bacterial metabolism. Eg. **Sulfonamides**

Susceptibility testing con't…

Table:Target sites for antimicrobial action (Summery)

Target	Antibioticst	Mechanism of action	Basis of selective toxicity
Bacterial cell wall	j3-Lactams	Inhibit peptidoglycan synthesis	None in mammalian cells
	Glycopeptides	Inhibit peptidoglycan synthesis	None in mammalian cells
	Cycloserine	Inhibits peptidoglycan synthesis	None in mammalian cells
	Isoniazid*	Inhibits mycolic acid synthesis	None in mammalian cells
	Ethambutol*	Inhibits arabinogalactan synthesis	None in mammalian cells
Bacterial ribosome function	Aminoglycosides	Distort 30S ribosomal subunit	No action on 40S subunit
	Tetracyclines	Block 30S ribosomal subunit	Excluded by mammalian cells
	Chloramphenicol	Inhibits peptidyl transferase	No action on mammalian equivalent
	Macrolides, azalides	Block translocation	No action on mammalian equivalent
	Fusidic acid	Inhibits elongation factor	Excluded by mammalian cells
	Mupirocin	Inhibits isoleucyl-tRNA synthesis	No action on mammalian equivalent
Chromosome function	Quinolones	Inhibit DNA gyrase	No action on mammalian equivalent
	Metronidazole (also**)	DNA strand breakage	Requires anaerobic conditions not present in mammalian cells
	Nitrofurantoin	DNA strand breakage	
	Rifampicin (also*)	Inhibits RNA polymerase	No action on mammalian equivalent
	5-Fluorocytosine***	Inhibits DNA synthesis	Converted to active form in fungi
Folate metabolism	Sulphonamides (also**)	Inhibit folate synthesis	Not present in mammalian cells
	Trimethoprim	Inhibits dihydrofolate reductase	Mammalian enzyme not inhibited
	Pyrimethamine**	Inhibits dihydrofolate reductase	Mammalian enzyme not inhibited
	Trimetrexate**/***	Inhibits dihydrofolate reductase	Toxicity overcome with leucovorin
Cytoplasmic membrane	Polymyxins	Disrupt bacterial membranes	Bind to LPS and phospholipids
	Polyenes***	Disrupt fungal membranes	Bind preferentially to ergosterol
	Imidazoles and triazoles***	Inhibit ergosterol synthesis	Pathway not in mammalian cells
	Naftidine***	Inhibits ergosterol synthesis	Pathway not in mammalian cells

t All antibacterial except: *antimycobacterial agent; **antiprotozoal agent; ***antifungal agent.
LPS, lipopolysaccharide.

Antimicrobial Resistance

- Most of the antimicrobial resistance which is now making it difficult to treat some infectious diseases is due to the extensive use and misuse of antimicrobial drugs which have favored the emergence and survival of resistant strains of micro– organisms.
- Bacteria become resistant to antimicrobial agents by a number of mechanisms, the commonest are:

1.Production of enzymes which inactivate or modify antibiotics.

E.g.Production of beta – lactamase enzyme that destroy the beta – lactam ring of penicillin's and cephalosporin's.

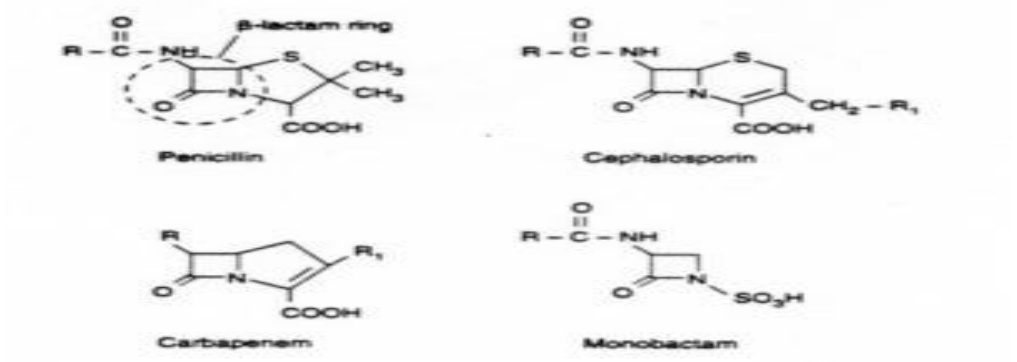


Figure: beta – lactam ring of penicillin's and cephalosporin's.

2. Changes in the bacterial cell membrane, preventing the up take of antimicrobial agent

   E.g.- Altering the permeability of the outer

   membrane of the bacterial cell wall which occurs in

   resistance to tetracycline's

   - Forming a natural barrier to amino glycosides

3. Modification of the target so that it no longer interacts with the antimicrobial agent

   E.g. Bacterial mutations. i.e. They change their DNA make up, this brings in change in protein synthesis, and this in turn brings change in receptors for drugs. As a result the drug will not be effective.

Susceptibility testing con't…

4. Development of metabolic pathways by bacteria which enables the site if antimicrobial action to be by passed. E.g. Resistance to sulphonamides and trimethoprim.

5. Increasing the production of a certain bacterial enzyme, e.g., overcoming drugs that resemble substrates and tie-up bacterial enzymes.

- Antibiotic resistance either plasmid mediated or maintained on the bacterial chromosome.
- The most important mechanism of resistance to the penicillins and cephalosporins is antibiotic hydrolysis mediated by the bacterial enzyme beta-lactamase.
- The expression of chromosomal beta-lactamase can either be induced or stably depressed by exposure to beta-lactam drugs.

Susceptibility testing con't…

Control of Resistance

- understanding of the mechanisms and spread of resistance indicate that certain principles can help keep the drug resistance problem under control:

1. Use antimicrobics conservatively and specifically in therapy.

2. Use an adequate dosage and duration of therapy to eliminate the infecting organism and reduce the risk of selecting resistant variants.

3. Select antimicrobics according to the proved or anticipated known susceptibility of the infecting strain whenever possible.

4. Use narrow-spectrum rather than broad-spectrum antimicrobics when the specific etiology of an infection is known, if possible.

5. Use antimicrobic combinations when they are known to prevent emergence of resistant

Susceptibility testing con’t…

6. Use antimicrobics prophylactically only in situations in which it has been proven valuable and for the shortest possible time to avoid selection of a resistant flora.

7. Avoid environmental contamination with antimicrobics.

8. Rigidly apply careful, aseptic and handwashing procedures to help prevent spread of resistant organisms.

9. Use containment isolation procedures for patients infected with resistant organisms that pose a threat to others, and use protective precautions for those who are highly susceptible.

10. Epidemiologically monitor resistant organisms or resistance determinants in an institution and apply enhanced control measures if a problem develops.

11. Restrict the use of therapeutically valuable antimicrobics for nonmedical purposes.

ANTIMICROBIAL SENSITIVITY (SUSCEPTIBILITY) TESTING

- The test is used to measure the ability of the drug to inhibit or kill pathogens in vitro. I.e. it is used to select effective antimicrobial drugs.
- **Sensitivity test is performed:**

➢For organisms with variable antibiotic sensitivity (un predictable sensitivity) E.g. Shigella

➢For non responding patients after taking adequate therapy.

➢For patients whose immune system is depressed

➢For relapsing cases (reappearance of disease)

- **Sensitivity test is not performed**

➢If the bacteria is a normal flora contaminant

➢If the culture is mixed. Sensitivity is performed on pure culture and

➢For organisms with predictable sensitivity

E.g. - *S. Pyogens* & *N. meningitis* are sensitive to penicillin

- *Proteus* species are generally resistant to tetracyclines, so no need of sensitivity testing

Sensitivity Testing Techniques

Laboratory antimicrobial sensitivity testing can be performed using:

1. A dilution technique and
2. A disk diffusion technique

1. Dilution technique for sensitivity test

- This technique can be done either on Agar or broth media.

Principle: Graded amounts of antimicrobial agents are incorporated into liquid or solid bacteriology media. The media are subsequently inoculated with test bacteria and incubated.

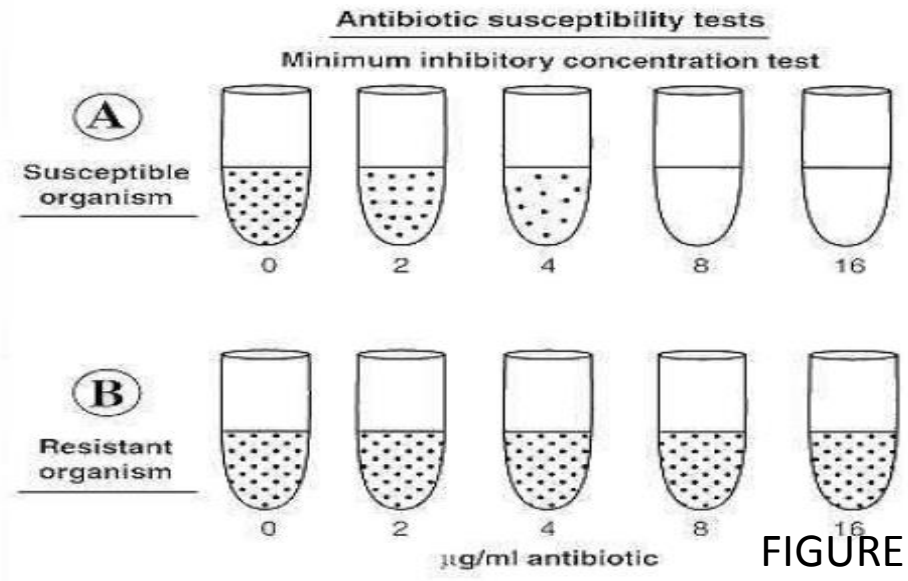
- The end point is taken as that amount of antimicrobial agent required to inhibit the growth of the test bacteria (MIC-minimum inhibitory concentration) or to kill the test bacterial (MBC-minimum bactericidal concentration).

Susceptibility testing con't…

Procedure

1. Prepare liquid or solid media
2. Add graded amount of antimicrobial agent (drug)
3. Inoculate with the pure culture
4. Read and take the last tube with no growth as MIC.

- This MIC or MBC value is then compared with known concentration of drug obtainable in the serum or other body fluids, and then the likely clinical response can be assessed.



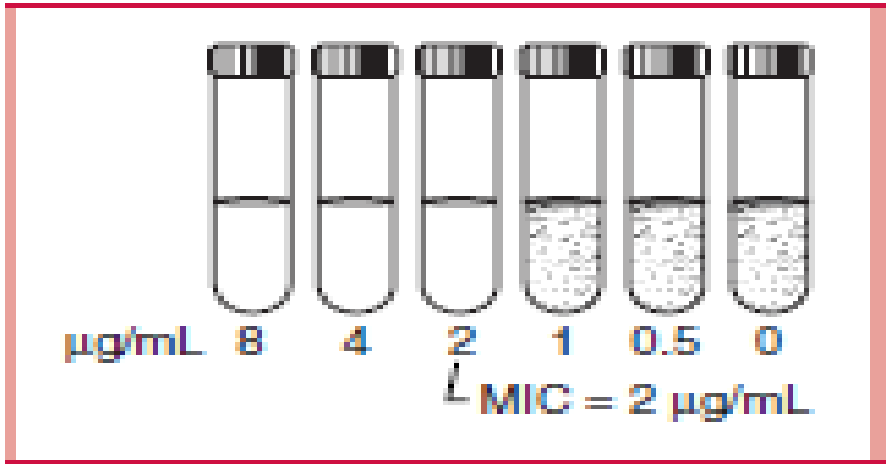


FIGURE: Broth dilution susceptibility test. The stippled tubes represent turbidity produced by bacterial growth. The MIC is 1.0 g/mL.

2. Disk Diffusion Sensitivity Testing

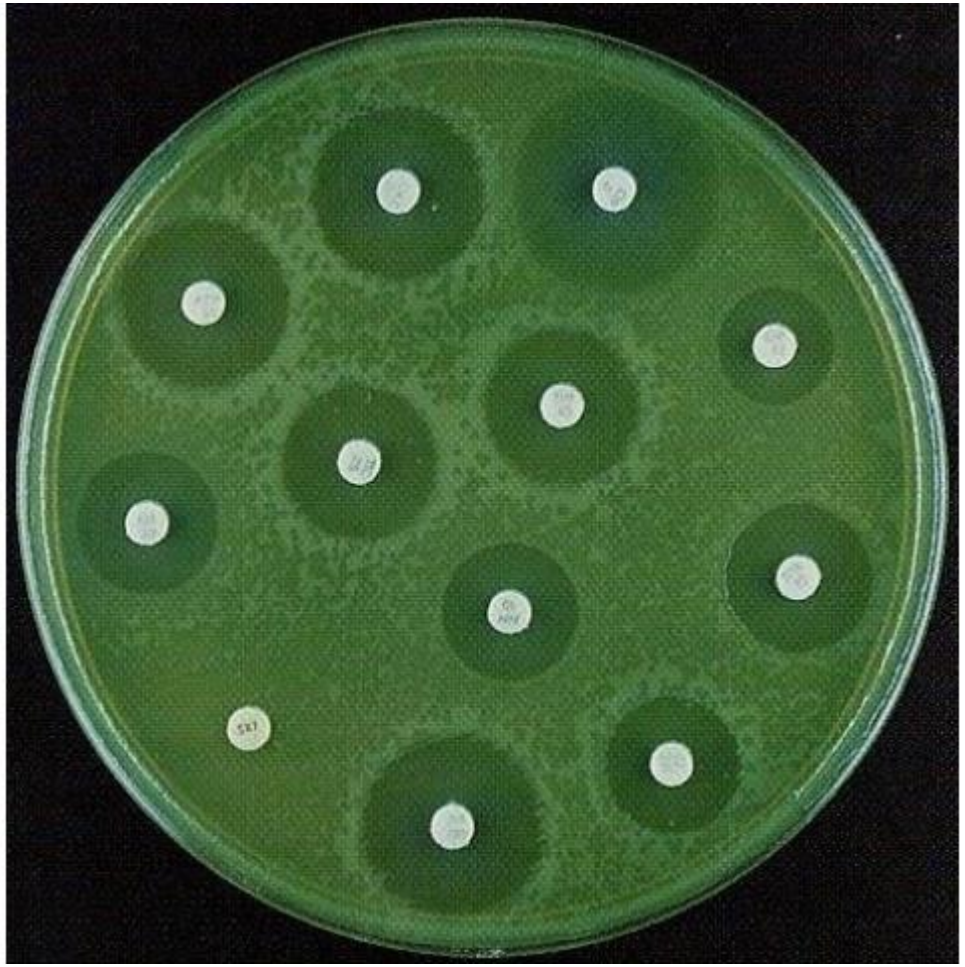
- Disk diffusion techniques are used by most laboratories to test routinely for antimicrobial sensitivity. (Because it is simple, economical & reproducible).

- **Principle**: A disk of blotting paper is impregnated with a known volume and appropriate concentration of an antimicrobial, and this is placed on a plate of sensitivity testing agar uniformly inoculated with the test organism. The antimicrobial diffuses from the disc into the medium and the growth of the test organism is inhibited at a distance from a disc that is related to the sensitivity of the organism.

- Strains sensitive to the antimicrobial agent are inhibited at a distance from the disc where as resistant strains have smaller zones of inhibition or grow up to edge of the disc.

Susceptibility testing con’t…

- -The zone of inhibition is measured by clippers or ruler and values are matched (compared) with the predetermined standard values and reported as susceptible, Intermediate and resistant.



Susceptibility testing con't…

Kirby-Bauer NCCLS Modified Disk Diffusion

- The validity of this technique depends on use of reliable Muller Hinton agar, discs of correct antimicrobial content and turbidity standard equivalent to McFarland's.

Mueller Hinton Sensitivity testing Agar

- Prepare the medium as instructed by the manufacturer.
- The PH of the medium should be 7.2 – 7.4.
- Pour into 90mm diameter sterile Petri dishes to a depth of 4mm. (i.e. 25ml of MHAP)
- Care must be taken to pour the plates on a level surface so that the depth of the medium is uniform
- Control each new batch of agar by testing it with a control strain.

  E.g. *E. faccalis* (ATCC 29122 or 33186) and co–trimoxazole disc. The zone of inhibition should be 20mm or more in diameter.
- The plates should be stored at 2 – 8^{o}C for up to 2 weeks

  NB: Un modified Mueller Hinton agar is not suitable for sensitivity testing *H. influenzae*, *S. pneumonia*, *N. gonorrhoeae*

Susceptibility testing con't…

Antimicrobial Discs

- The choice of antimicrobials to be included in sensitivity tests will depend on the pathogen, the range of locally available antimicrobials and local prescribing polices.
- Most paper discs can be used for 1 year or longer from the date of manufacture if stored properly (2 – 8^{o}C for working stock).
- About 1 hour before use, the working stock of discs should be allowed to warm to room temperature.

Turbidity standard equivalent to McFarland 0.5

- This is a barium sulphate standard against which the turbidity of the test and control inocula can be compared

Susceptibility testing con't…

Preparation of turbidity standard

1. Prepare a 1% v/v solution of sulphuric acid by adding 1ml of concentrated sulphuric acid to 99ml of distilled water.
2. Prepare a 1% w/v solution of barium chloride by dissolving 0.5 g of dehydrate barium chloride (BaCl2. $2H_2O$) in 50ml of distilled water.
3. Add 0.6 ml of the barium chloride solution to 99.4 ml of the sulphuric acid solution, and mix

Susceptibility testing con't…

Procedure:

1. Using a sterile wire loop, touch 3 – 5 well – isolated colonies of similar appearance to the test organism and emulsify in 3 – 4 ml to sterile physiological saline or nutrient broth.
2. In a good light match the turbidity of the suspension to the turbidity standard (mix the standard before use)
3. Using a sterile swab, inoculate a plate of Muller Hinton agar. Remove excess fluid by pressing and rotating the swab against the side of the tube above the level of the suspension.

   - Streak the swab evenly over the surface of the medium in three directions rotating the plate approximately 60^{o} to ensure even distribution.

Susceptibility testing con't…

4. With the Petri dish lid in place, allow 3 – 5 minutes for the surface of the agar to dry.

5. Using sterile forceps or multi disc dispenser, place the appropriate antimicrobial discs evenly distributed on the inoculated plate.

   NB. The discs should be about 15mm from the edge of the plate and no closer than 25mm from disc to disc. No more than 6 discs should be applied in 90mm dish. Each disc should be lightly pressed down to ensure its contact with the agar. It should not be moved once in place.

6. Within 30 minutes of applying the discs, invert the plate and incubate it aerobically at 35^{o}C for 16 – 18 hours.

7. After over night incubation, examine the control and the test plates. Using a ruler measure the diameter of each zone of inhibition in mm on the underside of the plate. the end point of inhibition is where growth starts.

Susceptibility testing con't…

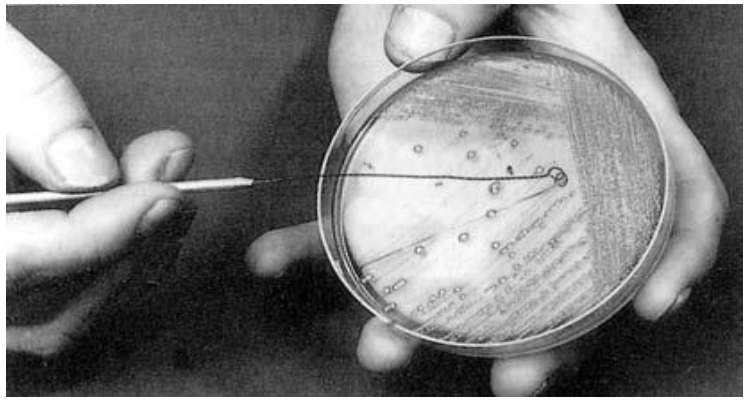


Figure: Removing colonies from a primary culture plate to make a suspension of the test organism.

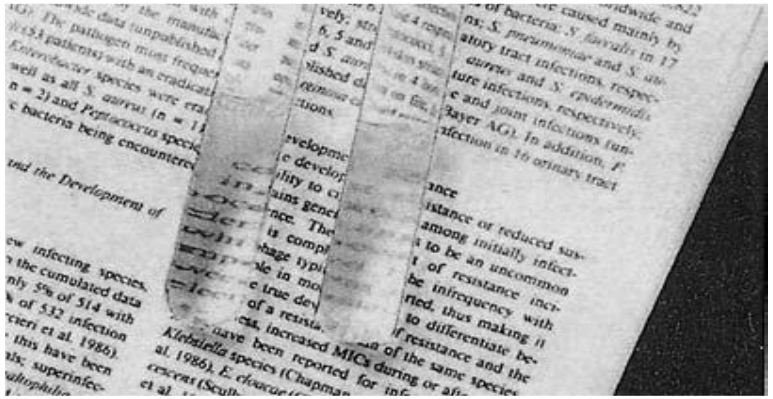


Figure: Checking the turbidity of the test suspension against the turbidity of a chemical standard.

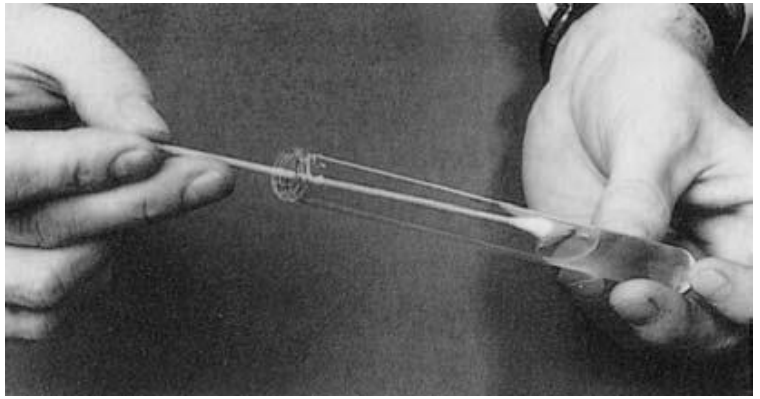


Figure: Avoiding using too much inoculum by pressing and rotating the swab against the side of the tube

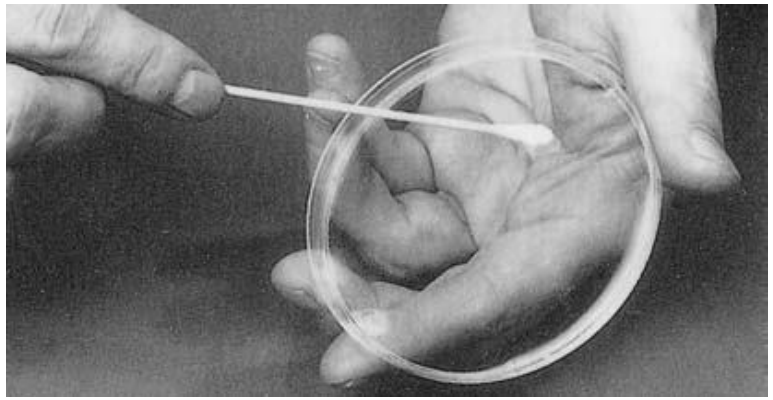


Figure: Swabbing the surface of the susceptibility testing agar. The plate is swabbed in three directions, rotating the plate approximately 60° to ensure even distribution.

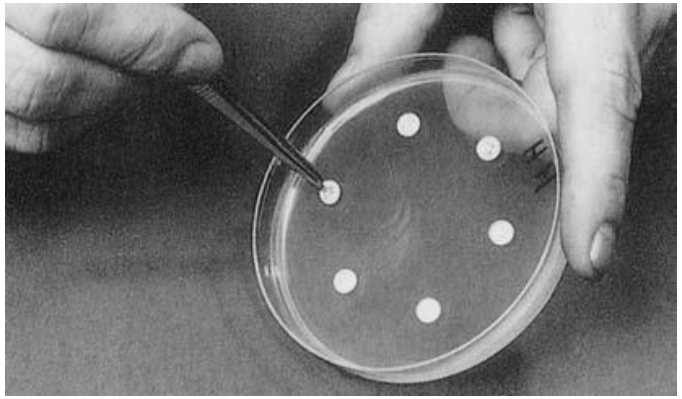


Figure: Placing antimicrobial discs on the inoculated plate.

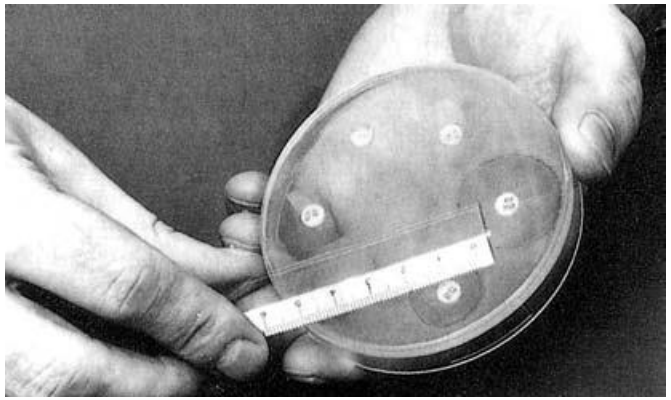


Figure: Measuring the zones of inhibition in mm. The end of inhibition is where growth starts.

Susceptibility testing con't…

Interpretation of Zone Size

Using the interpretative chart, interpret the zones sizes of each antimicrobial and report the organisms as 'Resistant', Intermediate (moderately sensitive) or 'Sensitive' (susceptible).

Resistant: implies that the infection it has caused will not respond to treatment with the drug to which it is resistant irrespective of dose or site of infection.

Intermediate: the infection it has caused is likely to respond to treatment when the drug is used in larger doses than normal or when the drug is concentrated at the site of infection. Consideration should be given to using other drugs that may provide more optimal therapy.

Sensitive (Susceptible):- the infection it has caused is likely to respond to treatment when the drug is used in normal recommended doses.

NB: It is necessary to report the first and second choice of antibiotics for a patient's infection unless the strain is resistant.

Susceptibility testing con't…

Table: Zone Size Interpretive Chart for Bauer-Kirby Test

Antimicrobial agent	Disc code	R= mm or less	I= mm	MS= mm	S= mm or more
Amikacin	AN-30	≤15	15-16	-	≥16
Amoxicillin/ Clavulanic Acid - *Staphylococcus* -other organisms	AmC-30	 ≤19 ≤13	 - 14-17	 - -	 ≥20 ≥18
Ampicillin - *Staphylococcus* -G- enterics	AM-10	 ≤28 ≤11	 - 12-13	 - -	 ≥29 ≥14
Azlocillin	AZ-75	≤14	15-17	-	≥13
Aztreonam	ATM-30	≤15	-	16-21	≥22
Carbenicillin -*Enterobacteriaceae* *Pseudomonas*	CB-100	 ≤17 ≤13	 18-22 14-16	 - -	 ≥23 ≥17
Cefamandole	MA-30	≤14	15-17	-	≥18
Cefazolin	CZ-30	≤14	15-17	-	≥18
Cefonicid	CID-30	≤14	15-17	-	≥18
Cefoperazone	CFP-75	≤15	-	16-20	≥21
Cefotaxime	CTX-30	≤14	-	15-22	≥23
Cefotetan	CTT-30	≤12	-	13-15	≥16
Cefoxitin	FOX-30	≤13	-	15-17	≥18
Ceftazidime	CAZ-30	≤14	15-17	-	≥18
Ceftizoxime -*Pseudomonas* -other organisms	ZOX-30	 ≤10 ≤14	 - -	 ≥11 15-19	 - ≥20
Ceftriaxone	CRO-30	≤13	-	14-20	≥21
Cefuroxime	CXM-30	≤14	15-17	-	≥18
Cephalothin	CF-30	≤14	15-17	-	≥18
Chloramphenicol	C-30	≤12	13-17	-	≥18
Cinoxacin	CIN-100	≤14	15-18	-	≥19
Ciprofloxacin	CIP-5	≤15	16-20	-	≥21
Clindamycin	CC-2	≤14	15-20	-	≥21

R = Resistant
I = Intermediate
MS = Moderately Susceptible
S = Susceptible

Susceptibility testing con't…

Table: Zone Size Interpretive Chart for Bauer-Kirby Test

Antimicrobial agent	Disc code	R= mm or less	I= mm	MS= mm	S= mm or more
Doxycycline	D-30	≤12	13-15	-	≥16
Erythromycin	E-15	≤13	14-22	-	≥23
Gentamicin	GM-10	≤12	13-14	-	≥15
Imipenem	IPM-10	≤13	14-15	-	≥16
Kanamycin	K-30	≤13	14-17	-	≥18
Methicillin - *Staphylococcus*	DP-5	≤9	10-13	-	≥14
Mezlocillin	MZ-75	≤12	13-15	-	≥16
Minocycline	MI-30	≤14	15-18	-	≥19
Moxalactam	MOX-30	≤14	-	15-22	≥23
Nafcillin - *Staphylococcus*	NF-1	≤10	11-12	-	≥13
Nalidixic Acid	NA-30	≤13	14-18	-	≥19
Netilmicin	NET-30	≤12	13-14	-	≥17
Nitrofurantoin	F/M-300	≤14	15-16	-	≥17
Norfloxacin	NOR-10	≤12	13-16	-	≥17
Oxacillin - *Staphylococcus*	OX-1	≤10	11-12	-	≥13
Penicillin - *Staphylococcus*	P-10	≤28	-	-	≥29
Piperacillin/Tazobactum -*Enterobactereaceae* - *Staphylococcus* or *P. aeruginosa*	TZP-110	≤17 ≤17	18-20 -	- -	≥21 ≥18
Sulfamethoxazole + Trimethoprim	SXT	≤10	11-15	-	≥16
Tetracycline	Te-30	≤14	15-18	-	≥19
Ticarcillin	TIC-75	≤11	12-14	-	≥15
Ticarcillin/ Clavulanic Acid	TIM-85	≤11	12-14	-	≥15
Tobramycin	NN-10	≤12	13-14	-	≥15
Vancomycin	Va-30	≤9	10-11	-	≥12

R = Resistant
I = Intermediate
MS = Moderately Susceptible
S = Susceptible

Reading of Unclear Result

1.Some proteus strains make swarming growth in the area of inhibition zone.

i.e. There is major inhibition zone and a thin growth of bacterial in the inhibited zone.

► in this case ignore the thin growth and measure the size of the major inhibition zone

2. In case sulfonamides and co-trimoxazole discs a small growth may be seen in the inhibition zone.

►Ignore the small growth and measure the size of the inhibition zone

3. Colonies in the inhibition zone

This is seen either in mixed culture or due to presence of few resistant strains of the test organism.

►In this case repeat the test.

4. Over lapping of inhibition zone

This may be due the error during the placement of the discs.

►Repeat the test and place the disc properly.

Factors Influencing Zone Size in Disc Diffusion Test

1. Inoculum density (bacterial density)

- If the inoculum is heavy – the zone size will be falsely reduced so sensitive strains will be falsely reported as resistance.
- If the inoculum is light – the zone size will become falsely large and resistant species are reported as falsely sensitive.

∴ The turbidity of the inoculum should be exactly equal to the McFarland's standard.

2. Time of disc application

- The discs should be placed 3 – 5 minutes after inoculating the media.

Incase of delay (>3 – 5') the result will be reduced zone size. So sensitive species are reported as resistant.

3. Temperature:

- At higher temperature the bacterial growth is retarded and the whole media will appear as falsely sensitive. And if the temperature is low the bacterial growth is inhibited and the result is falsely increased zone of inhibition.

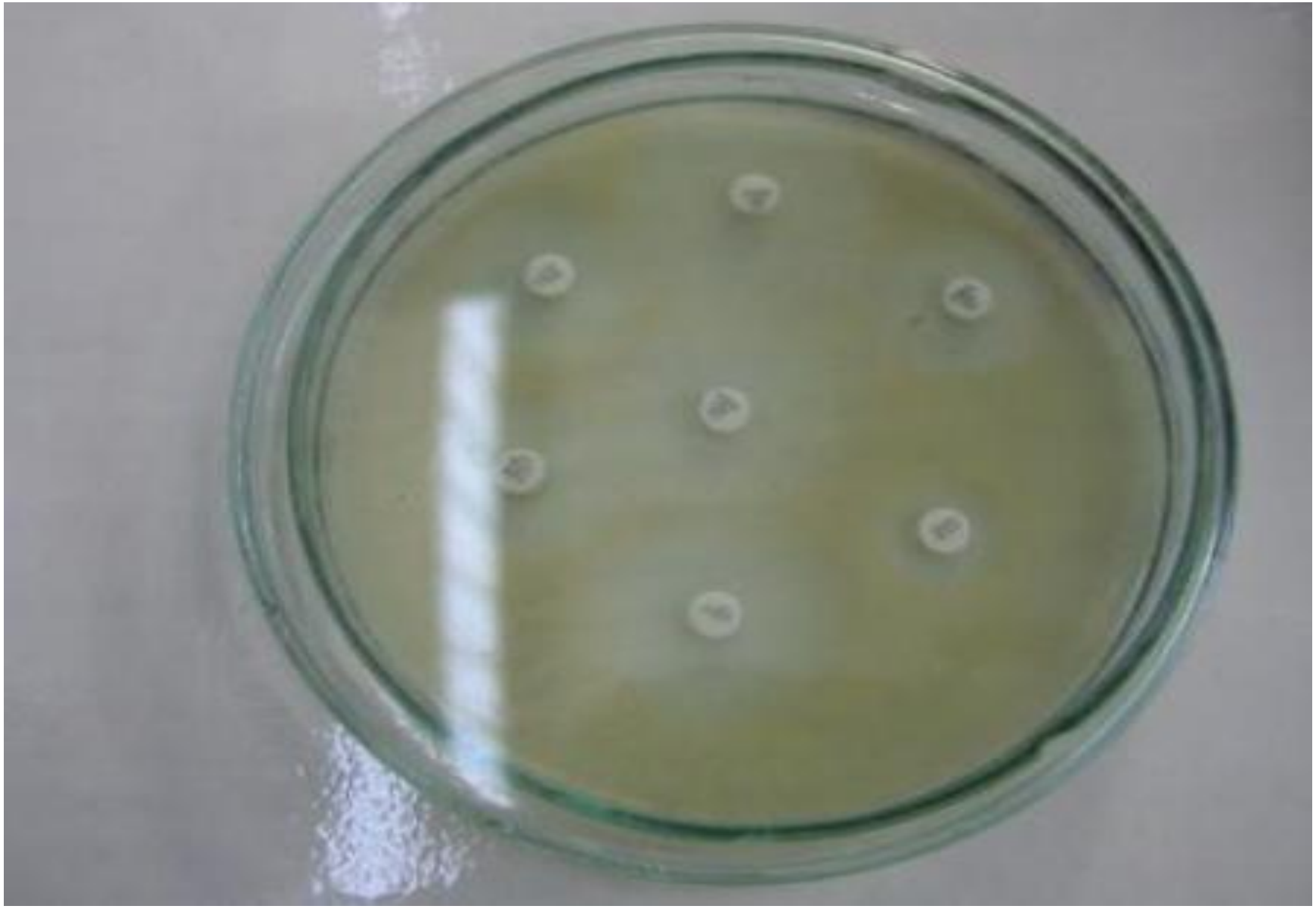
4. Depth of the agar

- When the depth of the agar is less than 4 mm there will be large zone of inhibition and when the media is thick or greater than 4mm then there will be reduced zone of inhibition.

5. Potency of the disk: - is the strength of the antimicrobial agent in the disc. If the potency is deteriorated due to different factors then there will be smaller zone of inhibition which leads to falsely resistant result.

Quality control when performing Kirby – Bauer disc diffusion technique

- Purchase Mueller Hinton agar from a reliable source. Check its pH, Ensure plates contain the correct amount of agar. Check new batches using E. faecalis control strain (ATCC 29212 or 33186) and co – trimoxazole disc.
- Prepare carefully the Inoculum of the test and control organisms to ensure growth is confluent. Renew the turbidity standard every few months.
- Use discs containing the correct amount of antimicrobilel stores discs correctly and do not use them beyond their expiry date. Alternatively, use stable antimicrobial tablets
- Place discs or tablets correctly on the plate, not too close to each other or to the rim of the plate.
- Use appropriate control strains
- Regularly check the temperature of the incubator to ensure test are incubated at 35^{o} C.
- Measure inhibition zones carefully
- Zone diameters with control strains should be within the limits published by NCCLS.



THANK YOU

Chapter four
Basic principles of *Immunologic and Serologic* reactions

- *Learning Objectives*

At the end of the lesson, the students should be able to:

1. List different types of serological tests
2. State the principle of serological tests
3. Describe the application of different serological techniques
4. Identify the advantages and drawbacks of different serological techniques
5. Identify factors, which affect antigen-antibody reaction.

Out line

- **Introduction**
  - Overview on immune system, antibodies, and complement system
  - Definitions of terms
- **Immunological technique**
  - primary binding tests
    - Immunoflurecence tests
    - ELISA
    - RIA
  - Secondary binding tests
    - Agglutination tests
    - Precipitation tests
    - Complement fixation tests
  - Tertiary binding tests
- **Factors affecting antigen antibody reactions**

Introduction

Definitions

- **Serology** is the scientific study of blood serum. In practice, the term usually refers to the diagnostic identification of antibodies in the serum.
  - Such antibodies are typically formed in response to an infection (against a given microorganism)
  - against other foreign proteins (in response, for example, to a mismatched blood transfusion), or to one's own proteins (in instances of autoimmune disease).

- Immune system: the structures, cells, and soluble constituents that allow the host to recognize and respond to foreign stimulus.
- Secondary immune response: the cellular and humoral events that occur when an antigen is encountered for a second or subsequent time.
- Serum: the fluid portion of the blood after the blood clots.
- Specificity: the special affinity between an antigen and its corresponding antibody.
- Susceptibility: having little resistance.

Definitions

- **Antigen (Ag) :**
  - is a substance when introduced parentally into the body stimulates the production of an antibody with which it reacts specifically and in an observable manner.
  - The word originated from the notion that they can stimulate **anti**body **gen**eration

- **Immunogen:**
  - A substance that induces a specific immune response.
- **Epitope or Antigenic Determinant:**
  - The portion of an antigen that combines with the products of a specific immune response.

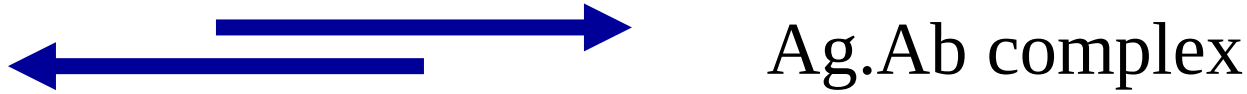
Antigen - Antibody interactions

- The binding follow the **law of mass action** and is a reversible process.
- This union complies with the principles of a chemical reaction that has reached equilibrium.
- When Ag and Ab combines, an immune complex is produced.
- The amount of Ag - Ab complex formation is determined by the association constant of the reaction .
- When the forward reaction rate is faster than the reverse reaction rate Antigen-Antibody complex formation is favored.
- Therefore a higher association constant influences greater immune complex formation at equilibrium

Application of serologic tests

- Serological tests performed for diagnostic purposes when an infection is suspected, in rheumatic illnesses, and in many other situations, such as checking an individual's blood type.
- Serology blood tests help to diagnose patients with certain immune deficiencies associated with the lack of antibodies. In such cases, tests for antibodies will be consistently negative.

- Antibody molecules combine reversibly with antigens to form immune complexes.



- The detection and measurements of these reactions form the basis of serology a sub discipline of immunology.

❖ **Therefore;**

Serology - is the science of measuring antibody or antigen in body fluids.

Immunological techniques

- **Three** groups of immunological techniques are used to detect and measure antigen-antibody combination.
  - ***Primary binding tests***
  - ***Secondary binding tests and***
  - ***Tertiary binding tests.***

Primary binding tests

Examples

- Enzyme linked Immunosorbent assay (ELISA) tests and
- Radioimmunoassay (RIA)
- Western blotting
- Northern blotting
- Southern blotting
- Fluorescence tests

- Widely used in the serological diagnosis of
  - *bacterial,*
  - *viral,*
  - *fungal, and*
  - *parasitic diseases.*
- They are usually sensitive and give reproducible results.

- ELISA

ELISA (*Enzyme Linked Immunosorbent Assay*)

- used in the diagnosis of microbial infections
- they are specific, sensitive, and require only a small amount of specimen
- Reagents used in the ELISA are stable and have a long shelf life which makes for easy distribution to district laboratories
- The results of qualitative ELISA techniques can be read visually
- Large numbers of specimens can be tested at one time and the ELISA can be easily automated for use in epidemiological surveys.

❖There are two main ways of performing ELISA

- *Double antibody technique, to detect antigen*
- *Indirect technique, to detect and assay antibody.*

ELISA Con't…

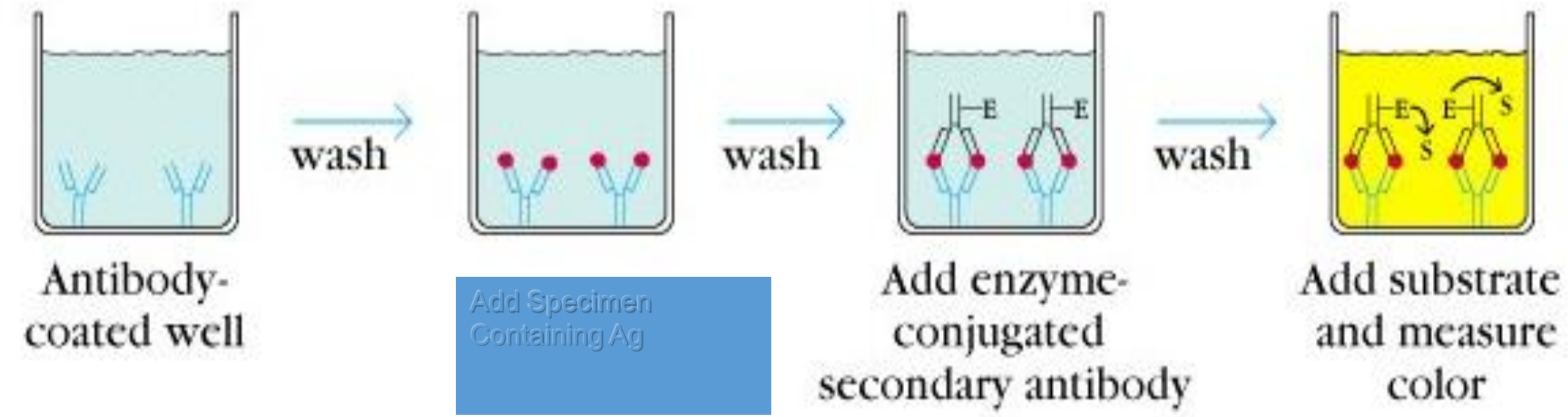
A. Double antibody ELISA

1. Specific antibody is coated on the surface of the well of a microtitration plate (or a test tube), and the specimen is added.
2. After a period of incubation during which the antibody takes up (captures) the antigen from the specimen, the well is washed leaving the antigen attached to the antibody.
3. Enzyme labeled specific antibody (often the same antiserum as that coating the well except it is enzyme linked) is added to detect the presence of the antigen.
4. After a further period of incubation during which the enzyme labeled antibody combines with the antigen, the well is washed and a substrate is added. The enzyme acts on the substrate to give a color change in the fluid.
5. The enzyme activity is stopped by altering the pH of the reaction or denaturing the enzyme.

6. By measuring the color produced, the amount of attached antibody and therefore antigen in the specimen can be estimate.

Application

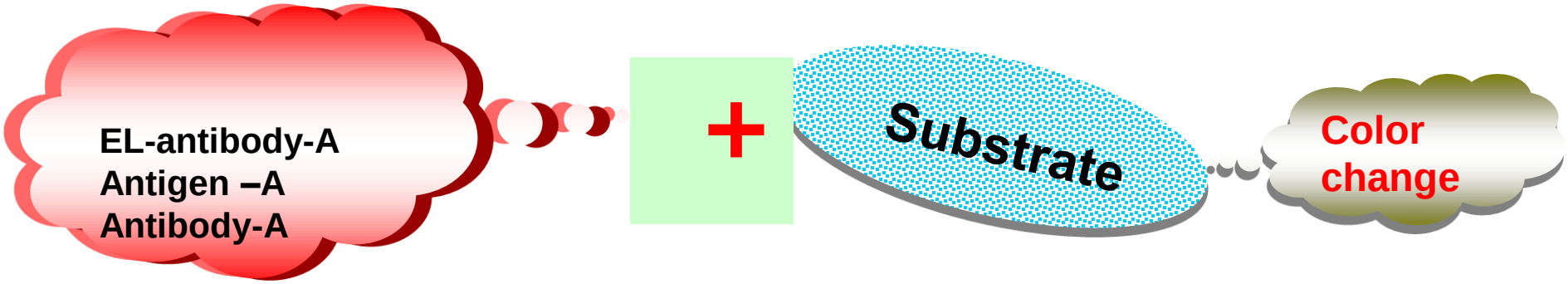
- In developing countries, an important application of the double antibody ELISA is in the diagnosis of
  - *Rotavirus infection in young children*



- *Double antibody ELISA (antigen test)*

Procedure

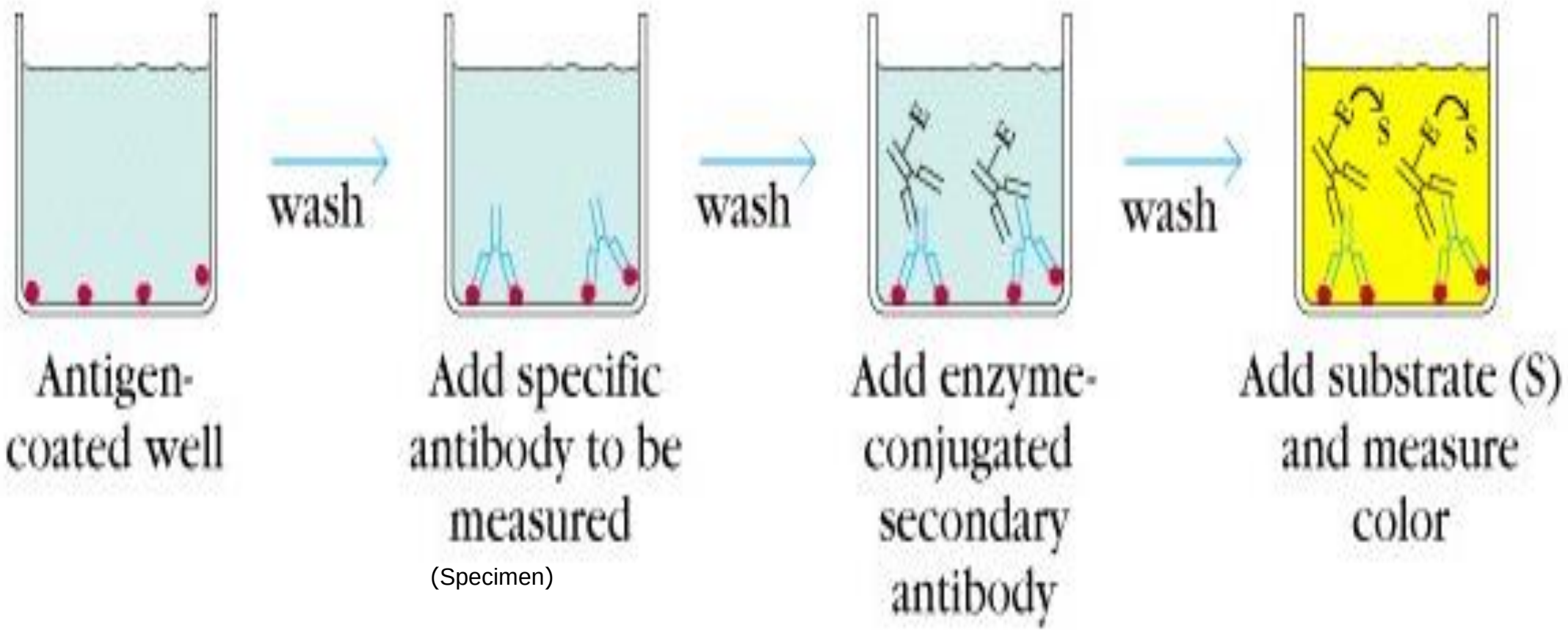
- Prepare all reagents and necessary materials
- Prepare serum from non-hemolyzed blood.
- Take micro titration well plates, which are coated with specific antibody.
- Add positive and negative control in 4micro titration well.
- Add specimens to the micro titration well of 96. If specimen containing antigen combines with antibody.
- Wash the plate by using automatic washer more than 4 times.
- Add enzyme labeled antibody, which attaches to the antigen.
- Wash the procedure.
- Add the substrate, which is hydrolyzed (broken down), gives color changes.
- Read the result.



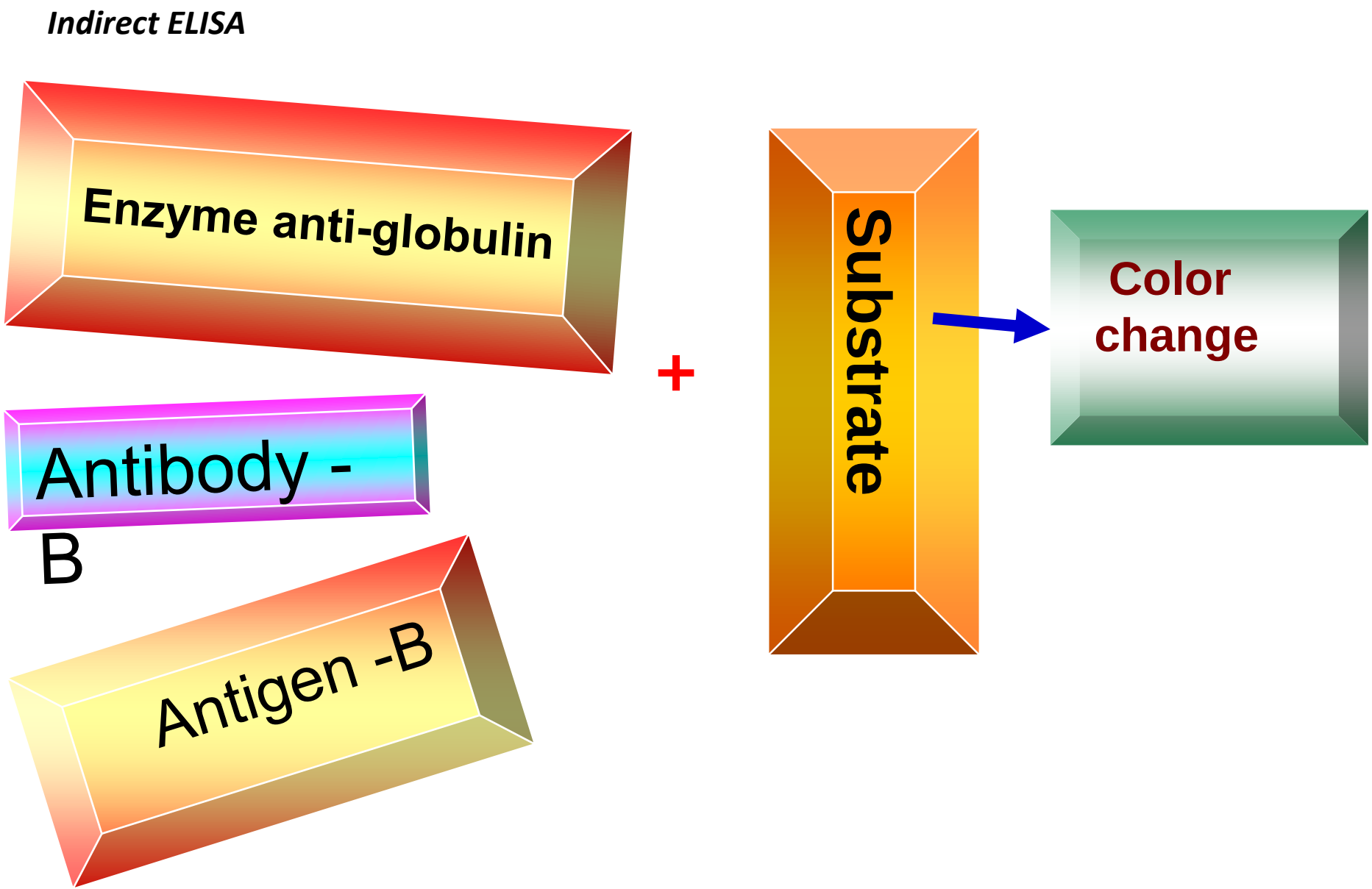
- **B. Indirect ELISA**

- In this technique, known antigen is attached to the inside surface of the well and patient's serum is added. After incubation and washing, enzyme labeled antihuman globulin is reacted with the antibody that has attached to the antigen.
- The presence and concentration of antibody that has reacted with the antigen is shown by a change in color when the substrate is added.
- The intensity of the color is directly proportional to the concentration of antibody in the serum.

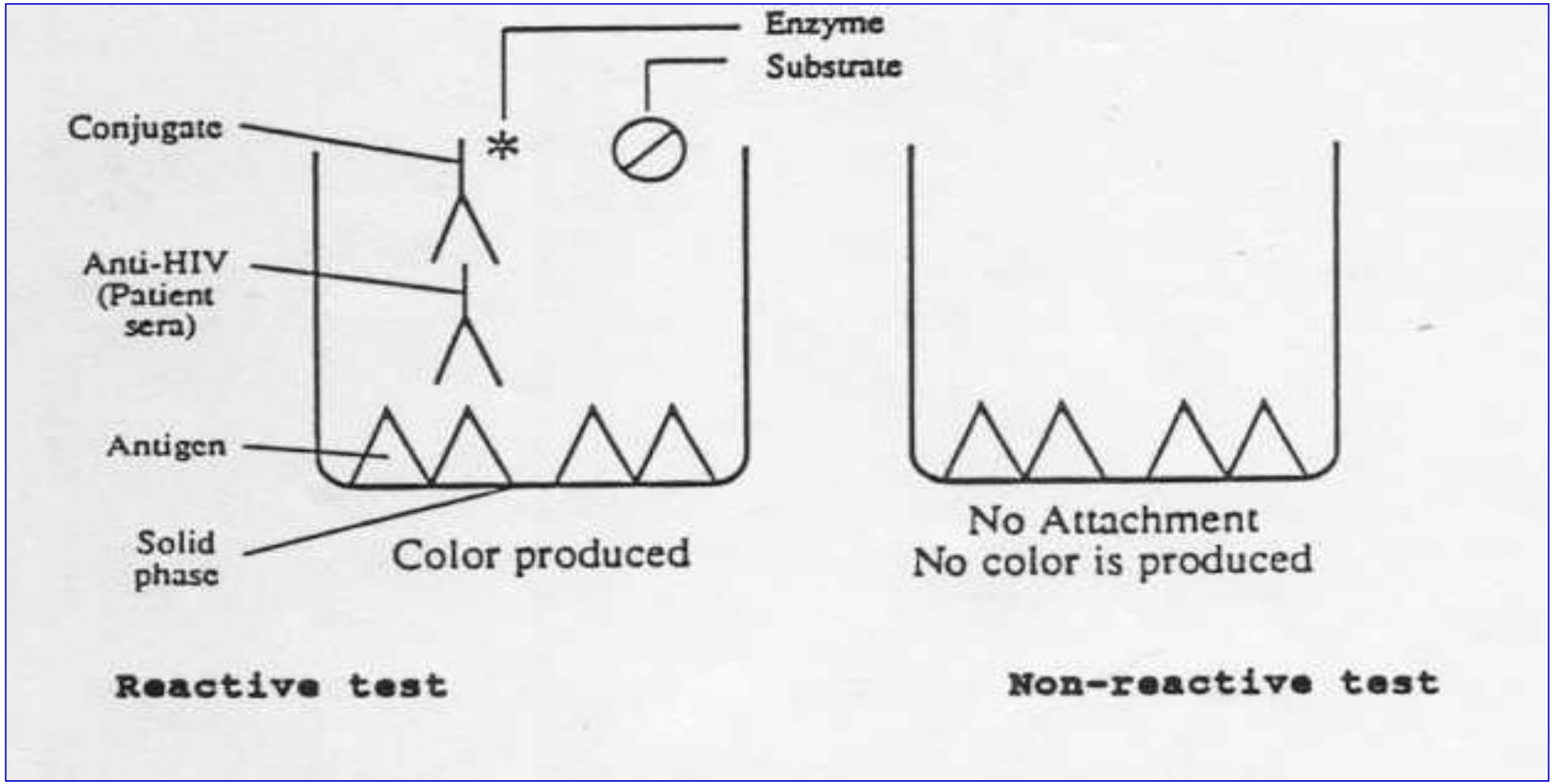
ELISA Con't…


wash
wash
wash
E
S
Antigen-coated well
Add specific antibody to be measured
(Specimen)
Add enzyme-conjugated secondary antibody
Add substrate (S) and measure color

Indirect ELISA principle



Indirect ELISA principle



ELISA Con't…

Procedure

- A micro titration well plate is coated with known antigen.
- Add patent's serum. If the serum contains antibody it combine with antigen.
- Wash carefully by using automatic washer more than 4 times.
- Add enzyme labeled antihuman globulin, which attaches to the antibody.
- Wash carefully.
- Add the substrate, which is hydrolyzed (broken down) by the enzyme to give a color change.
- Read the ELISA results. If it is qualitative ELISA
    - Read by naked eye.
        - The presence or absence of antigen is seen as a simple color change.
    - If it is quantitative antibody techniques
        - Read either
            - by measuring the intensity of color in a spectrometer (spectrophotometer) or
            - by testing dilutions of the test serum and determining the highest dilution that shows a color change.

Radio Immunoassay (*RIA*)

- One of the most sensitive techniques for detecting antigen or antibody is ***RIA***
- 1st developed by two endocrinologist called
  - S.A. Berson and Rosalyn Yalow, in 1960 to determine levels of insulin-anti-insulin complexes in diabetics.

A. Conventional RIA

- is a competitive immunologic procedure
- It measures very low concentrations of antigens (or antibodies) by using radioactively labeled antigens as competitors.
- Radioactive isotopes such as ^{3}H , ^{14}C, ^{35}S, ^{30}P or ^{125}I can be used for labeling
- *Radioactive isotopes* are molecules with unstable nuclei and therefore emit radiation spontaneously.

RIA Con't…

- It is highly sensitive method to detect low concentration of unknown (unlabeled) antigen
- RIA is used to assay:
  - ➢Hormones,
  - ➢Drugs,
  - ➢Enzymes,
  - ➢Microbial antigens

    e.g. Hepatitis B antigen, Carcinoembryonic and α- feto protein antigen.
- It also used to the detection of antibody,
- RIA technique utilizes **three** components
  - Patient antigen

     the specific compound we wish to determine.
  - Labeled antigen

     the same compound as above to which is attached a radioactive label.
  - Antibody

     specific for the sample and labeled antigen.

RIA Con't…

- There are ***two*** assay approaches in conventional *RIA*
  - *Liquid phase Assay*
  - *Solid phase Assay*

- ***Liquid phase assay:***
  - The sample, labeled antigen and the specific antibody are added to the mixture in a solution form.
  - After completion of incubation with the ligand of interest (analyte), a bound-free separation step is performed using different techniques.
- ***Solid phase assay:*** In this assay,

- the specific antibody is added either in a suspension or
- the antibody is covalently bound to the inside wall of the reaction tube.
- Separation of the bound-free fraction is realized by centrifugation or magnetic separation followed by decanting the supernatant or by simply pouring off the reaction mixture if coated tube is used.
- The bound fraction is then washed adequately with appropriate buffered wash solution and made ready for counting.

B. Immunoradiometric Assay (IRMA) (Sandwich Immunoassay)

- Developed with the objective of solving the problems associated with conventional RIA

  - ***Reading assignment (Read more about IRMA)***

- ***Western blot test (WB)***
- ***Southern blotting***
- ***Northern blotting***

You learn it in the chapter of molecular diagnostic techniques

Secondary binding tests

Agglutination tests

- Visible clumping of particulate Ags caused by interaction with a specific Ab
- In district laboratories, agglutination tests are frequently used
  - because compared with other serological tests, they are
    - simpler to perform
    - require no special equipment
    - usually less expensive.

- The fundamental and most commonly used reaction in medical serology laboratory
- Mostly used in clinical lab to demonstrate the interaction of antigen and antibody
- By broad definition, agglutination is simply the clumping of cells into aggregates, often as a result of the combination of an antibody's binding sites with antigen binding sites of the cells.

Agglutination tests Con't…

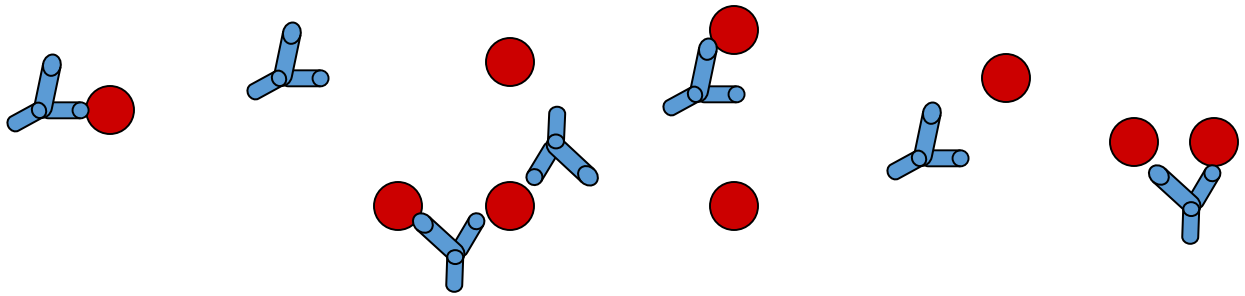
- Agglutination test occurs in two stages:
  - sensitization and
  - lattice formation

Agglutination tests Con't…

Stages of Ag-Ab Reaction…

A-Sensitization-the first phase

- represents the physical attachment of Ab molecules to Ags on the RBC membrane.



Agglutination tests Con't…

Factors affecting the sensitization phase

1. The antigen - antibody ratio

   For example : pro-zone phenomenon.

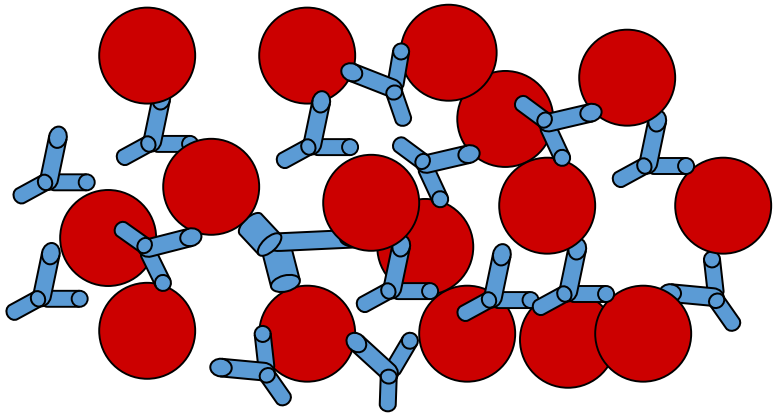
2. Physical conditions such as:
   - PH,
   - Temperature
   - Time of incubation
   - Ionic strength, and
   - Steric hindrance.

Agglutination tests Con't…

Stages of Ag-Ab Reaction…

B- Lattice formation – the second phase

- Is the establishment of cross links between sensitized particles and Abs resulting in clumping.



- Cross linking is influenced by Zeta potential

- Zeta potential
  - is the difference in electrostatic potential between the net charge at the cell membrane and the charge at the surface of shear.

Agglutination tests Con't…

The influence of Ab type on agglutination

- IgM antibodies are more efficient than IgG or IgA antibodies in exhibiting invitro agglutination
- IgG antibodies are less efficient due to:
    - The deep location of the antigen determinants and
    - Restricted movement of the hinge region causes them to be functionally monovalent.

Agglutination tests Con't…

Methods of enhancing agglutination

- Centrifugation
- Treatment with proteolytic enzyme,
- Use of colloids, and
- Addition of anti-human globulin (AHG) reagent.
- Others
  - Poly ethylene glycol PEG)
  - Low Ionic strength saline (LISS)

Agglutination Con’t…

- Agglutination tests can be performed:
  - *i. On slides or tiles*
  - *ii. In tubes*
  - *iii. In micro titration plates*

Agglutination Con't…

i. Slide or tiles agglutination tests

- ***Used:*** To identify bacteria from cultures are difficult to standardize and control and to checl auto-agglutination (false agglutination)
  - Agglutination can be either
  - Rapid and easily performable techniques
  - Gives a reaction in minutes or even seconds.
  - Not usually as sensitive as tube or micro titration techniques.
  - Specificity depends on the reagent used.
    - active or
    - passive.

Active agglutination slide tests

➢Direct agglutination of bacterial antigen with its corresponding antibody.

E.g. the slide agglutination of salmonella, shigella or vibrio cholera using specific antibody, o leptospiral antigen by leptospiral antibodies present in a patients serum in acute leptosprosis.

Agglutination Con't…

Passive agglutination slides and tile tests

- Specific antibody or known antigen is attached to inert particles or cells.
- When the known antigen or antibody combines with its corresponding-antibody or antigen in the specimens the particles or cells are used only to show that an antigen antibody reaction has occurred. Their role in these reactions is therefore passive.
- The substances and cells used as carriers in passive slide agglutination test include:
    - Latex particles
    - Carbon particles
    - Stabilized staphylococcal cells

Agglutination Con't…

ii. Tube agglutination tests

- In tube tests, agglutination occurs in a larger volume of fluid and therefore in an environment that can be more fully controlled.
- Tube tests are usually more sensitive than slide tests.

iii. Micro titration agglutination tests

- Are performed in micro iteration plates
- Now replaced several tube agglutination tests because they are more sensitive, more economical, and easier to perform, and usually give quicker results.

Precipitation tests

- A precipitation reaction may be defined as
  - ✓the visible result of an antigen antibody reaction between a ***soluble antigen*** and ***its antiserum.***
- In addition to these two substances, electrolytes are necessary to bring the process to its desired conclusion and pH and temperature of the mixture also have an effect.
- Antigen and antibody molecules are bound together in lattice of alternate molecules if the reaction is successful.

- Unlike agglutination rxns, ppt rxn involve a small, soluble antigen.
- When the antibody antigen rxn occurs, a few small, soluble lattice complexes form followed by a long period and are less visible than agglutination.

Precipitation Con't…

Precipitation techniques

- Are used to detect and identify antigens in
  - specimens
  - extracts and
  - cultures
- Are used to detect and quantify antibodies in serum.
- Compared with aggt. tests, ppt techniques require more experience in their performance and interpretation.
- Some tests have a low sensitivity.

❖Types of precipitation test

There are three main types of precipitin techniques:

- ➢ Tube precipitation test
- ➢ Gel diffusion tests
- ➢ Counter immunoelectrophoresis tests

Precipitation Con't…

Gel diffusion tests

- When an antibody and its antigen are placed in different regions of an agar gel, they diffuse toward each other and form an opaque band of precipitate at the junction of their diffusion fronts.

- When both antigen and antibody diffuse through the agar this is referred to as **double diffusion.**
- When only the antigen or antibody diffuse, with the corresponding antigen or antibody is being contained in the agar, this is called **single diffusion.**

Precipitation Con't…

Double gel diffusion (*Ouchterlony*)

➲Antigen and antibody diffuse towards each other and where they meet in optimal proportion a visible line of precipitation forms.

➲The thickness of the line of precipitation is a semi quantitative measure of the amounts of antigen and antibody that combine.

❖Example:

☯Elek gel technique

✓used to detect toxogenic strains of *C. diphtheria*

☯Biken test

✓used to detect toxin- producing fecal *E. coli* (ETEC)

Single gel diffusion

- In this technique, specific antibody is incorporated into the agar gel and wells are cut to contain the antigen, which diffuses radially.
- A ring of precipitation forms around a well that contains the corresponding antigen.
- The higher the concentration of antigen, the larger the ring of precipitation will be formed.

Tertiary binding tests

- Tertiary binding tests measure the consequences of immune responses *in vivo*.
- These tests are much more complex than primary and secondary tests but their results reflect the practical significance of the immune response.
  - E.g. measurement of the protective effects of antibody.

Factors affecting antigen antibody reaction

- specificity
- cross reactivity
- temperature
- pH
- ionic strength
- concentration
- intermolecular specificity

Review questions

1. Define:

   A. Antigen

   B. Antibody

   C. Immunogenicity

2. Identify some characteristics of the IgG subtypes

3. What are the characteristic differences between Natural and Immune antibodies?

4. Which classes of antibodies predominate during the primary immune response and secondary immune response?

5. List the factors that affect antigen and antibody interaction

Review questions Con’t…

6. Describe some of the laboratory techniques for infectious disease.

7.List the methods that are routinely used in the blood banking laboratory to enhance agglutination reaction.

8. Write the difference between precipitation and agglutination tests

9. Describe the principle and different types of ELISA techniques.

10. How does the zonal reaction affects test results?

11. Write the advantage and disadvantage of serological test compared with other

Common *Serologic* tests for bacterial and parasitic infections

- **Agglutination tests for Febrile diseases**

  - Salmonella
    - Serologic diagnosis
  - Rickettisia
    - Serologic diagnosis

❖Salmonella

- Are often pathogenic for humans or animals. Transmitted from animal and animal product to humans.
- Three main diseases are
  - Enterocolitis (enteritis)
  - Septicemia (systemic infection)
  - Enteric fever (typhoid fever)

Common *Serologic* tests Con't…

Morphology

- *Microscopic*
  - Vary in length
  - Gram –Ve
  - Rod shape bacilli
  - Most isolates are motile
  - has peritrichous flagella

Classification

- More than 2500 serotypes
- Four serotypes can cause enteric fever
  - *S. paratyphi* A (Serogroup A)
  - *S. paratyphi* B (serogroup B)
  - *S. choleraesusis* (serogroup C1)
  - *S. typhi* (serogroup D)

Antigenic variation

Salmonella has

O-antigen …… somatic

H-antigen…… flagellar antigen

Vi- antigen …… capsular antigen

- Organism may lose H antigens and become non-motile
- Loss of O antigen is associated with a change from smooth to rough colony form.

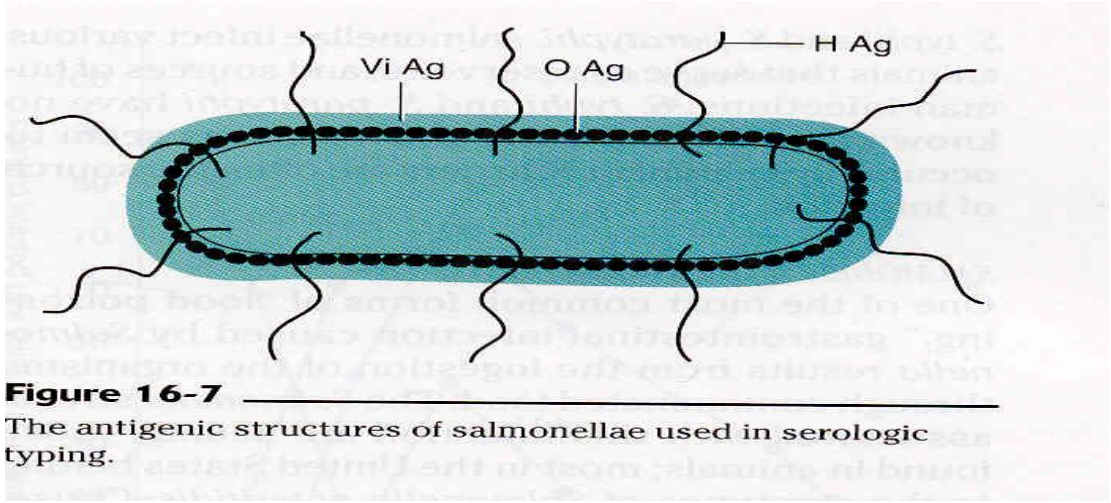


Figure 16-7
The antigenic structures of salmonellae used in serologic typing.

Common *Serologic* tests Con't…

Serological method

- ***Widal Test***

- **New serological methods**
  - **Typhidot (better)**
  - **Dipstick test**

Widal test **is a serological test widely used for diagnosis of enteric fever.**

▷**It is suspension of killed *S. typhi* as Ag. to detect ant-*S-typhi* antibody**

▷**it has many limitations as a diagnostic tool. However, it used to carry out sero-surveys in a community to know the endemicity of any infection**

- Widal test measures titres of serum agglutinins against somatic (O) and flagellar (H) antigens which usually begin to appear during the 2nd week.

- In the absence of recent immunization, a high titer of antibody to O antigen > 1:640 is suggestive but not specific.

Common *Serologic* tests Con't…

- The typhoid bacillus causes two types of agglutinins to be produced. The agglutinins are called:
  - *Flagella (H) agglutinins*
  - *Somatic (O) agglutinins.*
- The patient serum is tested for those O and H antibodies against the antigen suspensions.
- Salmonella antigen suspensions are commercially available from different manufacturer.
- The antigens are stable for rapid slide (screen) testing and tube testing.
- NB: Before use, the suspensions must be allowed to warm to room temperature and be well mixed. The test must be adequately controlled.

Common *Serologic* tests Con't…

- **Methods of widal tests**
  - Tube method
  - Slide method

Slide method

- Modification of tube test method by *Welch* and *Mickle* at 1936

Specimen: *serum/plasma*

- Take clean slide
- Add a drop of serum, which is obtained, from non-hemolyzed blood.
- Add a drop of antigen suspension, which is non-expired,
- Mix well antigen suspension and serum.
- Look for agglutination.

Common *Serologic* tests Con't…

Tube method

- Used to confirm slide test method

_ Sample: serum/plasma

Procedure

1. For each antigen arrange 10 small test tubes in a rack.
2. Place 0.9ml of saline in the 1st tube and 0.5ml in the remaining 9 tubes.
3. Add 0.1ml of fresh cell-free serum to the 1st tube.
4. Mix and transfer 0.5ml to tube 2,3,4,5,6,7,8,and tube 9. From tube 9 discard 0.5ml.
   - *Tube 10 will contain only saline and will serve as a negative control (antigen control)*
5. Mix antigens well and add 0.5ml to each tube. Mix by gently shaking the tubes.
6. The final dilutions are 1:20, 1:40, 1:80, 1:160, etc.
7. Incubate the tubes at 37°C for 2-4 hrs

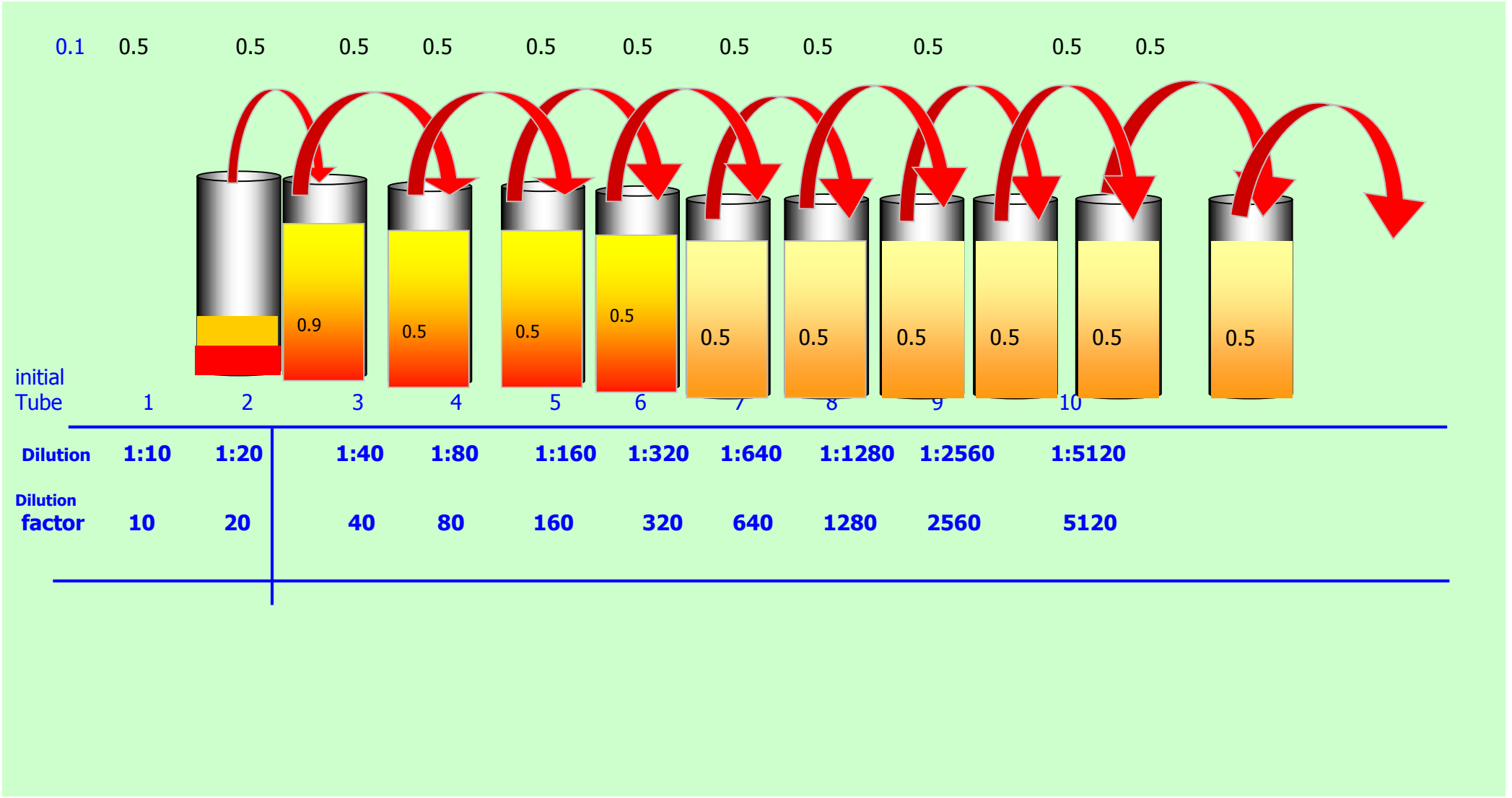
Common *Serologic* tests Con't…

8. Read the negative control at the end of the incubation period.
   - It should have no agglutination.
9. Read the test one row of antigen at a time. For reading a white light shining vertically above the tube is best and using a black background.
10. Shake the tubes gently.

    The H type of agglutination is easily broken up and may be missed. The agglutination is more granular and not so fragile.
11. Report the highest dilution with definite clumps.

Common *Serologic* tests Con't…

Tube dilution



Reporting of Widal Reactions

- The Widal test is reported by giving the titer for both O and H antibodies.
- The antibody titer is taken as the highest dilution of serum in which agglutination occurs.
- The type of agglutination seen with O reactions is granular while that seen with H reactions. Both slide and tube tests are more easily read against a dark background.
- If no agglutination occurs the test should be reported as:
- *S. typhi* O titer less than in 20 (O <1:20)
- *S. typhi* H titer less than in 20 (H <1:20)

Interpretation of the Widal Reaction

- A Negative test does not necessarily mean the patient is not infected. Reaction occurs in infected patients about
  - 50% during the 1st week
  - 80% in the 2nd week,
  - 90-95% in the 3rd or 4th week

Common *Serologic* tests Con't…

- Positive reactions with O antigen occur earlier in the disease than the reaction with the H antigen. H antigen reactions may remain sometimes for years.
- A positive reaction occurs after typhoid vaccination and lasts for 1-5 years.
- In endemic regions, natural agglutinins may be present in the serum.

Common *Serologic* tests Con't…

Causes of positive widal result

- Chronic salmonellosis, associated with Schistosomial infection
- Vaccination with typhoid vaccine
- the patient being tested has typhoid fever
- cross-reaction with non-typhoidal *Salmonella*.
- variability and poorly standardized commercial antigen preparation
- infection with malaria or other enterobacteriaceae

- other diseases such as dengueChronic liver disease
- Immunological disorder like
  - *rheumatic fever*,
  - *multiple mayeloma*,
  - *nephritic syndrome, and*
  - *ulcerative colitis*

Common *Serologic* tests Con't…

- Causes of negative Widal agglutination tests
  - absence of infection by *S typhi*
  - the carrier state
  - an inadequate inoculums of bacterial antigen in the host to induce antibody production
  - technical difficulty or errors in the performance of the test
  - previous antibiotic treatment
  - variability in the preparation of commercial antigens
- *Source of error*

- Hemolyzed blood
- Expired antigen suspension
- Dirty slides or tubes
- Failure in performing technique
- Failure in reading of technique or interpretation

Common *Serologic* tests Con't…

Significance of Widal test

- In enteric fever endemic areas Widal test are very important to diagnose *S. typhi.*
- *When facilities for culturing are not available, the Widal test if performed and interpreted with care can be of value in the diagnosis of typhoid fever.*
- Widal test for typhoid and paratyphoid fever is an *agglutination* test.

Common *Serologic* tests Con't…

- **Agglutination test for rikettsianceae**
  - The genus rickettsia contains several species and sub species.
  - Although classified as bacteria, rickettsia resemble viruses in that they are mostly obligate parasites and are unable to survive as free living organisms.
  - They are about the size of the largest viruses and can just be seen with the light microscope.
  - Rickettsia resembles bacteria also by virtue of their morphology and microscopic visibility.
  - Unlike viruses, however, rickettsia contains both RNA and DNA, multiply by binary fission, have cell wells that contain muramic acid, possess enzymes, and show sensitivity to antiseptic and antibodies.

Common *Serologic* tests Con't…

- Species in the genus rickettsia have been sub divided into three groups of antigenically related microorganisms:
  - typhus group
  - scrub group
  - spotted group

Serologic diagnosis

- The most reliable and useful serological technique for diagnosing rickettsial infections is the indirect fluorescent antibody (IFA) test. It is of value not only in diagnosing acute infections but also in serological epidemiological studies. Another important test is CFT test, which is not sensitive as IFA test.

Common *Serologic* tests Con't…

- **Serology of streptolysisn O and ASO**

Streptolysin O (SLO)

- Streptolysin O (SLO) is a bacterial toxin produced by virtually all strains of S. pyogenes.
- It is one of two extra cellular hemolysis (or cyto lysins), the other being Streptolysin S (SLS).
- SLO is released during infection as indicated by antibody production to it.
- The toxin is a protein with a molecular weight of approximately 70,000, which in its reduced state brings about the lysis of red and white blood cells.

Properties of Streptolysin O

- SLO is so called because of its oxygen liability, and it is quit distinct from SLS.
- It is hemolytically in active in the oxidized form and is characteristic of a group of cytolytic toxins known as the oxygen labile toxins.
- Toxins are produced by several different gram positive bacteria and possess a number of common properties they are activated by sulfhydry (SH) compounds.

Common *Serologic* tests Con't…

- The addition of SLO into culture causes hemomlysis of erythrocytes and toxic effect on mammalian cells.
- It also cardiotoxic.
- SLO may cause interstitial carditis in experimental animals

Importance of the Antistreptolysin O Reaction

- Streptolysin O is antigenic, eliciting the formation of antibodies that effectively neutralize its hemolytic action.
- Streptococcal infection particularly in cases of rheumatic fever and glomerulonephirits

Common *Serologic* tests Con't…

SLO	SLS
• SLO is so called because of its oxygen liability. • Its molecular weight is 70,000 • SLO is synthesized only by growing streptococci • In oxidized form, it is hemolytically inactive. • Several different gram-positive bacteria produce it. • Like other oxygen labile toxins, SLO is activated by SH compounds and is antigenically related, and its biologic activity is completely inhibited by low concentrations of cholesterol and certain related sterols. • SLO hemolyzes erythrocytes. • It is carditoxic • Only cells that contain membrane cholesterol are susceptible to the toxin; therefore, membrane cholesterol is the binding site of SLO. • It is inactivated by the membrane lipid fraction that contains cholesterol. • The mechanism that results in cell lysis is unknown.	• SLS is so called because of its oxygen stability. • It's molecular weight is 2,800 • Both growing and resting cells synthesize it. • SLS can be transferred among the various carriers and finally to the surface of mammalian cells. • SLS is inhibited by lecithin and beta lipoproteins, but not by cholesterol.

Common *Serologic* tests Con't…

Tests for antistreptolysin O

- The most widely used test for SLO is the neutralization test used to detect ASO in serum.
- ASO is important in the investigation of post-streptococcal diseases.

ASO latex slide agglutination test

Principle

- If polystyrene latex particles are coated with streptolysin O antigen visible agglutination will be exhibited in the presence of the corresponding antistreptolysin O antibody.
- If the patient's serum contains more than 200 lU/ml ASO antibody, the excess antibody will agglutinate the antigen in the latex reagent.
- If no agglutination occurs the antibody level is below 200lU/ml.
- When the antibody level is greater than 200lu/ml, further testing is required to estimate the approximate titer of the antibody.

Common *Serologic* tests Con't…

Procedure

Qualitative slide method

1. Allow each component to reach room temperature.
2. Gently shake the latex reagent to disperse the particles.
3. Place a drop of undiluted serum on to the circle of the test slide using the disposable pipettes provided.
4. Add one drop of the latex reagent next to the drop of serum.
5. Spread the latex reagent and serum sample over the entire area of the test circle.
6. See for agglutination by tilting the test slid for 2 min.

Interpretation

- Agglutination indicates a positive result which

  ≥200IU/ml and no agglutination indicates a negative result, which are 200IU/ml provided that the controls have given the expected results.

Common *Serologic* tests Con't…

ASO titration

Principle

- A constant amount of streptolysin O antigen reagent (reduced form) is added to a series of dilution of the patient's serum.
- Following a period of incubation, group O washed human or rabbit red cells is added.
- The tubes are then examined for lysis of the red cells.
- Hemolysisi occurs in those tubes in, which there is insufficient antibody to neutralize the antigen.
- The highest dilution of serum showing no hemolysis is the ASO titer (the titer of ASO antibody in the serum is directly proportional to the reciprocal of the serum dilution).

Procedure

- Always follow the manufacturer instructions, which is written over the manual

Results

- The titer is expressed as the reciprocal of the highest dilution showing microscopic agglutination.

ABO phenotyping

ABO phenotyping has two components:

- **Forward grouping or Direct (cell grouping)**
  - Testing of the red blood cells for the presence of ABO Antigens.
- **Reverse grouping or Indirect (serum grouping)**
  - Testing of serum or plasma for the expected ABO Antibodies

Principle

- When an antigen is mixed with its corresponding antibody under the right conditions it causes **agglutination** or **haemolysis** of the red cells.

Clinical significance

- For safe blood transfusion
- Prevention of Hemolytic Disease of the Fetus and Newborn (HDFN)

ABO phenotyping Reaction

Phenotype	**RBC reaction**		**Serum or plasma reaction**	
	Anti-A	Anti-B	**A-cells**	B-cells
A	+	O	O	+
B	O	+	+	O
AB	+	+	O	O
O	O	O	+	+

+= agglutination, O= no agglutination

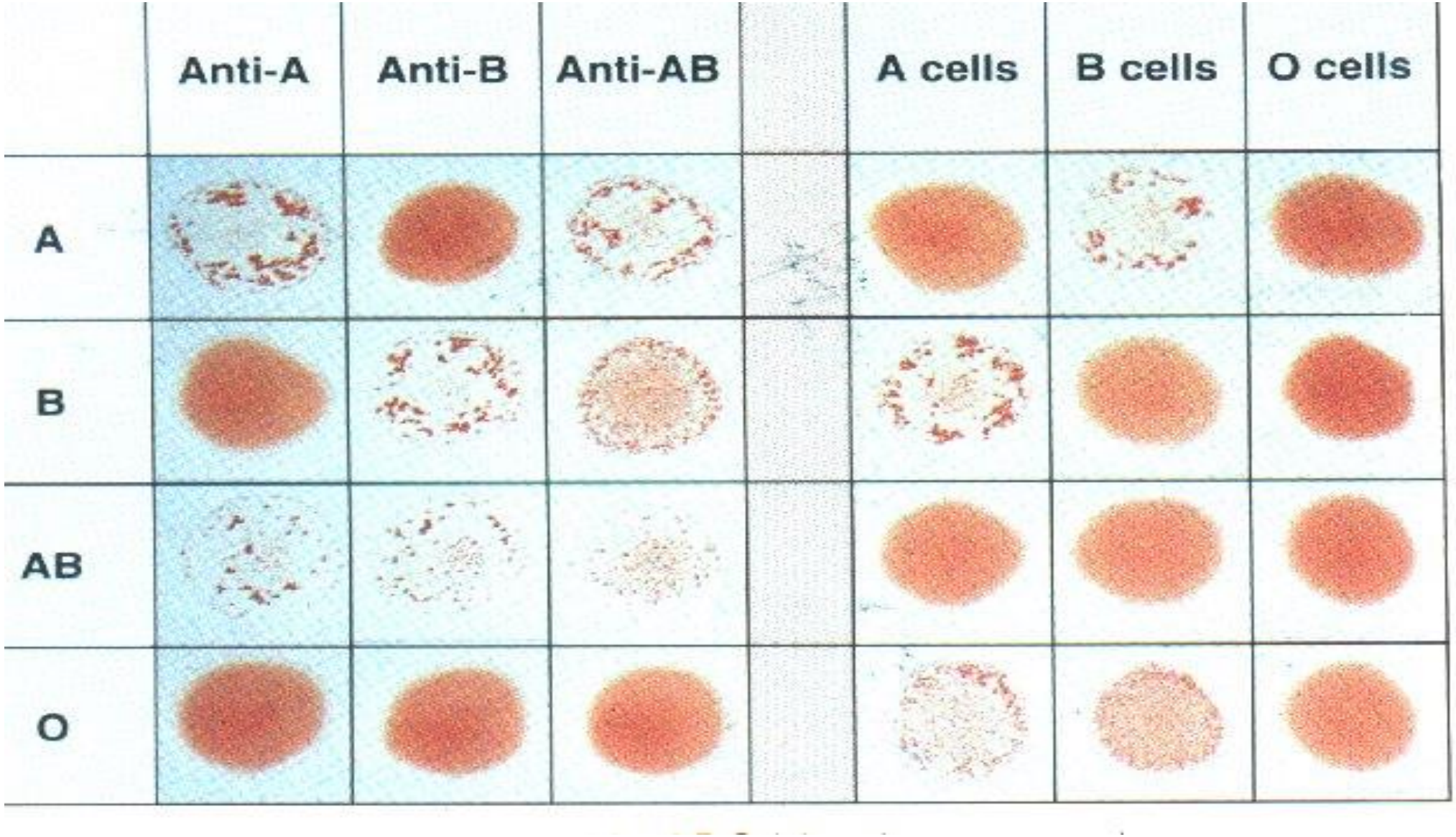
ABO phenotyping con’t…

Methods of Rh Grouping Technique

- Direct slide and
- Direct tube

- Reverse grouping cannot be done due to the absence of naturally occurring Rh antibodies in the serum of persons lacking the corresponding Rh antigen.

Illustration Of The Forward And Reverse Grouping Reaction Patterns Of the ABO groups



ABO phenotyping con’t…

Interpretation of the test result

-Agglutination of red cells ----------Rh positive.

-No agglutination of red cells------Rh negative.

Note: Check negative reactions microscopically**.**

ABO Review Question

1. Describe the general characteristics of human anti-A and anti-B antibodies.
2. Draw and label the structure of ABH antigen.
3. Describe the general characteristics of ABO antigen.
4. Discuss the inheritance and development of the ABO antigens.
5. Discuss how to perform direct and indirect method of ABO blood grouping.
6. List the basic technical errors during ABO grouping.

Common serologic tests for viral infection

- **HIV Serology**

☐ Several laboratory methods are available to screen blood, diagnose infection, and monitor disease progression in individuals infected by HIV.

- HIV tests can be classified into:
  - *Detect antibody*
  - *Identify antigen*
  - *Detect or monitor viral nucleic acids, and*
  - *Estimate of T-lymphocyte numbers (cell phenotyping).*

- The isolation of HIV, its nucleic acid and methods used to detect HIV antigen are mainly used to detect early HIV infection before antibodies develop

HIV Antibody Tests

- Based on a multi test algorithm for detecting antibodies to HIV by using screening and confirmatory tests.

Common *Serologic* tests Con't…

- Common HIV Antibody Tests

A. Enzyme Linked Immunosorbent Assays (ELISA)

B. Rapid Tests

C. Western Blot

Common *Serologic* tests Con't…

B. Rapid Tests

General Description

- Most HIV rapid tests contain antigens to HIV-1 and HIV-2 and detect antibodies to both.
- A positive test result is indicated by clumping, a spot dot or line depending on the test format.
- The sensitivity and specificity of the latest generation of rapid tests are similar to those of ELISA.
- Many rapid tests are under evaluation or are currently in use in developing countries for screening, diagnostic and surveillance purposes.

❖Characteristics of Rapid Tests

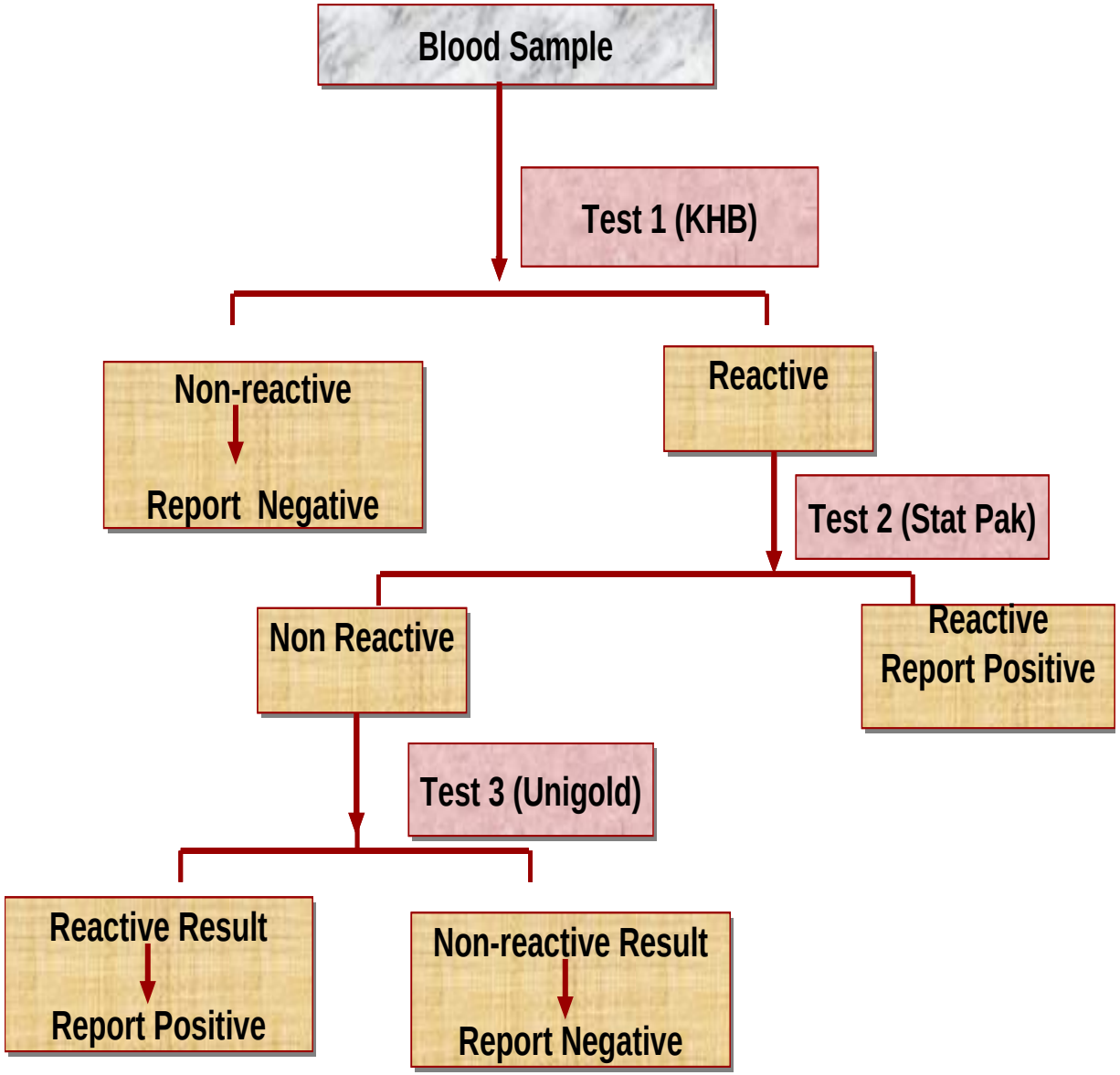
- Rapid tests are useful for small laboratories that routinely perform fewer than 100 HIV tests per day, for laboratories without electricity or equipment, and for geographic areas with limited laboratory infrastructure.
- In some instances, even if a laboratory performs more than 100 tests per day but only during a limited time in a year, rapid tests may be more appropriate than ELISA.

Common *Serologic* tests Con't…

❖Characteristics of Rapid Tests Con't…

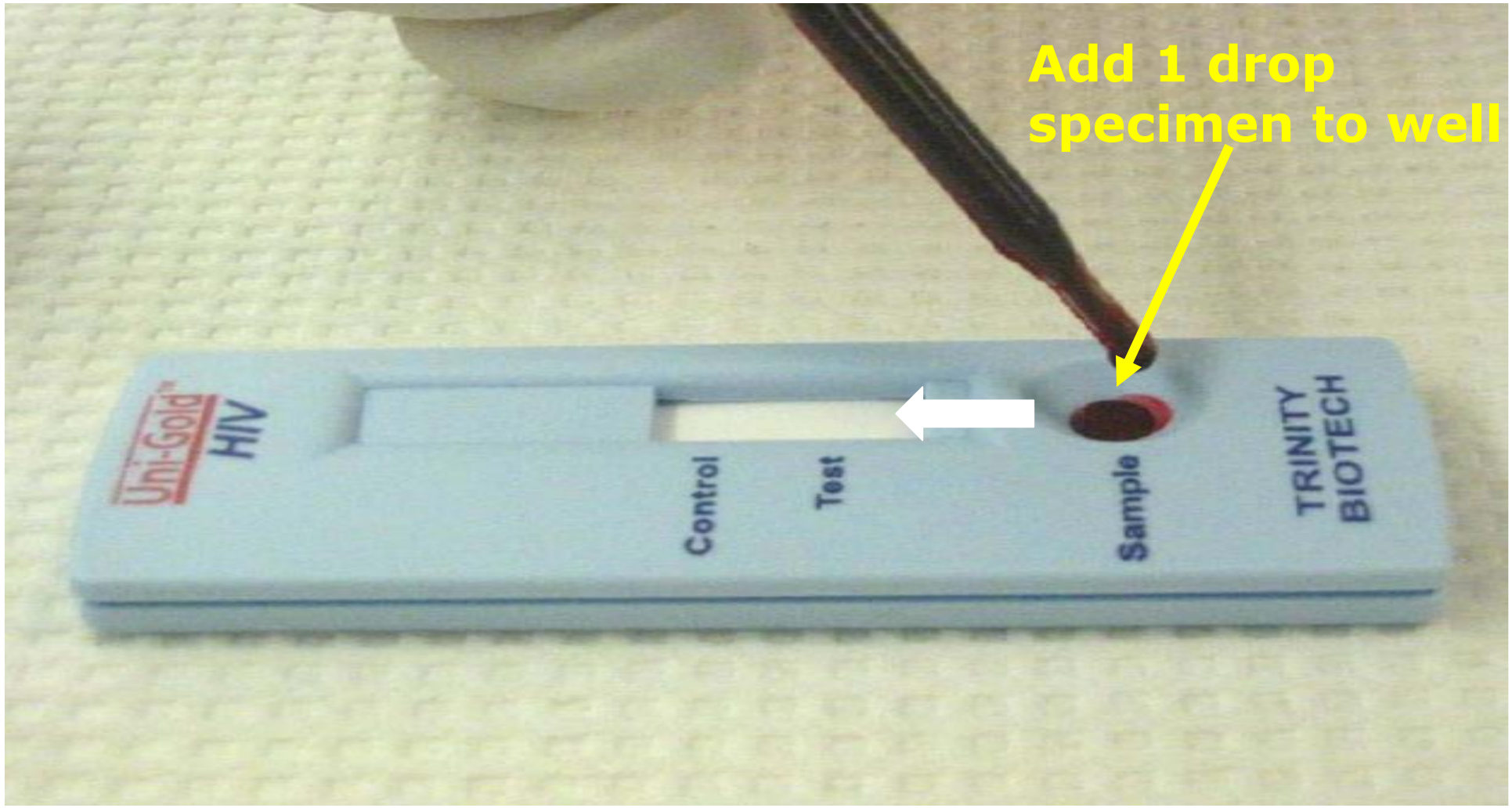
- A result can usually be obtained in less than 45 minutes, and it is easy to interpret.
- Training is required to correctly perform the test and interpret the results.
- The test kits generally contain all reagents needed to run the assay, no additional reagents or equipment is required.
- Many rapid tests do not require electricity, special equipment, refrigeration, or highly skilled staff although a few require refrigeration for heat- sensitive reagents.
- Sensitivity approaches 100%; specificity is >99%
- Negative tests can be reported as negatives
- Positive results should be confirmed
- Useful in situations where immediate results are important to manage decisions

Current HIV test algorithm

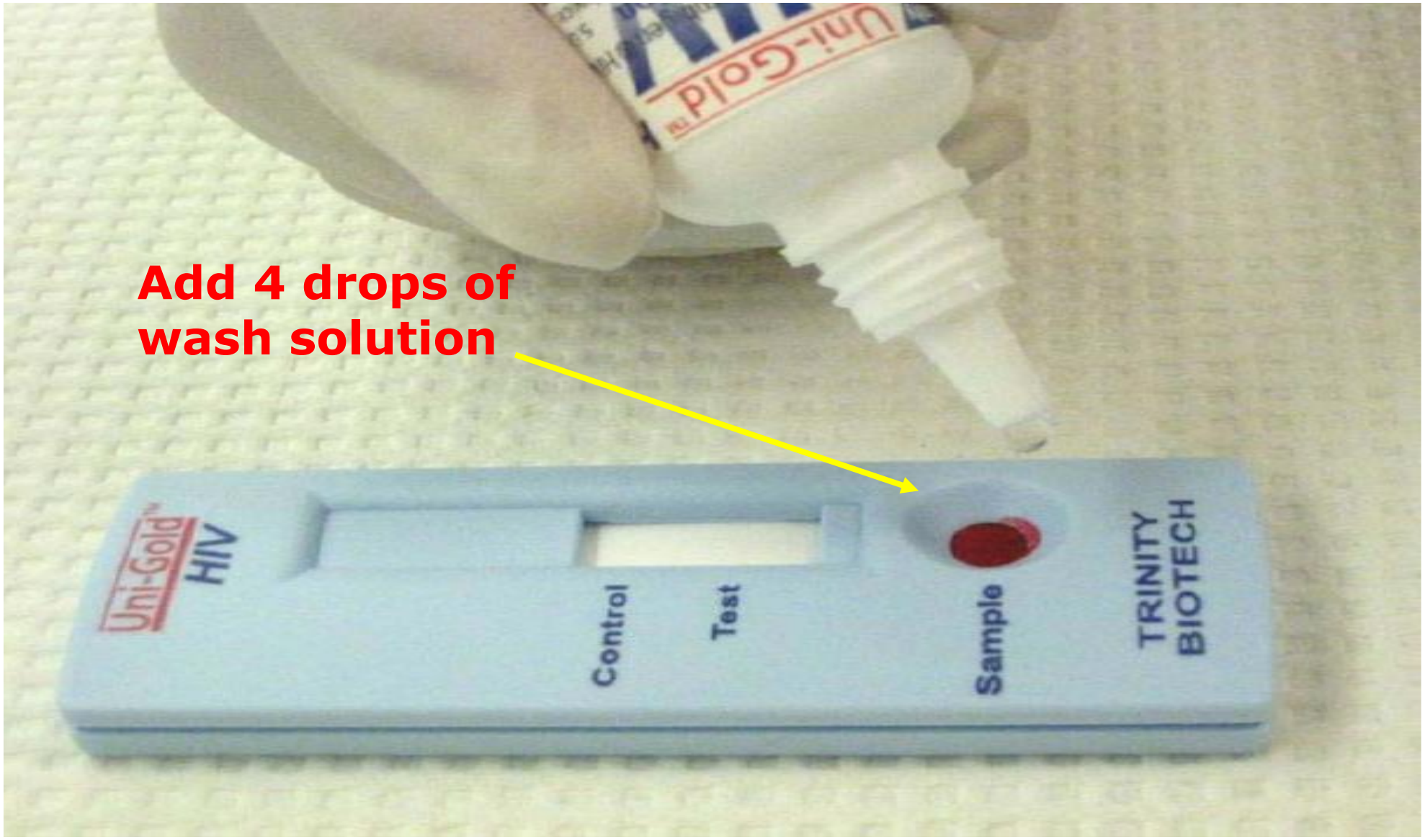


HIV test con’t…

HIV test con’t…



HIV test con't…



HIV test con't…

Serology of *H. pylori* tests

- H. pylori is a gram negative bacilli
- Found in patients with gastritis, peptic ulcers, gastric adenocarcinoma.
- It is highly motile cork screw shaped organism.
- They produce urase
- Colonize stomach
- Do not ferment carbohydrates
- Cultural growth requires a complex media
- humans , primates and pigs are reservoir host for H. pylorior
- Infection is more common in people with low socio economic class in developing ountries.
- Transmission is person to person (fecal- oral transmission)

Serology of *H. pylori Con't…*

- **Laboratory diagnosis**
- Histologic examination = gastric biopsy specimen stained with iemsa stain , hematoxylin –eosin or Gram stain , specificity 100%.
- Urease test = rapid way to detect h.pylori 100%specificity.
- Culture = insensitive method unless multiple biopsy is used.
- Serological diagnosis of H.pylori can be made by detection of antibodies in blood and /or fecal antigen
- Serological tests for detection of antibodies has false positive rate &it doesn't distinguish between active and passive infection

Serology of *hepatitis* tests

- Hepatitis is a generic term referring to an inflammation of liver
- Some viruses primarily infect the liver and are called hepatotrophic viruses.
- These includes hepatitis A, B, C, D, E,

Hepatitis B virus

- DNA virus
- Spread by- blood and contaminated needle

  - Sexual contact

- It has an incubation period of approximately 3 months
- It is more infectious than HIV

Serology of *hepatitis Con't…*

Hepatitis C virus

- RNA virus
- account 90% of transfusion associated hepatitis

4 Hepatitis D virus

- Defective virus that replicates in only HBV infected cells
- RNA virus
- Similar route of transmission with HBV

Serology of *hepatitis Con't…*

- Laboratory diagnosis

Markers for hepatitis B virus

- Hepatitis B surface antigen (HBsAg)
  - Detected during incubation period
  - Found during active phase of the disease
  - It persists for month and years, the individual is carrier and potentially infectious.
  - Produced in the cytoplasm of infected hepatocytes
  - It is the outer lipoprotein coat (envelope)

Serology of *hepatitis Con't…*

- Serological test for HBsAg
  - Agar gel diffusion
    - In gar gel preparation anti HBs or the patient sera is added to the control well.
    - Diffusion of reactants will form precipitation reaction

Advantage

- it demonstrates specificity by the formation of line of identity
- it is the simplest method

Reference

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2. Naville J. Bryant Laboratory Immunology and Serology 3rd edition. Serological services Ltd.Toronto,Ontario,Canada,1992
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