

## LECTURE NOTES

For Medical Laboratory Technology Students

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# *Medical Bacteriology*



**Ethiopia Public Health  
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In collaboration with the Ethiopia Public Health Training Initiative, The Carter Center,  
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# PREFACE

Text book on Medical Bacteriology for Medical Laboratory Technology students are not available as need, so this lecture note will alleviate the acute shortage of text books and reference materials on medical bacteriology.

Since it comprises most of the contents of course outline on medical bacteriology to nursing, pharmacy and environmental science students, it can be used as a main learning material to these category of students.

This lecture note gives emphasis on the knowledge and procedures of medical bacteriology to common pathogens in our country.

At last but not least, the quality of this lecture note is kept updated by continous comments made by users of this lecture note.

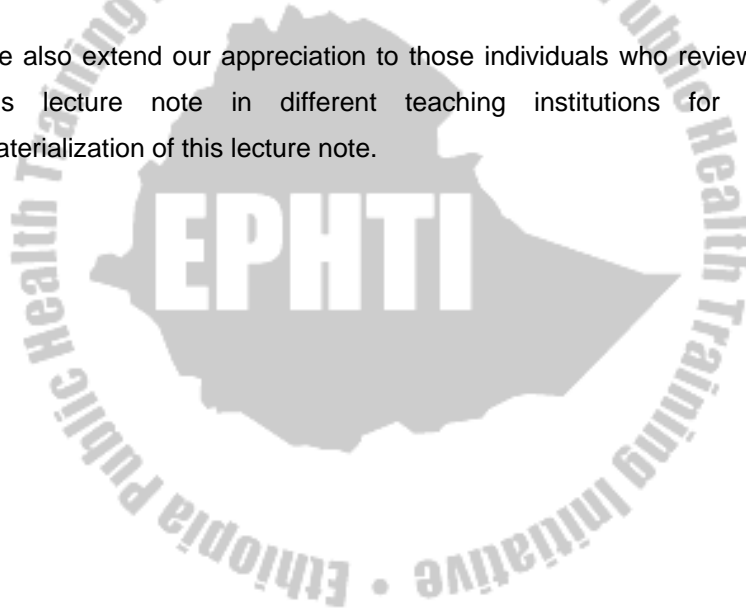
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# ABBREVIATIONS

. AIDS.....	Acquired immunodeficiency syndrome
. AFB .....	Acid fast bacilli
. ATP .....	Adenosine triphosphate
. CO <sub>2</sub> .....	Carbon dioxide
. CSF.....	Cerebrospinal fluid
. CNS .....	Central nervous system
. DNA .....	Deoxy ribonucleotide
. DNase .....	Deoxy ribonucleotidase
. GIT .....	Gastrointestinal tract
. HIV .....	Human immunodeficiency virus
. HPF .....	High power field
. IP .....	Incubation period
. LGV .....	Lymphogranuloma venereum
. NADase .....	Nicotinamide adenine dinucleotidase
. NB .....	Notta Bonne
. O <sub>c</sub> .....	Degree of Celsius
. P <sup>H</sup> .....	Hydrogen ion concentration
. RBC .....	Red blood cell
. RNA .....	Ribonucleotide
. RPR .....	Rapid plasma reagin
. SS agar .....	Salmonella-Shigella agar
. STD .....	Sexually transmitted disease
. UTI .....	Urinary tract infection
. VDRL .....	Venereal disease research laboratory test
. WBC .....	White blood cell

## CHAPTER ONE

### Learning Objective

- At the end of the lesson, the student should be able to:
  1. Identify the structure of bacterial cell
  2. Do simple and differential staining methods
  3. Describe the essential nutrients required for bacterial growth
  4. Describe the mechanisms of genetic variation in bacterial cell
  5. Identify the chemical meanses of sterilization and disinfection, and their effect on bacterial cell
  6. Do and interpret the result of anti-microbial sensitivity testing in vitro

### 1.1 INTRODUCTION TO MICROBIOLOGY

Microbiology is a subject which deals with living organisms that are individually too small to be seen with the naked eye.

It considers the microscopic forms of life and deals about their reproduction, physiology, and participation in the process of nature, helpful and harmful relationship with other living things, and significance in science and industry.

#### Subdivision of microbiology

Bacteriology deals about bacteria.

Mycology deals about fungi.

Virology deals about viruses.

### **History of Microbiology**

Man kind has always been affected by diseases which were originally believed to be visitations by the gods and meant to punish evil doers.

Hippocrates, father of medicine, observed that ill health resulted due to changes in air, winds, water, climate, food, nature of soil and habits of people.

Varro (117-26 BC) said a theory that disease was caused by animated particles invisible to naked eye but which were carried in the air through the mouth and nose into the body.

Fracastorius (1500 G.C.) proposed that the agents of communicable disease were living germs, that could be transmitted by direct contact with humans and animals, and indirectly by objects ; but no proof because of lacking experimental evidence.

Antony Van Leeuwenhoek (1632-1723 G.C.), father of Microbiology, observed "animalcules" using simple microscope with one lens.

He was the first who properly described the different shapes of bacteria.

Although Leeuwenhoek was not concerned about the origin of micro-organism; many other scientists were searching for an explanation for spontaneous appearance of living things from decaying meat, stagnating ponds, fermenting grains and infected wounds.

On the bases of this observation, two major theories were formulated.

1. Theory of Abiogenesis
2. Theory of Biogenesis

**Theory of Abiogenesis** deals with the theory of spontaneous generation; stating that living things originated from non-living things. Aristotle (384-322 BC): The founder of a theory spontaneous generation.

He observed spontaneous existence of fishes from dried ponds, when the pond was filled with rain.

Francesco Redi (1626-1697): He is the scientist who first tried to set an experiment to disprove spontaneous generation.

- He put the meat in a bottle and covered it with a gauze.
- He observed that the flies laid eggs from which the maggots developed.
- He said maggots did not developed from meat but from flies egg.

**Theory of Biogenesis** states that life comes from pre-existing life.

Louis Pasteur (1822-1895 GC) was the scientist who disproved the theory of abiogenesis.

He designed a large curved flask (Pasteur goose neck flask) and placed a sterile growth broth medium. Air freely moved through the tube; but dust particles were trapped in the curved portion of flask.

Microbial growth in the broth was not seen.

Therefore Pasteur proved that micro-organisms entered to substrates through the air and micro-organisms did not evolve spontaneously.

#### **Major contribution of Louis Pasteur**

1. Microbial theory of fermentation
2. Principles and practice of sterilization and pasteurization

3. Control of diseases of silk worms
4. Development of vaccines against anthrax and rabies.
5. Discovery of streptococci

### **The germ theory of disease**

The complete establishment of the germ theory of disease depended on the work of a German scientist, Robert Koch (1843-1910).

### **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 phenomenon
4. Koch's postulates

### **Koch's postulates: proof of germ theory of disease**

A micro-organism can be accepted as a causative agent of an infectious disease only if the following conditions are satisfied.

1. The micro-organism should be found in every case of the disease and under conditions which explain the pathological changes and clinical features.
2. It should be possible to isolate the causative agent in pure culture from the lesion.
3. When such pure culture is inoculated into appropriate laboratory animal, the lesion of the disease should be reproduced.
4. It should be possible to reisolate the bacterium in pure culture from the lesion produced in the experimental animal.
5. Now a days additional postulate is mentioned i.e.

Specific antibody to the bacterium should be detectable in the serum during the course of the disease.

It has not been possible to fulfil every one of Koch's postulates, but by adhering to them as closely as possible, serious errors have been prevented.

### **Exceptions to Koch's postulates**

1. Many healthy people carry pathogens but do not exhibit symptoms of the disease.
2. Some microbes are very difficult or impossible to grow in vitro (in the laboratory) in artificial media. Eg. *Treponema pallidum*
3. Many species are species specific. Eg. *Brucella abortus* cause abortion in animals but no report in humans.
4. Certain diseases develop only when an opportunistic pathogen invades immunocompromised host.

## **1.2. THE MICROBIAL WORLD**

### **TAXONOMIC CLASSIFICATION OF ORGANISMS**

TAXONOMY is the science of organisimal classification.

Classification is the assignment of organisms (species) into an organised scheme of naming. Ideally these schemes are based on evolutionary relationships (i.e. the more similar the name, the closer the evolutionary relationships). Thus, classification is concerned with:-

1. The establishment of criteria for identifying organisms & assignment to groups (what belongs where)



2. The arrangement of organisms into groups of organism of organism (e.g. At what level of diversity should a single species be split in to two or more species?).
3. Consideration of how evolution resulted in the formation these groups.

TAXON:-

- A group or category of related organisms.

Two key characteristics of taxa are:

-Members of lower level taxa (e.g. Species) are more similar to each other than are members of higher level taxa (eg.Kingdom or domain).

-Member of specific taxa are more similar to each other than any are to members of different specific taxa found at the same hierarchical level (eg. Humans are more similar to apes, i.e., comparison between species, than either is similar to, for example, Escherichia coli). Thus once you know that two individuals are member of the same taxon, you can infer certain similarities between the two organisms.

NOTE that taxa are dynamic, changing as our knowledge of organism and evolutionary relationships change

**BINOMIAL NOMENCLATURE**

- Organisms are named using binomial nomenclature ( viruses are exceptions)
- Binomial nomenclature employs the names of the two level taxa, genus and species, to name a specie. Binomial nomenclature includes:

- i. Genus comes before species (e.g., *Escherichia coli*)
- ii. Genus name is always capitalized (e.g., *Escherichia*)
- iii. Species name is never capitalized (e.g., *coli*)
- iv. Both names are always either italicized or underlined ( e.g *Escherichia coli* )
- v. The genus name may be used alone, but not the species name (i.e saying or writing "*Escherichia* " alone is legitimate while saying or writing "*coli*" is not)

#### Strain

- a) A strain in some ways is equivalent to a breed or subspecies among plants or animal. Strain is the level below the species
- b) Two members of the same strain are more similar to each other than either is to an individual that is a member of a different strain, even if all three organisms are members of the same species

#### Bacterial species

- A bacterial species is defined by the similarities found among its members. Properties such as biochemical reactions, chemical composition, cellular structures, genetic characteristics, and immunological features are used in defining a bacterial species. Identifying a species and determining its limits presents the most challenging aspects of biological classification for any type of organism.

- A formal means of distinguishing bacterial species is by employing a dichotomous key to guide the selection of test used to efficiently determine those bacterial properties most relevant to bacterial identification

The five kingdom system

The five kingdom system was first proposed in 1969 and is showing its age

The five kingdoms include:

- i. Plantae ( the plants)
- ii. Fungi ( the fungi)
- iii. Animalia ( the animals )
- iv. Protista ( the unicellular eukaryotes)
- v. Monera ( the prokaryotes)

Kingdom of Monera

Three categories:

- Eubacteria

Are our common, everyday bacteria, some of which are disease – causing; also the taxon from which mitochondria originated.

- Cyanobacteria

Are photosynthetic eubacteria, the taxon from which chloroplast originated

- Archaeobacteria

Are distinctive in their adaptation to extreme environments (e.g., very hot, salty, or acidic) though not all archaeobacteria live in extreme environments.

These distinctions are more phenotypic than they are evolutionary (i.e., a cyanobacteria is a eubacteria, and neither is an archaeobacteria).

Kingdom Protista

Protista like Monera consist mostly of unicellular organisms. Distinctively, however, the members of Kingdom Protista are all eukaryotic while the members of kingdom Monera are all prokaryotic. Some members of protista are multicellular, however Kingdom protista represents a grab bag, essentially the place where the species are classified when they are not classified as either fungi, animals or plants.

Kingdom Fungi

Unlike protists, the eukaryotic fungi are typically non – aquatic species. They traditionally are nutrients absorbers plus have additional distinctive features. They do exist unicellular fungi, which we call yeast

DOMAIN

The domain is a taxonomic category that, depending on point of view, is either above the level of kingdom or supercedes the kingdom. The domain system contains three members

- Eukaryotes ( domain Eukarya )
- Eubacteria ( domain Bacteria)
- Archaeobacteria ( domain Archaea)

#### Viral classification

Classification of viruses is not nearly as well developed as the classification of cellular organisms. Today viruses tend to be classified by their chemical, morphological and physiological attributes (e.g. genome = DNA vs RNA, virion particle = enveloped vs non enveloped and myriad details of their intracellular infection cycles). Binomial nomenclature is not employed to name viruses; instead viruses are named by their common names (e.g., Human Immunodeficiency Virus a.k.a HIV)

#### Dichotomous key

A means of assigning an organism to a specific taxonomic category typically involves the use of specific criteria that may posed as questions ( e.g. What does the organism look like etc. ). Relevant criteria may be arranged as a dichotomous key. In a dichotomous key questions are arranges hierarchically with more general questions are asked first, with questions becoming more specific asked subsequently

#### **EUKARYOTIC CELL**

Eu- true

Karyote- nucleus

The eukaryotic cell has a true membrane bound nucleus, usually containing multiple chromosomes, a mitotic apparatus, a well defined endoplasmic reticulum and mitochondria.

**PROKARYOTIC CELL**

Pro- primitive

Karyote- nucleus

The prokaryotic cell possesses naked DNA with out associated basic proteins, divides amitotically by binary fission and bounded by a semi rigid cell wall.

**Table 1.1. The distinguishing features between Eukaryotic cell and Prokaryotic cell**

<u>Features</u>	<u>Prokaryotic cell</u>	<u>Eukaryotic cell</u>
.Size	1µm	10µm
. Nuclear membrane	Absent	Present
. Chromosome	Single	Multiple
. Nucleolus	Absent	Present
. Histones	Absent	Present
. Sexual reproduction	Absent	Present
. Cytoplasmic ribosomes	70s	80s
. Mitochondria	Absent	Present
. Endoplasmic reticulum	Absent	Present
. Lysosomes	Absent	Present.

. Micro filaments and tubules	Absent	Present
. Site of oxidative phosphorylation	Cell membrane	Mitochondria
. Site of photosynthesis	Cell membrane	Chloroplast
. Peptidoglycan	Present	Absent
. Cell membrane composition	Phospholipids & Proteins	Sterols

### Bacterial Cell

General property:

- Typical prokaryotic cell
- Contain both DNA and RNA
- Most grow in artificial media
- Replicate by binary fission
- Almost all contain rigid cell wall
- Sensitive to antimicrobial agent

### 1.3. STRUCTURE OF BACTERIA

Bacterial structure is considered at three levels.

1. Cell envelope proper: Cell wall and cell membrane.
2. Cellular element enclosed within the cell envelope: Mesosomes, ribosomes, nuclear apparatus, polyamines and cytoplasmic granules.
3. Cellular element external to the cell envelope: Flagellum, Pilus and Glycocalyx.

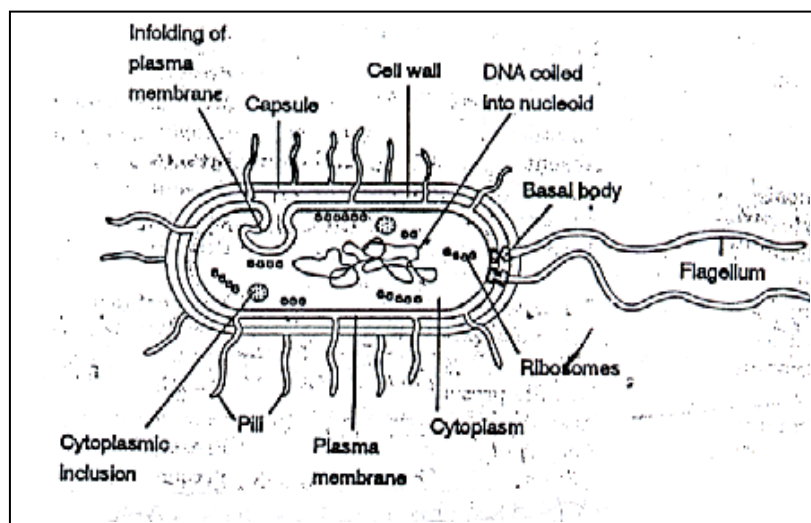


Fig. 1.1 Ultrastructure of Bacteria

## 1. Cell envelope proper

### A. Cell wall

Multi layered structure and constitutes about 20% of the bacterial dry weight.

Average thickness is 0.15-0.5  $\mu\text{m}$ .

Young and rapidly growing bacteria has thin cell wall but old and slowly dividing bacteria has thick cell wall.

It is composed of N-acetyl Muramic acid and N-acetyl Glucosamine back bones cross linked with peptide chain and pentaglycine bridge.

Components of cell wall of Gram negative bacteria

1. Peptidoglycan
2. Lipoprotein



3. Phospholipid
4. Lipopolysaccharide

Components of cell wall of Gram positive bacteria

1. Peptidoglycan
2. Teichoic acid

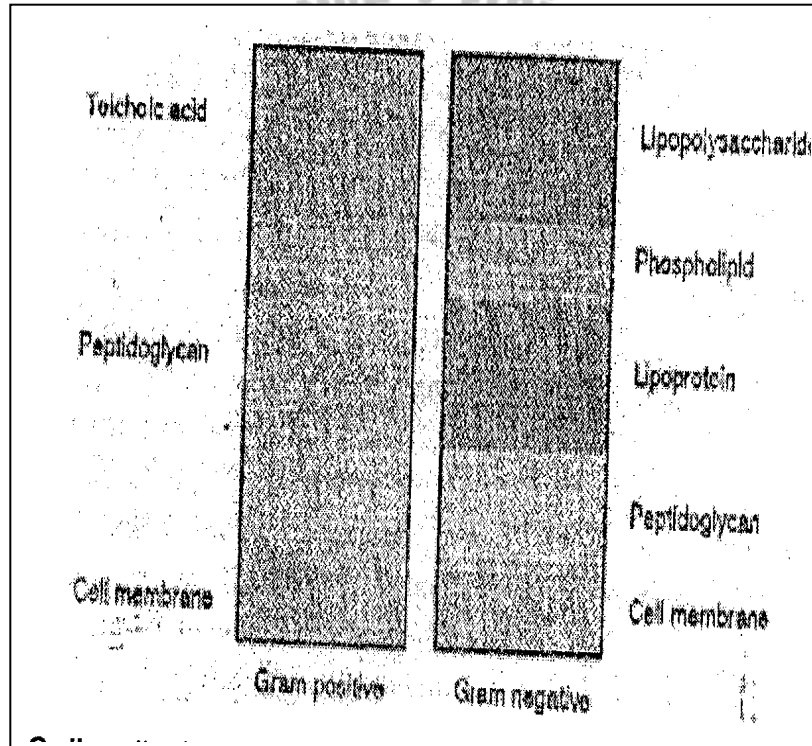


Fig. 1.2 Cell wall of Gram Positive & Gram Negative Bacteria

**Functions of cell wall**

1. Provides shape to the bacterium
2. Gives rigidity to the organism

3. Protects from environment
4. Provides staining characteristics to the bacterium
5. Contains receptor sites for phages/complements
6. Site of action of antibody and colicin
7. Contains toxic components to host

#### **Bacteria with defective cell walls**

Bacteria with out cell wall can be induced by growth in the presence of antibiotics and a hypertonic environment to prevent lysis.

They are of three types:

1. **Protoplasts:** Derived from Gram-positive bacteria and totally lacking cell walls; unstable and osmotically fragile; produced artificially by lysozyme and hypertonic medium: require hypertonic conditions for maintenance.
2. **Spheroplast:** Derived from Gram-negative bacteria; retain some residual but non-functional cellwall material; osmotically fragile; produced by growth with penicillin and must be maintained in hypertonic medium.
3. **L- forms:** Cell wall-deficient forms of bacteria usually produced in the laboratory but sometimes spontaneously formed in the body of patients treated with penicillin; more stable than protoplasts or spheroplasts , they can replicate in ordinary media.

## **B. Cell membrane**

Also named as cell membrane or cytoplasmic membrane

It is a delicate trilaminar unit membrane .

It accounts for 30% of the dry weight of bacterial cell.

It is composed of 60% protein, 20-30% lipids and 10-20% carbohydrate.

### **Function of cell membrane**

1. Regulates the transport of nutrients and waste products into and out of the cell.
2. Synthesis of cell wall components
3. Assists DNA replication
4. Secretes proteins
5. Carries on electron transport system
6. Captures energy in the form of ATP

## **2. Cellular element enclosed with in the cell envelope**

### **A. Mesosomes**

Convolute invagination of cytoplasmic membrane often at sites of septum formation.

It is involved in DNA segregation during cell division and respiratory enzyme activity.

### **B. Ribosomes**

Cytoplasmic particles which are the sites of protein synthesis.

It is composed of RNA(70%) and proteins(30%) and constitutes 90% of the RNA and 40% of the total protein.

The ribosome monomer is 70s with two subunits, 30s and 50s.

### **C. Polyamines**

They are of three types

- . Putrescin
- . Spermidine
- . Spermine

It is found in association with bacterial DNA, ribosomes and cell membrane.

### **Function of polyamines**

1. Antimutagenic.
2. Prevent dissociation of 70s ribosome into subunits.
3. Increase resistance of protoplast lysis.

### **D. Cytoplasmic granules**

. represent accumulated food reserves.

#### **Nature of granules**

- . Glycogen
- . Poly-beta hydroxy butyrate
- . Babes-ernst (Volutin)

### **E. Nuclear apparatus**

Well defined nucleus and nuclear membrane , discrete chromosome and mitotic apparatus are not present in bacteria ; so nuclear region of bacteria is named as nuclear body, nuclear apparatus and nucleoid.

Bacterial genome consists of single molecule of double stranded DNA arranged in a circular form.

Besides nuclear apparatus, bacteria may have extra chromosomal genetic material named as plasmids.

Plasmids do not play any role in the normal function of the bacterial cell but may confer certain additional properties(Eg. Virulence, drug resistance) which may facilitate survival and propagation of the micro- organism.

### **3. Cellular element external to the cell envelope**

#### **A. Glycocalyx (capsule and slime layer)**

Capsule is gel firmly adherent to cell envelope.

Slime is gel easily washed off from cell envelope.

All bacteria have at least a thin slime layer.

Capsule is composed of polysaccharide and protein(D-Glutamate of *Bacillus anthracis*)

#### **Features of capsule**

1. Usually weakly antigenic.
2. Not necessary for viability.
3. Endows virulence.
4. Protects from phagocytosis.
5. Capsulated strains are invariably non-motile.
6. Visualized by negative staining and capsule staining.
7. Detected by quellung phenomenon.

## **B. Flagellum**

It is the organ of locomotion in bacterial cell and consists of three parts. These are .The filament

- . The hook
- . The basal body

The basal body and hook are embedded in the cell surface while the filament is free on the surface of bacterial cell.

Their presence in bacterial cell is detected by

- . Hanging drop preparation
- . Swarming phenomenon on surface of plate agar
- . Motility media
- . Special staining methods
- . Silver impregnation methods
- . Dark –field microscopy
- . Electron microscopy

Size: 3-20 $\mu$ m in length and 0.01-0.013 $\mu$ m in diameter.

It is composed of protein named as flagellin.

The flagellar antigen in motile bacterium is named as H (Hauch) antigen.

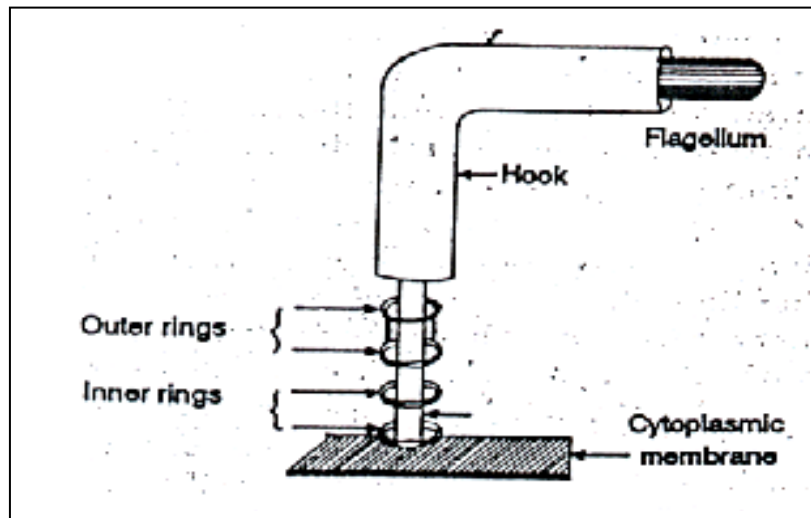


Fig. 1.3 Components of Bacterial Flagellum

#### Flagellar arrangements

1. Atrichous: Bacteria with no flagellum.
2. Monotrichous: Bacteria with single polar flagellum.
3. Lophotrichous: Bacteria with bunch of flagella at one pole.
4. Amphitrichous: Bacteria with flagella at both poles.
5. Peritrichous: Bacteria with flagella all over their surface.

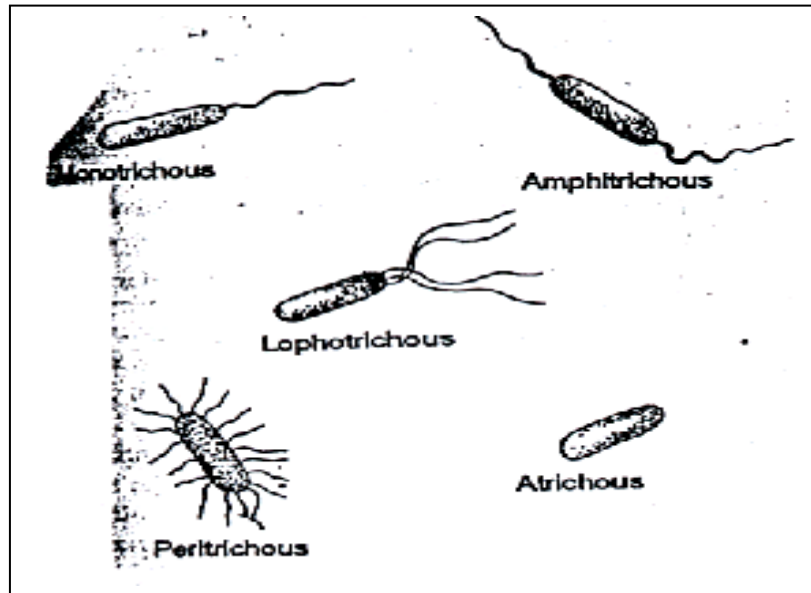


Fig. 1.4 Flagellar arrangements

**Endoflagella (axial filament)**

It is the organ of motility found in periplasmic space of spirochetes.

**C. Pili (fimbriae)**

It is hair like structure composed of protein (pilin)

**Two types (Based on function)**

- . Common pili: The structure for adherence to cell surface.
- . Sex pili: The structure for transfer of genetic material from the donor to the recipient during the process of conjugation.



**Table 1.2. Comparison between flagella and pili**

Character	Flagella	Pili
. Size	Large	small
. Thickness	+++	+
. Origin	Cell membrane	Cell wall
. Organ of locomotion	+	-
. Organ of adhesion	-	+
. Required for conjugation	-	+

#### **D. Spores**

Resting cells which are capable of surviving under adverse environmental conditions like heat, drying, freezing, action of toxic chemicals and radiation.

Bacterial spore is smooth walled and oval or spherical in shape.

It does not take up ordinary stains.

It looks like areas of high refractivity under light microscope.

It is significant in spread of disease and indicator of sterility of materials.

Spores are detected by

- . Simple staining methods
- . Special staining methods

#### **Arrangements of spores**

1. No bulging of cell wall
  - . Oval central
  - . Oval sub terminal

- . Spherical central
- 2. Bulging of cell wall
  - . Oval sub terminal
  - . Oval terminal
  - . Spherical terminal
  - . Free spore

#### 1.4. Classification of bacteria

Bacterial classification depends on the following characteristics.

1. Morphology and arrangement
2. Staining
3. Cultural characteristics
4. Biochemical reactions
5. Antigenic structure
6. Base composition of bacterial DNA

Morphology and staining of bacteria are the commonly used characteristics to classify bacteria.

##### 1. Morphology of bacteria

When bacteria are visualized under light microscope, the following morphology are seen.

1. Cocci (singular coccus): Round or oval bacteria measuring about 0.5-1.0 $\mu$ m in diameter. They are found in single, pairs, chains or clusters.

2. Bacilli (singular bacillus): Stick-like bacteria with rounded, tapered, square or swollen ends; with a size measuring 1-10 $\mu$ m in length by 0.3-1.0 $\mu$ m in width.
3. Coccobacilli (singular coccobacillus): Short rods.
4. Spiral: Spiral shaped bacteria with regular or irregular distance between twisting.  
Eg. Spirilla and spirochaetes

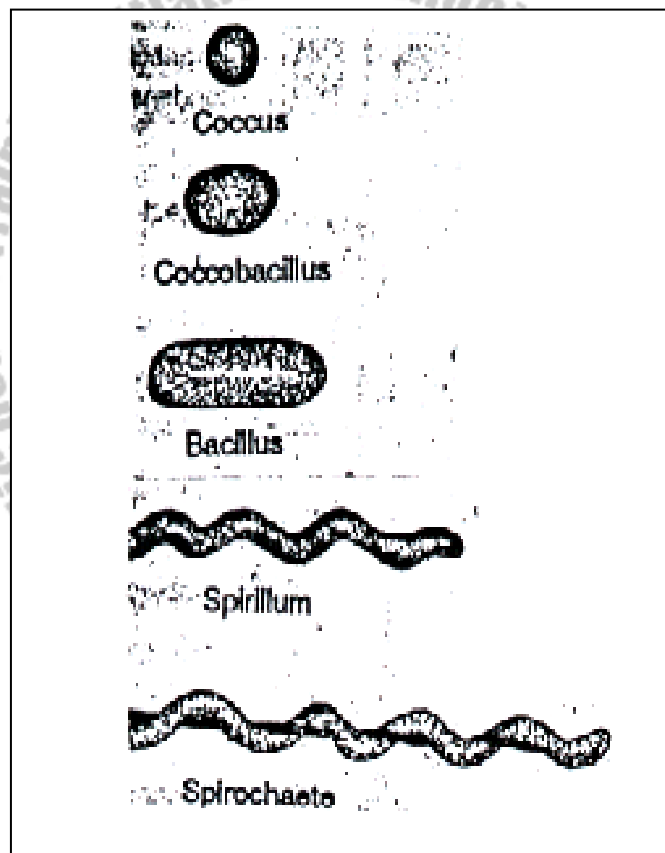


Fig. 1.5 Morphology of Bacteria

## 2. Staining of bacteria

Bacterial staining is the process of coloring of colorless bacterial structural components using stains (dyes). The principle of staining is to identify microorganisms selectively by using dyes, fluorescence and radioisotope emission.

Staining reactions are made possible because of the physical phenomena of capillary osmosis, solubility, adsorption, and absorption of stains or dyes by cells of microorganisms.

Individual variation in the cell wall constituents among different groups of bacteria will consequently produce variations in colors 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.

There are many types of affinity explaining this attraction force:

1. hydrophobic bonding
2. reagent-cell interaction
3. reagent-reagent interaction
4. ionic bonding
5. hydrogen bonding
6. covalent bonding

Why are stains not taken up by every microorganism?

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)

### Properties of dyes

Why dyes color 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 colorless. E.g., acid fuschin absorbs blue green and transmit red.

### General methods of staining

#### 1. Direct staining

Is the process by which microorganisms are stained with simple dyes. E.g., methylene blue

#### 2. Indirect staining – is the process which needs mordants.

A mordant is the substance which, when taken up by the microbial cells helps make dye in return, serving as a link or bridge to make the staining recline possible.

It combines with a dye to form a colored "lake", which in turn combines with the microbial cell to form a " cell-mordant-dye-complex".

It is an integral part of the staining reaction itself, without which no staining could possibly occur. E.g., iodine.

A mordant may be applied before the stain or it may be included as part of the staining technique, or it may be added to the dye solution itself.

An accentuator, on the other hand is not essential to the chemical union of the microbial cells and the dye. It does not participate in the staining reaction, but merely accelerate or hasten the speed of the

staining reaction by increasing the staining power and selectivity of the dye.

**Progressive staining**

- is the process whereby microbial cells are stained in a definite sequence, in order that a satisfactory differential coloration of the cell may be achieved at the end of the correct time with the staining solution.

**Regressive staining**

- with this technique, the microbial cell is first over stained to obliterate the cellular details, and the excess stain is removed or decolorized from unwanted part.

**Differentiation (decolorization)**

- is the selective removal of excess stain from the tissue from microbial cells during regressive staining in order that a specific substance may be stained differentially from the surrounding cell.

Differentiation is usually controlled visually by examination 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.

Biological stains are dyes used to stain micro-organisms.

**Types of microbiological stains**

- . Basic stains
- . Acidic stains
- . Neutral stains

**NB:** This classification is not based on P<sup>H</sup> of stains.

**Basic stains** are stains in which the coloring substance is contained in the base part of the stain. The acidic part is colorless. Eg.

**Acidic stains** are stains in which the coloring substance is contained in the acidic part of the stain. The base part is colorless. It is not commonly used in microbiology laboratory.

Eg. Eosin stain

**Neutral stains** are stains in which the acidic and basic components of stain are colored.

Neutral dyes stain both nucleic acid and cytoplasm. Eg. Giemsa stain

### **Types of staining methods**

1. Simple staining method
2. Differential staining method
3. Special staining method

#### **1. Simple staining method**

It is type of staining method in which only a single dye is used.

Usually used to demonstrate bacterial morphology and arrangement

Two kinds of simple stains

1. Positive staining: The bacteria or its parts are stained by the dye.

Eg. Carbol fuchsin stain

Methylene blue stain

Crystal violet stain

**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 like 1% methylene blue, 1% carbolfuchsin or 1% gentian violet for 1 minute.
  - . Wash off the stain with water.
  - . Air-dry and examine under the oil immersion objective.
2. Negative staining: The dye stains the background and the bacteria remain unstained. Eg. Indian ink stain Negrosin stain

**2. Differential staining method**

Multiple stains are used in differential staining method to distinguish different cell structures and/or cell types. Eg. Gram stain and Ziehl-Neelson stain

**A. Gram staining method**

Developed by Christian Gram.

Most bacteria are differentiated by their gram reaction due to differences in their cell wall structure.

Gram-positive bacteria are bacteria that stain purple with crystal violet after decolorizing with acetone-alcohol.

Gram-negative bacteria are bacteria that stain pink with the counter stain (safranin) after losing the primary stain (crystal violet) when treated with acetone-alcohol.



**Required reagents:**

- . Gram's Iodine
- . Acetone-Alcohol
- . Safranin

**Procedure:**

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

**Interpretation:**

- . Gram-positive bacterium .....Purple
- . Gram-negative bacterium .....Pink

### **B. Ziehl-Neelson staining method**

Developed by Paul Ehrlich in 1882, and modified by Ziehl and Neelson

Ziehl-Neelson stain (Acid-fast stain) is used for staining Mycobacteria which are hardly stained by gram staining method.

Once the Mycobacteria is stained with primary stain it can not be decolorized with acid, so named as acid-fast bacteria.

#### **Reagents required:**

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

#### **Procedure for Ziehl-Neelson staining method**

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 spirit 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.

Interpretation:

Acid fast bacilli.....Red  
Back ground.....Blue

Reporting system

0 AFB/100 field .....No AFB seen  
1-2 AFB/ 300 field..... Scanty  
1-10 AFB/100 field.....1+  
11-100AFB/100 field.....2+  
1-10 AFB/field.....3+  
>10 AFB/field.....4+

NB: AFB means number of acid fast bacilli seen.

### 3. Special stains

- a. Spore staining method
- b. Capsule staining method

#### a. Spore staining method

**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. Apply 1% safranin for 30 seconds.

5. Wash with clean water.
6. Dry and examine under the oil immersion objective.

**b. Capsule staining method: Welch method**

**Procedure:**

1. Prepare smear of capsulated bacteria.
2. Allow smear to air-dry; do not fix the smear.
3. Cover the smear with 1% aqueous crystal violet for 1 minute over steaming water bath.
4. Wash with 20% copper sulfate solution. Do not use water.
5. Dry and examine under the oil immersion objective.

**1.5. CULTIVATION OF BACTERIA IN CULTURE MEDIA**

**Culture media**

It is the media containing the required nutrients for bacterial growth.

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

**Common ingredients of culture media**

- . Peptone
- . Meat extract
- . Yeast extract
- . Mineral salts
- . Carbohydrates
- . Agar

. Water

**Peptone:** Hydrolyzed product of animal and plant proteins: Free amino acids, peptides and proteoses (large sized peptides).

It provides nitrogen; as well carbohydrates, nucleic acid fractions, minerals and vitamins.

**Meat extract:** supply amino acids, vitamins and mineral salts.

**Yeast extract:** It is bacterial growth stimulants.

**Mineral salts:** these are: Sulfates as a source of sulfur.

. Phosphates as a source of phosphorus.

. Sodium chloride

. Other elements

**Carbohydrates:** Simple and complex sugars are a source of carbon and energy.

. Assist in the differentiation of bacteria.

Eg. Sucrose in TCBS agar differentiates vibrio species.

Lactose in MacConkey agar differentiates enterobacteria.

**Agar:** It is an inert polysaccharide of seaweed.

It is not metabolized by micro-organism.

**Property**

. It has . high gelling strength

. high melting temperature (90-95 °C)

. low gelling temperature

. It forms firm gel at 1.5% W/V concentration.

. It forms semisolid gel at 0.4-0.5% W/V concentration.

Uses:

. Solidify culture media

. May provide calcium and organic ions to inoculated bacteria.

### **Water**

Deionized or distilled water must be used in the preparation of culture media.

### **Types of culture media**

#### **1. Basic /Simple / All purpose media**

It is a media that supports the growth of micro-organisms that do not require special nutrients.

Uses :

- . To prepare enriched media
- . To maintain stock cultures of control bacterial strains
- . To subculture pathogenic bacteria from selective/differential medium prior to performing biochemical or serological tests.

Eg. Nutrient Broth

Nutrient Agar

#### **2. Enriched media**

Media that are enriched with whole blood, lyzed blood, serum, special extracts or vitamins to support the growth of pathogenic bacteria.

Eg. Blood Agar

Chocolate Agar

### **3. Enrichment media**

Fluid media that increases the numbers of a pathogen by containing enrichments and/or substances that discourage the multiplication of unwanted bacteria.

Eg. Selenite F broth media

Alkaline peptone water

### **4. Selective media**

Media which contain substances ( Eg. Antibiotics) that prevent or slow down the growth of bacteria other than pathogens for which the media are intended.

Eg. Modified Thayer –Martin Agar

Salmonella-Shigella( SS) agar

#### **1. Differential media**

Media to which indicator substances are added to differentiate bacteria.

Eg. TCBS Agar differentiates sucrose fermenting yellow colonies of *Vibrio cholerae* to non-sucrose fermenting blue colonies other *Vibrio* species.

NB: Most differential media distinguish between bacteria by an indicator which changes color when acid is produced following carbohydrate fermentation.

#### **2. Transport media**

Media containing ingredients to prevent the overgrowth of commensals and ensure the survival of pathogenic bacteria when specimens can not be cultured soon after collection.

EG. Amies transport media

Stuart media

Kelly-Blair media

### **Choice of culture media**

The selection culture media will depend 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 sites having normal microbial flora.
3. The cost, availability and stability of media.
4. The training and experience of laboratory staff in preparing, using and controlling culture media.

### **Forms of culture media**

1. solid culture media
2. semisolid culture media
3. Fluid culture media

#### **1. solid culture media**

- . Plate cultures in petri dishes
- . stab/slope cultures in tubes and bottles

**Uses:** Description of bacterial colonies

- size : diameter in mm
- Out line : circular, entire, wavy, indented



- Elevation: flat, raised, low convex and dome shaped.
- Transparency: transparent, opaque, and translucent.
- Surface: smooth (muroid) and shiny, rough and dull.
- Color: colorless, white, pink, and pigmented
- changes in medium

Eg. Hemolysis in Blood Agar

Blackening of medium due to hydrogen sulfide production.

## 2. Semisolid culture media

### Uses:

- . as an enrichment media
- . as motility media

## 3. Fluid culture media

Bacterial growth in fluid media is shown by a turbidity in the medium.

### Uses :

- . as an enrichment media
- . as biochemical testing media
- . as blood culture media

## Preparation of culture media

Culture media contains essential ingredients for microbial growth requirements.

For successful isolation of pathogens, culture media must be prepared carefully.

Most culture media are available commercially in ready –made dehydrated form.

The major processes during preparation of culture media

- Weighing and dissolving of culture media ingredients
- Sterilization and sterility testing
- Addition of heat-sensitive ingredients
- Dispensing of culture media
- pH testing of culture media
- Quality assurance of culture media
- Storage of culture media

#### **1. Weighing and dissolving of culture media ingredients**

Apply the following while weighing and dissolving of culture media ingredients

- Use ingredients suitable for microbiological use.
- Use clean glass ware, plastic or stainless steel equipment.
- Use distilled water from a glass still.
- Do not open new containers of media before finishing previous ones.
- Weigh in a cool, clean, dry and draught-free atmosphere.
- Weigh accurately using a balance.
- Wear a facemask and glove while weighing and dissolving toxic chemicals.
- Do not delay in making up the medium after weighing.

- Add powdered ingredients to distilled water and mix by rotating or stirring the flask.
- Stir while heating if heating is required to dissolve the medium.
- Autoclave the medium when the ingredients are dissolved.

## 2. Sterilization and sterility testing

Always sterilize a medium at the correct temperature and for the correct length of time as instructed in the method of preparation.

Methods used to sterilize culture media

- A) . Autoclaving
- B) . Steaming to 100 °C
- C) . Filtration

### A) Autoclaving

Autoclaving is used to sterilize most agar and fluid culture media.

### B) Steaming at 100 °C

It is used to sterilize media containing ingredients that would be inactivated at temperature over 100 °C and re-melt previously bottled sterile agar media.

### C) Filtration

It is used to sterilize additives that are heat-sensitive and can not be autoclaved.

### **Sterility testing**

The simplest way to test for contamination is to incubate the prepared sample media

At 35-37 °C for 24 hours. Turbidity in fluid media and microbial growth in solid media confirm contamination.

### **3. Addition of heat-sensitive ingredients**

Refrigerated-heat sensitive ingredients should be warmed at room temperature before added to a molten agar medium.

Using an aseptic technique, the ingredients should be added when the medium has cooled to 50 °C, and should be distributed immediately unless further heating is required.

### **4. pH testing**

The pH of most culture media is near neutral, and can be tested using pH papers or pH meter.

### **5. Dispensing of culture media**

Media should be dispensed in a clean draught-free room using aseptic technique and sterile container.

#### **Dispensing agar media in petridish**

- Lay out the sterile petridishes on a level surface.
- Mix the medium gently by rotating the flask or bottle.
- Flame sterilize the neck of flask or bottle.
- Pour 15 ml of medium in each petridish.
- Stack the plates after the medium has gelled or cooled.

- Store the plates in a refrigerator.

NB: Agar plates should be of an even depth and of a firm gel.

The surface of the medium should be smooth and free from bubbles.

## 6. Quality control

- Inoculate quarter plates of the medium with a five hours broth culture for each control organism.
- Use a straight wire to inoculate and wire loop to spread the inoculum.
- Depending on the species, incubate aerobically, CO<sub>2</sub>-enriched atmosphere and anaerobically at 35-37 °C for 24 hours.
- Examine for the degree of growth, morphology and other characteristics of microbial colonies.
- Record the result of each control species and compare to your standard reading.

## Storage of culture media

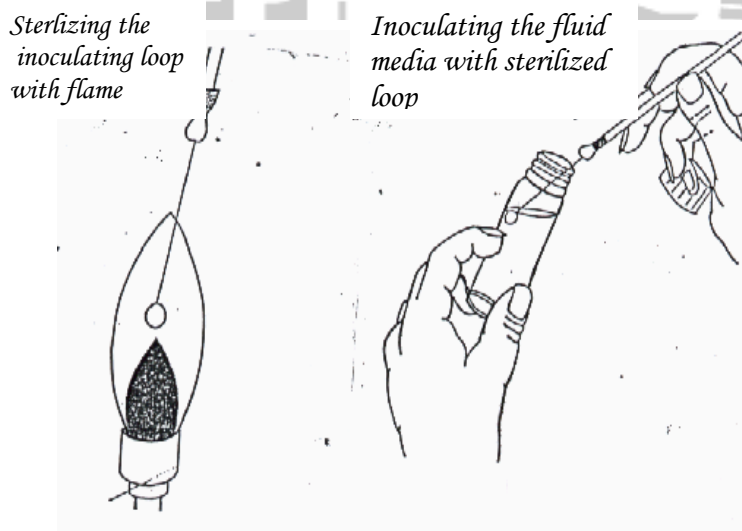
- Dehydrated culture media and dry ingredients should be stored at an even temperature in a cool dry place away from direct light.
- Plates of culture media, and additives like serum, blood and antimicrobials in solid form require storage at 2-8 °C.
- Antimicrobials in solution form should be stored at -20 °C.
- All culture media and additives should be labeled with the name and date of preparation.

### Inoculation of culture media

When inoculating culture media, an aseptic technique must be used to prevent contamination of specimens and culture media, and laboratory worker and the environment.

#### **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.



**Fig. 1.6** Aseptic inoculation technique

### Inoculation of media in petridishes

The inoculation of media in petridishes is named as 'plating out' or 'looping out'.

Before inoculating a plate of culture media, dry the surface of the media by incubating at 37 °C for 30 minutes.

To inoculate a plate, apply the inoculum to a small area of the plate ('the well') using sterile wire loop and then spread and thin out the inoculum to ensure single colony growth.

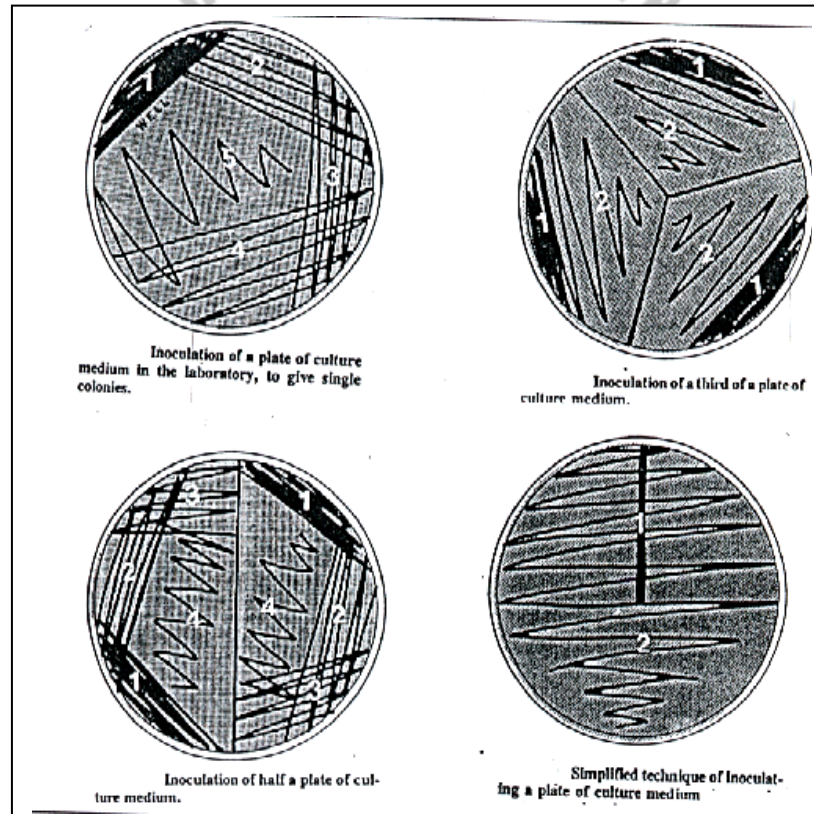


Fig. 1.7 Methods of inoculating solid culture media in petridishes

### **Inoculation of butt and slant media**

To inoculate butt and slant media, use a sterile straight wire to stab into the butt and then streak the slant in a zigzag pattern.

### **Inoculation of slant media**

To inoculate slant media, use a straight wire to streak the inoculum down the center of the slant and then spread the inoculum in a zigzag pattern.

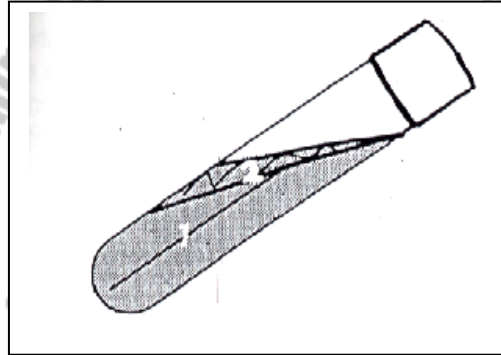


Fig. 1.8 Inoculation of slant and bath media

### **Inoculation of stab media**

To inoculate stab media, use a straight wire to stab through the center of the medium and withdraw the wire along the line of inoculum.



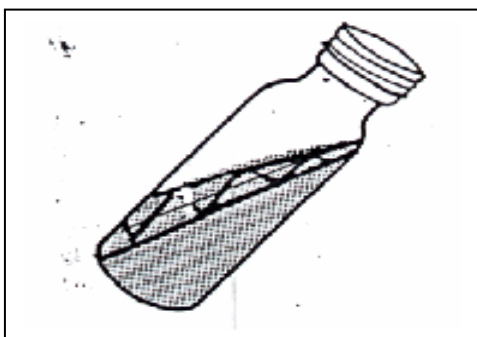


Fig. 1.9 Inoculation of slant media

### **Inoculation of fluid media**

To inoculate fluid media, use straight wire or wire loops.

### **Incubation of cultures**

Inoculated media should be incubated as soon as possible.

Optimal temperature, humidity and gaseous atmosphere should be provided for microorganisms to grow best.

The temperature selected for routine culturing is 35-37 °C.

Some pathogens require CO<sub>2</sub>-enriched atmosphere to grow in culture media, and the simplest way to provide CO<sub>2</sub>-enriched atmosphere is to enclose a lighted candle in an airtight jar which provides 3-5% CO<sub>2</sub> by the time the candle is extinguished.

Anaerobic atmosphere is essential for the growth of strict anaerobes, and the techniques for obtaining anaerobic conditions are the following:

- . Anaerobic jar with a gas generating kit.
- . Reducing agents in culture media.

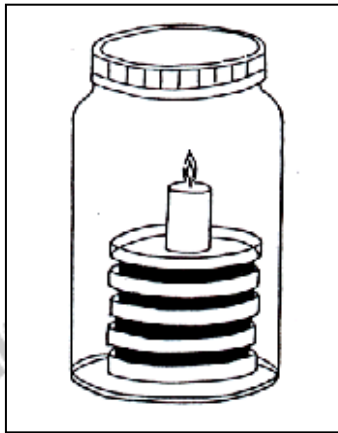


Fig. 1.10 Co2-enriched atmosphere

## 1.6. BACTERIAL NUTRITION

***Bacteria, like all cells, require nutrients for the maintenance of their metabolism and for cell division.***

Bacterial structural components and the macromolecules for the metabolism are synthesized from the elements. The four most important elements of bacteria are carbon, hydrogen, oxygen and nitrogen.

### **Carbon**

Organisms require a source of carbon for the synthesis of numerous organic compounds that comprise protoplast.

Depending on their requirements, bacteria can be classified as

1. Autotrophs: Free-living, non-parasitic bacteria which use carbondioxide as carbon source.

The energy needed for their metabolism can be obtained from:

- . Sun light-Photoautotrophs
- . Inorganic compounds by oxidation-Chemoautotrophs

2. Heterotrophs: Parasitic bacteria require more complex organic compounds as their source of carbon and energy.

Human pathogenic bacteria are heterotrophs.

The principal source of carbon is carbohydrate which are degraded either by oxidation, in the presence of oxygen, or by fermentation, in the absence of oxygen, to provide energy in the form of ATP.

### **Hydrogen and oxygen**

- Obtained from water.
- Essential for the growth and maintenance of cell.

### **Nitrogen**

- Constitutes 10% of dry weight of bacterial cell.
- Obtained from organic molecules like proteins and inorganic molecules like ammonium salts and nitrates.

NB: Main source of nitrogen is ammonia, in the form of ammonium salt.

### **Growth factors**

Growth factors are organic compounds that are required by micro-organisms in small amounts which the cell can not synthesize from other carbon source.

These are aminoacids, purines and pyrimidines, and vitamins.

Prototrophs: Wild-type bacteria with normal growth requirements.

Auxotrophs: Mutant bacteria, which require an additional growth factor not needed by the parental or wild type strain.

## **1.7. BACTERIAL GROWTH**

It is an orderly increase in all the components of an organism.

It is an increment in biomass.

It is synchronous with bacterial cell reproduction.

### **Generation time**

It is the time taken for the size of a bacterial population to double.

Bacteria grow by taking nutrients and incorporate them into cellular components; then bacteria divide into two equal daughter cells and double the number.

### **Bacterial growth phases**

The pattern in cell numbers exhibited by bacterial population obtained after inoculation

Of a bacterium into a new culture medium.

The normal bacterial growth curve has four phases.

#### **1. Lag phase**

The period of adaptation with active macro molecular synthesis like DNA, RNA, various enzymes and other structural components.

It is the preparation time for reproduction; no increase in cell number.

#### **2. Exponential(log) phase**

The period of active multiplication of cells.

Cell division precedes at a logarithmic rate, and determined by the medium and condition of the culture.

### **3. Maximal stationary phase**

The period when the bacteria have achieved their maximal cell density or yield.

There is no further increase in viable bacterial cell number.

The growth rate is exactly equal to the death rate.

A bacterial population may reach stationary growth when one of the following conditions occur:

1. The required nutrients are exhausted
2. Inhibitory end products are accumulated
3. Physical conditions do not permit a further increase in population size

### **4. Decline phase**

The period at which the rate of death of bacterial cells exceeds the rate of new cell formation.

There is drastic decline in viable cells.

Few organisms may persist for so long time at this period at the expense of nutrients released from dying micro-organisms.

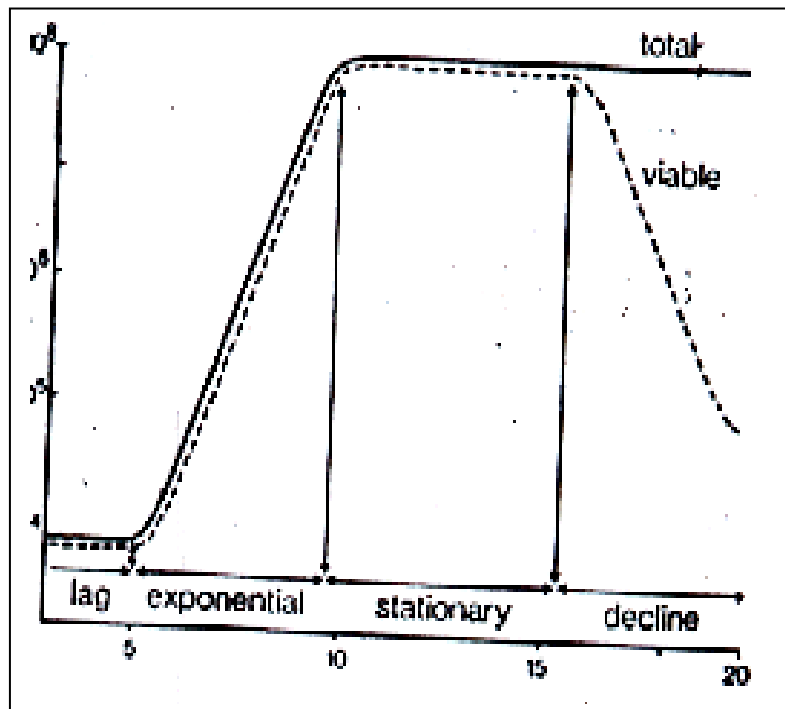


Fig. 1.11 Bacterial growth curve

### Quantitative measurement of bacterial growth

Bacterial growth is measured by determining number of bacteria.

The common measuring methods are

1. Viable plate count
2. Direct count
3. Turbidimetric method

### 1. Viable plate count

The most common method of estimating bacterial growth which involves counting the number of bacterial colonies grown on solid media after incubation of the inoculated media for 18-24 hours.

#### Procedure

- The sample is serially diluted.
- The suspension is inoculated on solid media by surface spread technique i.e. the suspension is spread
- The plate is incubated for 18-24 hrs to allow the bacteria to grow and form colonies.
- The concentration of bacteria in the original sample can be determined by counting the visible colonies multiplied by the dilution factor.

$$\text{Number of colonies} = \frac{\text{Number of colonies} \times \text{dilution factor}}{\text{Volume of sample}}$$

NB: The statistically significant plate count is between 30 and 300 colonies.

Less than 30 colonies on a plate are not accepted for statistical reasons.

Greater than 300 colonies on a plate are too close to distinguish as an individual colony forming unit (too numerous to count).

Limitation of viable plate count: It selectively in favor of a certain group of bacterial population.

## 2. Direct count

It involves direct microscopic counting of bacteria in the sample using counting chamber.

It is relatively quick and does not need the sample to be incubated.

### Procedure

- . Serial dilution of the sample
- . Fill known area and volume of the counting chamber with the sample
- . Total number of bacteria in the sample per unit volume is equal to  
No. of bacteria in the sample  $\times$  the No. of squares  $\times$  dilution factor.

## 3. Turbidimetric method

It is the method of determination of bacterial growth in liquid media.

Bacterial growth increases the turbidity of liquid to absorb light.

The turbidity of the suspension is determined by spectrophotometer.

### Factors influencing bacterial growth in vitro

Not all bacterial species grow under identical environmental conditions. Each bacterial species has a specific tolerance range for specific environmental parameters.

Out side the tolerance range environmental conditions for a bacteria to reproduce, it may survive in dormant state or may lose viability.

Rates of bacterial growth are greatly influenced by the following environmental parameters.

- . Nutrition
- . Temperature



- . Oxygen
- . p<sup>H</sup>
- . Salinity
- . Pressure
- . Light radiation

### 1. Nutrition

The following nutrients must be provided for optimal bacterial growth.

- Hydrogen donors and acceptors
- Carbon source
- Nitrogen source
- Minerals: sulfur and phosphorus, trace elements
- Growth factors: amino acids, purines, pyrimidines and vitamins.

### 2. Temperature

Temperature tolerance range: The minimum and maximum temperature at which a micro-organism can grow; which is different in different species of bacteria.

Optimal growth range of temperature: The temperature at which the maximum growth rate occurs; and results in the shortest generation time of bacteria.

Based on different optimal growth temperature requirement, bacteria are divided into:

### Optimal growth temperature

- . Psychrophilic bacteria .15-20 °c; grow best at low T<sup>0</sup> range
- . Mesophilic bacteria .30-37<sup>0</sup>c; grow best at middle T<sup>0</sup> range
- . Thermophilic bacteria .50-60<sup>0</sup>c; grow best at high T<sup>0</sup> range

NB: Most human pathogens and many of the normal flora of human bodies have an optimal temperature of 37<sup>0</sup>c; There fore they are mesophilic bacteria.

### 3. Oxygen

Base on oxygen requirements and tolerance, bacteria are divided classified as:

- . Obligate aerobes
  - . Obligate anaerobes
  - . Facultative anaerobes
  - . Microaerophiles
- Obligate aerobic bacteria grow only when free oxygen is available to support their respiratory metabolism.  
They obtain ATP by using oxygen as a final electron acceptor in respiration.
  - Obligate anaerobic bacteria grow in the absence of oxygen; exposure to oxygen kills anaerobes.
  - Facultative anaerobic bacteria grow in the presence or absence of oxygen.

They obtain ATP by fermentation or anaerobic respiration

- Microaerophilic bacteria grow best at reduced oxygen tension; high oxygen tension is toxic to them.

#### 4. Hydrogen ion concentration

It is a measure of acidity and alkalinity.

$P^H < 7$  is acidic

$P^H = 7$  is neutral

$P^H > 7$  is alkaline

- Neutrophilic bacteria grow best at near neutral  $P^H$  value.
- Acidophilic bacteria prefer to grow at low  $P^H$  value (acidic medium).
- Alkaliphilic bacteria prefer to grow at high  $P^H$  value (alkaline medium).
- Most pathogenic bacteria grow best at  $P^H$  of 6-8.

#### 5. Salinity

Salt content of the medium affects bacterial growth.

Halophilic bacteria grow best at high salt concentration.

- . Moderate halophiles require 3% salt concentration.
- . Extreme halophiles require 15% salt concentration.

Most bacteria can not tolerate high salt concentration. High salt concentration disrupts membrane transport systems and denatures proteins of bacteria but halophiles have adaptive mechanisms to tolerate high salt concentration.

## 6. Pressure

**Osmotic pressure:** The pressure exerted on bacterial cell surface as a result of difference in solute concentration between the inside and out side of a cell.

Osmotolerant bacteria can grow in solutions with high solute concentration.

Osmophilic bacteria grow best at high hydrostatic pressure.

**Hydrostatic pressure:** The pressure exerted by the weight of a water column.

High hydrostatic pressures more than 200 atmosphere generally inactivates enzymes and disrupts membrane transport process.

Barotolerant bacteria can grow at high hydrostatic pressure.

Barophilic bacteria grow best at high hydrostatic pressure.

## 7. Light radiation

Photosynthetic bacteria require light in the visible spectrum to carry out photosynthesis.

## COMMON BIOCHEMICAL TESTS

### Litmus milk reduction test:

Required: Litmus milk medium

Wire loop

Bunsen burner

Test bacteria

Method:

- . Inoculate 0.5 ml of sterile litmus milk medium with the test bacteria.
- . Incubate at 35-37 °c for up to 4 hrs.

- . Observe for changes in color every 30 min.

Results:

- . Change in color of medium from pink to white or pale is suggestive of enterococci

**CAMP test** (Christie, Atkins , Munich Paterson )

Principle: *S.agalaciae* produce protein named as camp factor, which interacts with staphylococci  $\beta$ -hemolysin on sheep red blood cell.

Method:

- . Streak *S.aureus* isolate across sheep blood agar plate.
- . Inoculate the test bacteria at right angle to staphylococci with out touching it.
- . Incubate over night at 35-37 °c.
- . Formation of an arrow-head shaped area of hemolysis indicates interaction of camp factor with staphylococci hemolysin.

**Bacitracin test**

Principle: *Streptococcus pyogenes* is sensitive to bacitracin but other kinds of streptococci are resistant to bacitracin.

Method:

- . Streak a blood agar plate with the isolated organism.
- . Place bacitracin disc in the streaked area.
- . Incubate the plate for 24 hours at 37 °c.

- . Examine the plate for a zone of no-growth around the disc.

No-growth around the disc..... S.pyogenes

Growth around the disc ..... Other streptococci

### Optochin test

Principle: S. pneumoniae is sensitive to optochin disc unlike other alpha-hemolytic streptococci.

Method:

- . Streak a blood agar plate with the isolated organism.
- . Place optochin disc in the streaked area.
- . Incubate the plate for 24 hours at 37 °C.
- . Examine the plate for a zone of no-growth around the disc.

No-growth around the disc ..... S.pneumoniae

Growth around the disc ...other Alpha-hemolytic streptococci

### Carbohydrate utilization test

Method:

- . Prepare saline suspension of test bacteria
- . Add 0.1ml of bacterial suspension into each of four test tubes containing glucose, lactose, maltose and sucrose carbohydrate discs.
- . Incubate in a water bath at 37°C and examine at 30 min intervals for 5 hrs for change in color.

Result: Change in color from red to yellow-orange indicates carbohydrate utilization.

### **BILE SOLUBILITY TEST**

This helps to differentiate *S.pneumoniae*, which is soluble in bile and bile salts, from viridans streptococci which are insoluble.

#### **Principle**

A heavy inoculum of the test organism is emulsified in physiological saline to give a turbid suspension. The bile salt sodium deoxycholate is then added. The test can also be performed by adding the bile salt to a broth culture of the organism. The bile salt dissolves *S.pneumoniae* as shown by a clearing of the turbidity within 10-15 minutes. Viridans streptococci are not dissolved and therefore there is no clearing of the turbidity.

#### **Required**

Sodium deocholate 100g/l

Physiological saline (sodium chloride, 8.5g/l)

#### **Method**

- Emulsify several colonies of the test organism in a tube containing 2ml of sterile physiological saline, to give a turbid suspension.
- Divide the organism suspension between two tubes.
- To one tube, add 2 drops of the sodium deoxycholate reagent and mix.

- To the other tube, add 2 drops of sterile distilled water and mix.
- Leave both tubes for 10-15 minutes.
- Look for a clearing of turbidity in the tube containing the sodium deoxycholate.

### Results

Clearing of turbidity ----- Probably  
S.pneumoniae

No clearing of turbidity -----organism is probably  
Not S.pneumoniae

There should be no clearing of turbidity in the tube to which distilled water was added. If there is, repeat the test.

**Note:** Some strains of S.pneumoniae are not dissolved by bile salts, and very occasionally some strains of viridans streptococci give a positive tests.

### Controls

Bile solubility positive control: Streptococcus pneumoniae.

Bile solubility negative control: Streptococcus faecalis.

### 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 into contact with hydrogen peroxide. Bubbles of oxygen are released if the organism is a catalase producer. The culture should not be more than 24 hours old.

Care must be taken if 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 colony, a false positive reaction will occur. It is usually recommended, therefore, that catalase testing be performed from a blood free culture medium such as nutrient agar.

### **Required**

- a. Hydrogen peroxide, 3% H<sub>2</sub>O<sub>2</sub>

Note: Shaking the reagent before use will help to expel any dissolved oxygen. False positive reactions may occur if the hydrogen peroxide contains dissolved oxygen.

### **Method**

- Pour 2-3ml of the hydrogen peroxide solution into a test tube.

- Using a sterile wooden stick or a glass rod, remove a good growth of the test organism and immerse it in the hydrogen peroxide solution.

**Note:** A nichrome wire loop must not be used because this may give a false positive reaction.

- Look for immediate bubbling.

### Results

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

**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.

**Caution:** performing the test on a slide is not recommended because of the risk of contamination from active bubbling.

If the rapid slide technique is used, the hydrogen peroxide solution 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.

### Controls

Positive catalase control: Staphylococcus species.

Negative catalase control: Streptococcus species.

## CITRATE UTILIZATION TESTS

This test is one of several techniques used to assist in the identification of enterobacteria. The test is based on the ability of an organism to use citrate as its only source of carbon and ammonia as its only source of nitrogen.

### Principle

The test organism is cultured in a medium which contains sodium citrate, an ammonium salt, and the indicator bromo – thymol blue. Growth in the medium is shown by turbidity and a change in colour of the indicator from light green to blue, due to the alkaline reaction, following citrate utilization.

### Required

Koser's citrate medium or Simmon's citrate agar.

### Method

Using a sterile straight wire, inoculate 3-4ml of sterile Koser's citrate medium with a broth culture of the test organism.

**Note:** Care must be taken not to contaminate the medium with carbon particles, such as from a frequently flamed wire.

Incubate the inoculated broth at 35 – 37°C for up to 4 days, checking daily for growth.

### Results

Turbidity and blue colour ----- Positive test  
Citrate

utilized

No growth ----- Negative test  
Citrate not utilized

### Controls

Positive citrate control: *Klebsiella pneumoniae*

Negative citrate control: *Escherichia coli*.

### COAGULASE TEST

This test is used to differentiate *Staphylococcus aureus* which produces the enzyme coagulase, 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.

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.

### Required

- a. Undiluted human plasma (preferably pooled) or rabbit plasma. The plasma should be allowed to warm to room temperature before being used.

Plasma from EDTA (ethylenediamine – tetra – acetic acid) or citrate anticoagulated blood is usually used.

**Note:** Occasionally citrate-utilizing organisms such as *Klebsilla* can cause the clotting of citrated plasma in the tube test. This can be prevented by adding heparin to the citrated plasma. It is also possible for human plasma to contain inhibitory substances which can interfere with coagulase testing. Adequate controls must be included for both slide and tube tests.

### Method for slide test (to detect bound coagulase)

Place a drop of physiological saline on each end of a slide, or on two separate slides.

Emulsify a colony of the test organism 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 drop of plasma to one of the suspensions, and mix gently. Look for clumping of the organisms within 10 seconds.

No plasma is added to the second suspension. This is used to differentiate any granular appearance of the organism from true coagulase clumping.

### Results

Clumping within 10 secs ----- S.aureus

No clumping within 10 secs-----No bound coagulase produced.

### Controls

Positive coagulase control: Staphylococcus aureus.

Negative coagulase control: Escherichia coli or  
Staphylococcus epidermidis

### Method for tube test (detect free coagulase)

Dilute the plasma 1 in 10 in physiological saline (mix 0.2 ml of plasma with 1.8ml of saline).

Take three small test tubes and label:

T = Test organism (18-24h broth culture)

Pos = Positive control (18-24h staph. Aureus broth culture)

Neg = Negative control (sterile broth)

A suitable broth is brain heart infusion

Pipette 0.5ml of the diluted plasma into each tube.

Add 5 drops (about 0.1ml) of the test organism culture to the tube labeled 'T'.

Add 5 drops of the staph. Aureus culture to the tube labeled 'Pos'.

Add 5 drops of sterile broth to the tube labeled 'Neg'.

After mixing gently, incubate the three tubes at 35-37°C. Examine for clotting after 1 hour. If no clotting has occurred, examine at 30minute intervals for up to 6 hours.

When looking for clotting, gently tilt each tube.

Most Staph, aureus strains produce a fibrin clot within 1 hour of incubation. There should be no fibrin clot in the negative control tube.

### **Results**

Fibrin clot ----- S. aureus

No fibrin clot ----- No free coagulase produced

### **DEOXYRIBONUCLEASE (DNase) TEST**

This test is used to differentiate Staph. Aureus which produces the enzyme DNase from other staphylococci which do not produce DNase. It is particularly useful if plasma is not available to perform a coagulase test or when the results of 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 indicating DNA hydrolysis.

### Required

- a. DNase agar plate  
Up to six organisms may be tested on the same plate.
- b. Hydrochloric acid, 1 mol/l

### 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 clearly labeled.

Incubate the plate at 36-37°C overnight.

Cover the surface of the plate with 1mol/l hydrochloric acid solution. Tip off the excess acid.

58. Look for clearing around the colonies within 5minutes of adding the acid.

### Results

Clearing around the colonies -----DNAse positive strain.

No clearing around to colonies -----DNAse negative strain.

### Controls

Positive DNase control: staphylococcus aureus .

Negative DNase control: staphylococcus epidermidis.



### **HYDROGEN SULPHID (H<sub>2</sub>S) PRODUCTION**

The detection of hydrogen sulphide gas (H<sub>2</sub>S) is used mainly to assist in the identification of enterobacteria and occasionally to differentiate other bacteria such as Bacteroides and Brucella species. H<sub>2</sub>S is produced when sulphur – containing amino acids are decomposed.

#### **Use of Kligler iron agar (KIA) to detect H<sub>2</sub>S**

This medium is suitable for detecting H<sub>2</sub>S production by enterobacteria. H<sub>2</sub>S is detected by the ferric citrate contained in the medium.

Inoculate the test organism into KIA and incubate it at appropriate temperature over night.

Observe blacking of the medium

#### **Lead acetate paper test to detect H<sub>2</sub>S**

When a sensitive technique for detecting H<sub>2</sub>S production is required, the lead acetate paper test is recommended.

Inoculate a tube or bottle of sterile peptone water or nutrient broth with the test organism.

Insert a lead acetate paper strip in the neck of the bottle or tube above the medium, and stopper well.

Incubate the inoculated medium at 35-37<sup>o</sup>C, and examine daily for a blackening of the lower part of the strip.

### Results

Blackening ----- Positive test H<sub>2</sub>S produced  
No blackening ----- Negative test No H<sub>2</sub>S produced.

### Controls

Positive hydrogen sulphide control: proteus vulgaris

Negative hydrogen sulphide control: shigella species

### INDOLE TEST

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 break down the amino acid tryptophan with the release of indole.

### 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)-dimethylaminobenzaldehyde. This reacts with the indole to produce a red coloured compound. In the following method the use of the combined motility indole urea (MIU) medium is described. A Kovac's reagent paper strip is inserted in the neck of the tube, and indole production is indicated by a reddening of the strip. Indole is a volatile substance (easily vaporized). The tube must be well stoppered during incubation.

The indole test can also be performed by culturing the organism in tryptone water or peptone water containing tryptophan, and

detecting indole production by adding Kovac's or Ehrlich's reagent to an 18-24h culture.

**Required**

Motility indole urea (MIU) medium

MIU medium indicates whether an organism is motile or non-motile, indole positive or negative, and urease positive or negative.

**Method**

Using a sterile straight wire, inoculate 5ml of sterile MIU medium with a smooth colony of the test organism.

Place an indole paper strip in the neck of the MIU tube above the medium, and stopper the tube, incubate at 35-37°C overnight.

Examine for indole production by looking for a reddening of the lower part.

**Results**

Reddening of strip -----Positive test

Indole produced

No red colour -----Negative test

No Indole produced

Note: If the reaction is weak, confirm the result by adding 1ml of Kovac's reagent to the culture. Examine for a red colouring of the surface layer within 10 minutes.

### **Controls**

Positive indole control: *Escherichia coli*

Negative indole control: *Enterobacter aerogenes*.

### **Motility Test**

This is shown by a spreading turbidity from the stab line or a turbidity throughout the medium (compare with an uninoculated tube).

### **Urease production**

This is shown by a red-pink colour in the medium.

### **NITRATE REDUCTION TEST**

This test is used to differentiate members of the Enterobacteriaceae that produce the enzyme nitrate reductase, from Gram negative bacteria that do not produce the enzyme.

The test is also helpful in differentiating *Mycobacterium* species as explained.

### **Principle**

A heavy inoculum of the test organism is incubated in a broth containing nitrate. After 4 hours, the broth is tested for the reduction of nitrate to nitrite by adding sulphanilic acid reagent. If nitrite is present, the acid reagent is diazotized and forms a pink-red compound with alpha-naphthylamine. When nitrite is not detected it

is necessary to test whether the organism has reduced the nitrate beyond nitrite. This is done indirectly by checking whether the broth still contains nitrate. Zinc dust is added which will convert any nitrate to nitrite. If no nitrite is detected when the zinc dust is added, it can be assumed that all the nitrate has been reduced beyond nitrite to nitrogen gas or ammonia by a nitrate reducing organism.

### Required

- a. Nitrate broth
- b. Sulphanilic acid reagent
- c. Alphanaphthylamine reagent
- d. Zinc dust

### Method

Inoculate 0.6 ml of sterile nitrate broth with a heavy growth of the test organism.

Incubate at 35-37°C for 4 hours.

Add 1 drop of sulphanilic acid reagent and 1 drop of alphanaphthylamine reagent.

Shake to mix and look for a red colour.

### Results

Red colour ----- Positive test

Nitrate reduced

If no red colour is produced, add a very small amount (knife point) of zinc dust powder. Look again for a red colour and interpret as follows:

Red colour ----- Negative test  
No reduction of nitrate

No red colour ----- Positive test  
Nitrate reduced

### **Controls**

Positive nitrate reduction control: *Escherichia coli*. Negative nitrate reduction control: *Pseudomonas aeruginosa*.

### **OXIDASE TEST (Cytochrome Oxidase)**

The oxidase test is used to assist in the identification of *pseudomonas*, *Neisseria*, *Vibrio*, and *Pasteurella* species, all of which produce oxidase enzymes.

### **Principle**

A piece of filter paper is soaked with a few drops of oxidase reagent. A colony of the test organism is then smeared on the filter paper. If 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 be useful, however, 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.

**Important:** Acidity inhibits oxidase enzyme activity. The oxidase test must not be performed, therefore, on colonies that produce fermentation on carbohydrate – containing media, such as sucrose fermenting *V.cholerae* colonies on TCBS medium, Subinoculation on nutrient agar is required before the oxidase test can be performed reliably. Non – fermenting colonies, however, can be tested. Colonies tested from a medium that contains nitrate may give unreliable oxidase test results.

### Required

- **Oxidase reagent**

*Freshly prepared*

This is a 10g/l solution of tetramethyl –p-phenylenediamine dihydrochloride.

Note: Oxidase reagent is easily oxidized. When oxidized, it is blue in colour and must not be used.

### Method

Place a piece of filter paper in a clean petri dish and 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.

### Results

Blue-purple colour ----- Positive test  
(within 10 seconds) Oxidase produced

No blue – purple colour ----- Negative test  
(within 10 seconds) No oxidase produced

Note: ignore any blue – purple colour that develops after 10 seconds.

### Controls

Positive oxidase control: *Pseudomonas aeruginosa*.

Negative oxidase control: *Escherichia coli*.

### OXIDATION – FERMENTATION (O-F) TEST

This test is used to differentiate those organisms that oxidize carbohydrates (aerobic utilization) such as *Pseudomonas aeruginosa*, from those organisms that ferment carbohydrates (anaerobic utilization) such as members of the *Enterobacteriaceae*.

### Principle

The test organism is inoculated into two tubes of a tryptone or peptone agar medium containing glucose (or other carbohydrate) and the indicator bromothymol blue. The inoculated medium in one tube is sealed with a layer of liquid paraffin to exclude oxygen.



Fermentative organisms utilize the carbohydrate in both the open and sealed tubes and the colour of the medium changes from green to yellow. Oxidative organisms, however, are able to use the carbohydrate only in the open tube. There is no carbohydrate utilization in the sealed tube (medium remains green).

Although most genera of aerobic bacteria are either carbohydrate oxidizers or fermenters, the production of acid may be slow and therefore cultures are usually incubated for 7-14 days.

#### **Required**

- a. Oxidation fermentation (O-F) medium  
Glucose, maltose, and sucrose O-F media are the most commonly used.
- b. Sterile paraffin oil (liquid paraffin)

#### **Method**

Using a sterile straight wire, inoculate the test organism to the bottom of two bottles (or more if testing several carbohydrates) of sterile O-F medium. Use a heavy inoculum.

Cover the inoculated medium in one of the tubes (or one from each carbohydrate pair) with a 10mm deep layer of sterile paraffin oil or molten wax.

Incubate the tubes at 35-37°C for up to 14 days. Examine daily for carbohydrate utilization as shown by acid production.

## Results

Open tube	Sealed tube	Interpretation
Yellow	Green	Oxidative organism
Yellow	Yellow	Fermentative organism
Green or blue	Green	No utilization of carbohydrate

## Controls

Oxidative control: *Pseudomonas aeruginosa*.

Fermentative control: *Escherichia coli*.

## PHENYLALANINE DEAMINASE TEST

The test, which is also referred to as the Phenylpyruvic acid (PPA) test, is used mainly to assist in the identification of enterobacteria. It is based on the ability of bacteria such as *Proteus* species and some *Providencia* strains to break down phenylalanine (by oxidative deamination) with the production of phenylpyruvic acid. *Y. enterocolitica* (urease – producer is a phenylalanine negative.

## Principle

The test organism is cultured on a slope of phenylalanine medium. After overnight incubation, the deamination of phenylalanine to phenyl – pyruvic acid is detected by adding iron III chloride (ferric chloride) which produces a green colour on the surface of the culture.

**Required**

- a. Phenylalanine agar
- b. Iron III chloride (ferric chloride),  
100g/l (10% w/v). The reagent must be freshly prepared.

**Method**

- . inoculate a slope of phenylalanine agar with the test organism, and incubate at 35-37<sup>o</sup>c overnight.
- . Add 4 or 5 drops of the freshly prepared iron III chloride reagent to the culture, allowing the reagent to run down the slope.
- . Look for a green colour on the slope.

**Results**

Green colour -----	Positive test
(Within 5 minutes)	Phenylalanine deaminated
No green colour -----	Negative e test
	No deamination of phenylalanine

**Controls**

Positive PPA control: Proteus species  
Negative PPA control: Escherichia coli.

### TWEEN 80 HYDROLYSIS TEST

This test is used mainly to differentiate slow-growing Mycobacterium species as described in 44:1 species that hydrolyze the detergent Tween 80 with the production of oleic acid are listed in Chart.

#### Principle

The test organism is incubated in a Tween 80 buffered substrate that contains the indicator neutral red. Tween hydrolysis is detected by a change in colour of the indicator from amber to pink – red due to the production of oleic acid.

#### Required

Tween 80 phosphate buffered substrate with neutral red.

\*The substrate requires storage at 4°C

#### Method

- . Inoculate 4 ml of sterile Tween 80 phosphate buffered substrate with a loopful of growth of the test organism.
- . Incubate at 35-37 °C for up to 18 days. Examine at 5, 10, and 18 days for a change in colour of the substrate from amber to pink-red, as shown in colour.

#### Results

Pink-red substrate ----- Positive test  
Tween 80 hydrolyzed

No change in colour ----- Negative test  
No hydrolysis of Tween 80

### **Controls**

Positive Tween hydrolysis control: *Mycobacterium kansasii*.

Negative Tween hydrolysis control: Use an uninoculated tube of substrate.

### **UREASE TEST**

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 °C). *Salmonellae* and *shigellae* do not produce urease.

### **Principle**

The test organism is cultured in a medium which contains urea and the indicator phenol red. If 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 red-pink.

The method described is that which uses the combined motility indole urea (MIU) medium, urease production can also be detected by culturing the organisms in Christensen's urea broth.

### **Required**

- a. Motility indole urea (MIU) medium.

### **Method**

- . Using a sterile straight wire, inoculate a tube of sterile MIU medium with a smooth colony of the test organism.

- . Place an indole paper strip in the neck of the MIU tube above the medium. Stopper the tube and incubate at 35-37 °c overnight.
- . Examine for urease production by looking for a red-pink colour in the medium as shown in colour.

### Results

Red-pink medium-----	Positive test
	Urease produced
No red-pink colour -----	Negative test
	No urease produced

### Controls

Positive urease control: *Proteus vulgaris*.

Negative urease control: *Escherichia coli*.

**Note:** After overnight incubation, *Y. enterocolitica* gives a weak urease reaction and is non-motile at 35-37 °c. At room temperature (22-29°C), the species is motile and shows a stronger urease reaction.

### VOGES – PROSKAUER (V-P) TEST

This test is occasionally used to assist in the differentiation of enterobacteria. *K. pneumoniae*, *Vibrio cholerae* biovar el tor, and some strains of *Enterobacter*, ferment glucose with the production of acetylmethylcarbinol (acetoin) which can be detected by an oxidation reaction.

**Principle**

The test organism is cultured in a glucose phosphate peptone water for 48 hours. Sodium hydroxide and a small amount of creatine are then added. Under alkaline conditions and exposure to the air, the acation produced from the fermentation of the glucose is oxidized to diacetyl which forms a pink compound with the creatine.

**Required**

- a. Glucose phosphate peptone water.
- b. Sodium hydroxide, 400g/l.
- c. Creatine powder.

**Method**

- . Inoculate 2ml of sterile glucose phosphate peptone water with the test organism. Incubate at 35-37 °c for 48hours.
- . Add a very small amount (knife point)of creatine and mix.
- . Add about 3ml of the sodium hydroxide reagent and shake well,

Caution: The sodium hydroxide reagent is corrosive, therefore handle with care and do not mouth – pipette.

- . Remove the bottle cap, and leave for 1 hour at room temperature. Look for the slow development of a pink – red.

**Results**

Pink – red colour -----	Positive test
	Acetoin produced
No pink – red colour -----	Negative test
	No acetoin produced

### **Controls**

V-P Positive control: Enterobacter aerogenes or  
Klebsiella pneumoniae

V-P Negative control: Escherichia coli.

### **1.8. BACTERIAL GENETICS**

Genetics is the study of inheritance. Bacterial inherited characteristics are encoded in DNA.

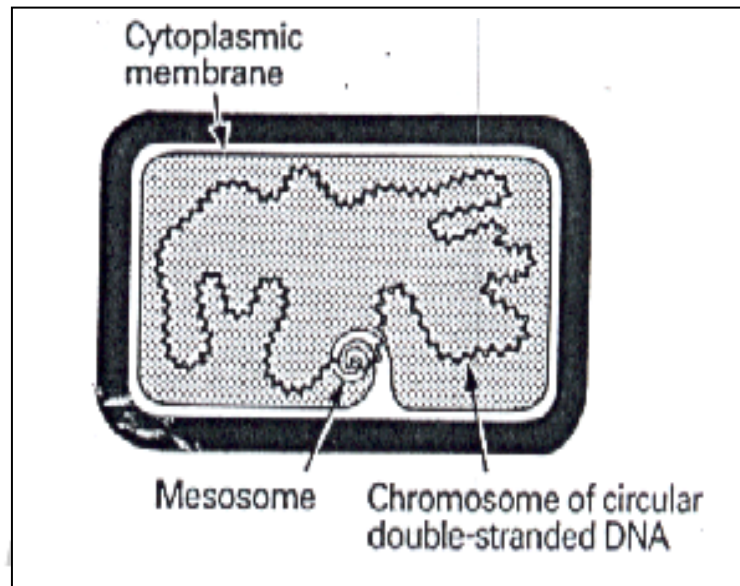
Bacteria have two types of DNA that contain their genes. These are :

- . Chromosome
- . Extra chromosome: Plasmid

**The bacterial chromosome** is circular, double stranded DNA attached to bacterial cell membrane.

DNA replication in bacteria is semi-conservative i.e. each strand of DNA is conserved intact during replication and becomes one of the two strands of the new daughter molecules.





**Fig. 1.12** Bacterial chromosome

**Plasmids** are self-replicating extra chromosomal DNA molecules. It multiplies independent of the host cell. Multiple copies of the same plasmid may be present in each bacterial cell. Different plasmids are also often present in the same bacterial cell.

#### **Plasmid types**

There are many types of plasmid types. The following are examples.

- a. R factors: Plasmids which contain genes that code for antibiotic resistance.

- b. Col factors: Plasmids which contain genes that code for extracellular toxin (colicines) production that inhibit strains of the same and different species of bacteria.
- c. F(fertility) factors: Plasmids that can recombine itself with the bacterial chromosome.

It promotes transfer of the chromosome at a high frequency of recombination into the chromosome of a second (recipient) bacterial cell during mating.

### **Genetic variation in Bacteria**

**Mechanisms:** Mutation and Gene transfer

**1. Mutation:** It is due to a chemical alteration in DNA.

It could be spontaneous or induced by chemical and physical means

Mutants are variants in which one or more bases in their DNA are altered; which are heritable and irreversible

#### **Types of mutation**

- 1. Substitution: Change of a single base.
- 2. Deletion: Loss of a base.
- 3. Insertion: Addition of a base.

#### **2. Gene transfer**

There are three types of gene transfer that alter the DNA gene content of bacteria.

These are:

- . Transformation
- . Transduction
- . Conjugation

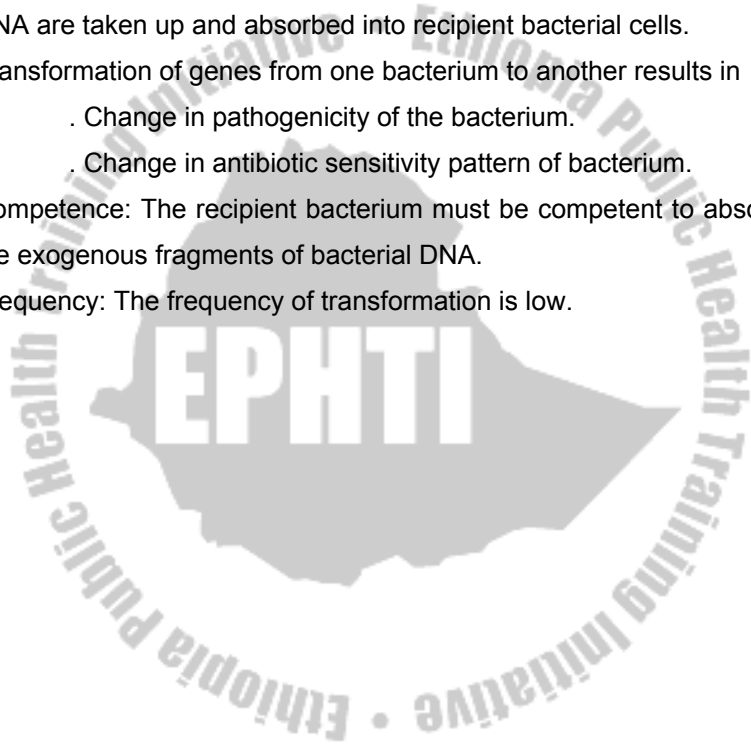
1. **Transformation** occurs when fragments of exogenous bacterial DNA are taken up and absorbed into recipient bacterial cells.

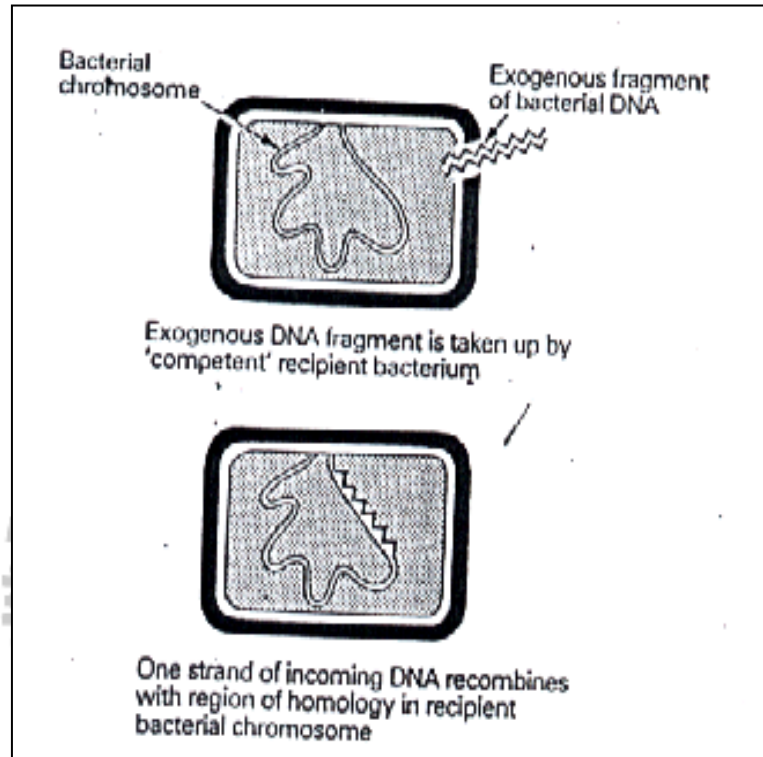
Transformation of genes from one bacterium to another results in

- . Change in pathogenicity of the bacterium.
- . Change in antibiotic sensitivity pattern of bacterium.

Competence: The recipient bacterium must be competent to absorb the exogenous fragments of bacterial DNA.

Frequency: The frequency of transformation is low.





**Fig. 1.13** Transformation; gene transfer by the uptake & subsequent recombination of a fragment of exogenous bacterial DNA

2. **Transduction** occurs when fragments of chromosomal DNA is transferred or transduced into a second bacterium by phage. During phage replication, the bacterial DNA may be accidentally enclosed instead of the normal phage DNA, and when this particle which enclosed the bacterial DNA infects a second bacterial cell, the DNA from the first bacterium is released and incorporated into The chromosome of the second bacterium.

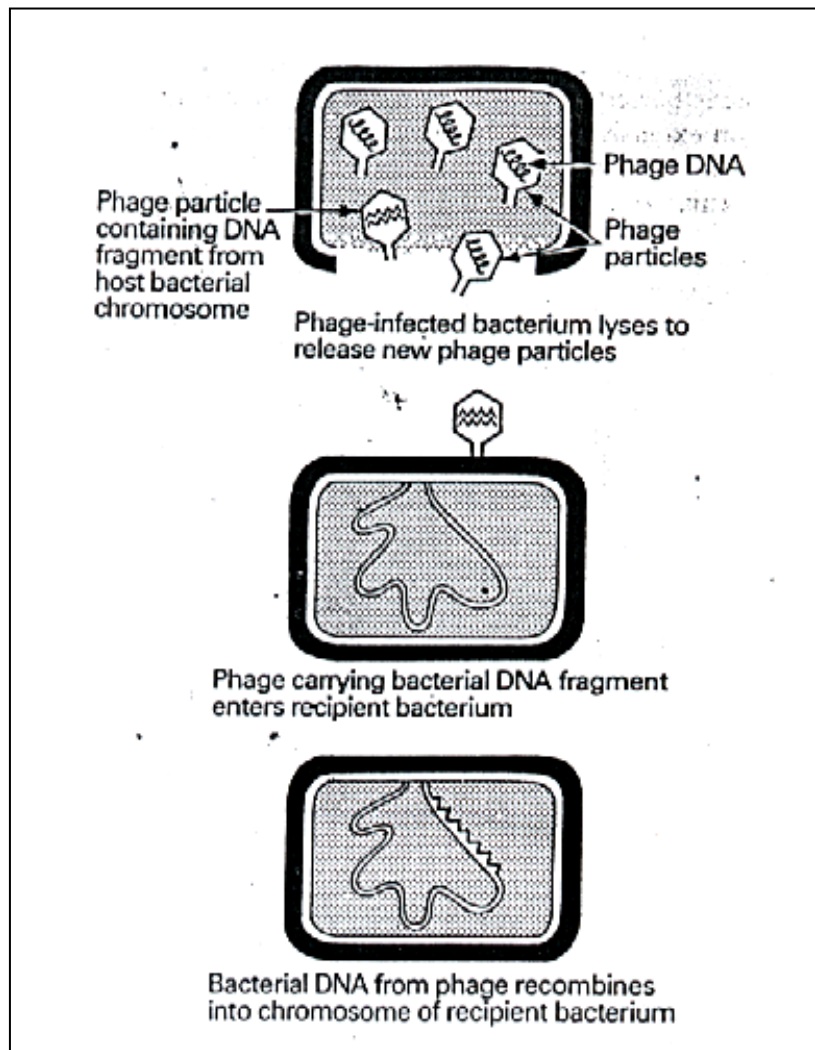
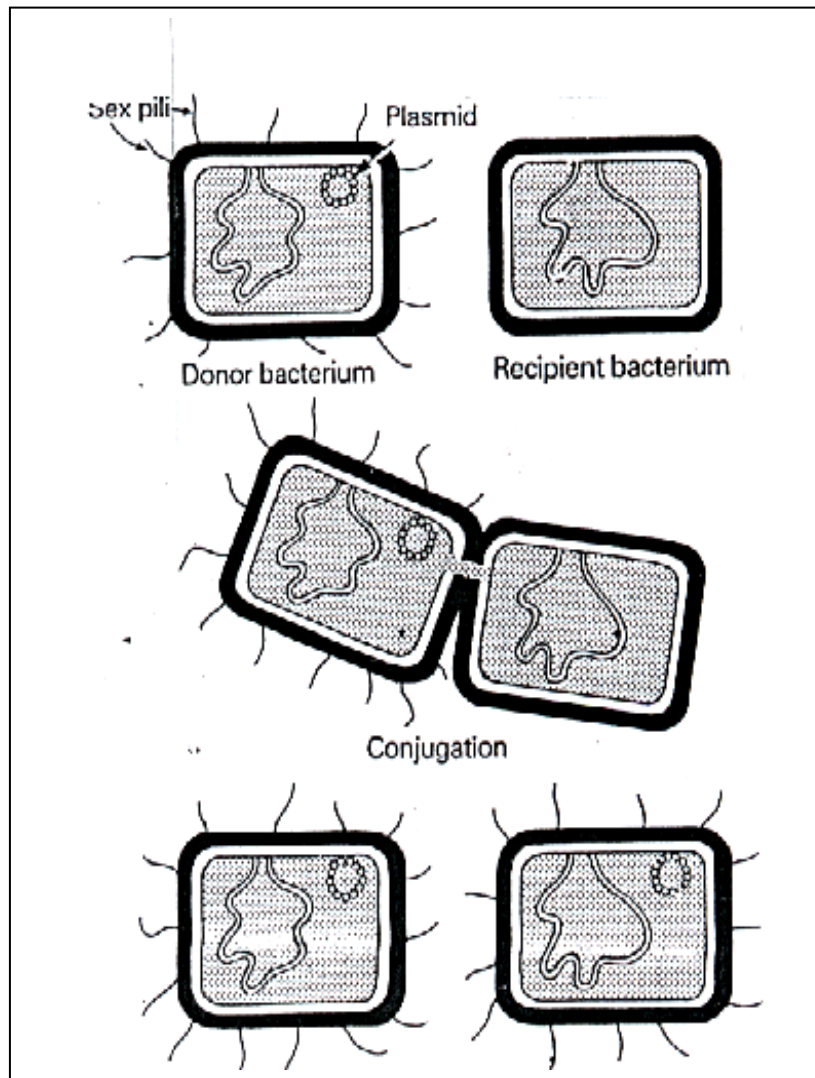


Fig. 1.14 Transduction; gene transfer from one bacterium to another via phage.

3. **Conjugation** occurs when plasmid DNA is transferred from donor to recipient bacterium by direct contact via a sex pilus.



**Fig. 1.15** Conjugation; plasmid gene transfer by conjugation

#### 4. Transposition

Mechanism which enhances genetic flexibility among plasmids and bacterial chromosomes.

Transposons(Jumping genes) are segments of DNA that can transpose or move extremely readily, from plasmid to plasmid or from plasmid to chromosome(and viceversa).In this way, plasmid genes become part of the chromosomal component of genes.

When transposons transfer to a new site, it is usually a copy of the transposon that moves, the original transposon remaining in situ.

Transposons code for toxin production, resistance to antibiotics as well as other functions.

#### 1.9. STERILIZATION AND DISINFECTION

**Sterilization:** Destruction of all forms of microbial life including spores.

**Disinfection:** Destruction of microbes that cause disease; may not be effective in killing spores.

**Antisepsis:** destruction or inhibition of microorganisms in living tissue there by limiting or preventing the harmful effect of infection.

Sterilizing and disinfecting agents are divided into two groups.

These are:

1. Chemical methods of sterilization and disinfection
2. physical methods of sterilization and disinfection

### 1.9.1. Chemical methods of sterilization and disinfection

These chemical agents destroy any type of microbes without showing any form of selectivity unlike antibiotics.

The efficacy of these agents depends on the following factors.

1. Concentration of the agent

There is a relationship between the concentration of the agent and the time required to kill a given fraction of the microbial population.

2. Time of exposure

Microbes are killed with a reasonable length of time with chemical agents.

3.  $p^H$  of the medium where action is to take place

Hydrogen ion concentration determines degree of ionization of the chemical and bacterial surface charge.

The non-ionized form passes through the bacterial cell membrane more readily than the ionized form.

4. Temperature

Bactericidal potency of the chemical agent increases with an increase in temperature.

An increase in  $10^{\circ}C$  doubles the bacterial death rate.

5. Nature of the organism

- . Species of the bacteria
- . Growth phase of bacteria in culture
- . Presence of capsule, spore and other special structures
- . Number of bacteria in test system

6. Presence of extraneous materials

Organic materials like serum, blood or pus makes chemicals inert that are highly active in their absence.



## **Classification of chemical methods of sterilization and disinfection**

1. Chemical agents that damage the cell membrane
  - . Surface active agents
  - . Phenols
  - . Organic solvents
2. Chemical agents that denature proteins
  - . Acids and alkalis
3. Chemical agents that modify functional groups of proteins and nucleic acids
  - . Heavy metals
  - . Oxidizing agents
  - . Dyes
  - . Alkylating agents

### **1. Chemical agents that damage the cell membrane**

#### **Surface active agents**

- a. Cationic agents
  - . Quaternary ammonium compounds (Quates)

It causes loss of cell membrane semi permeability leading to loss of nutrients and essential metabolites. It as well denatures protein.

  - More active in Gram-positive bacteria than in Gram-negative bacteria.
  - More active at alkaline P<sup>H</sup>

- Inactivated by organic materials.
- b. Anionic agents
- . Soaps and fatty acids
- It causes gross disruption of cell membrane lipoprotein framework.
- . More active in Gram-positive bacteria than in Gram-negative bacteria.
  - . Active at acidic P<sup>H</sup>

### **Phenolic compounds**

Phenol is highly effective in Gram positive bacteria.

Clinically no more used because of its neurotoxic effect.

Currently used as a standard for measuring bactericidal potency of new chemicals i.e. phenol coefficient.

Phenol coefficient is the ratio of the concentration of the new chemical agent being tested to the concentration of the reference standard (phenol) required to kill in a specific time.

If phenol coefficient is less than one, the new chemical agent is less effective than phenol.

If phenol coefficient is equal to one, the new chemical agent is equal to phenol in efficacy.

If phenol coefficient is more than one, the new chemical agent is more effective than phenol.

Derivatives of phenol:

- . Cresols e.g. Lysol, Creolin
  - . Halogenated diphenyl compounds eg. Hexachlorophene
- It is more active on Gram-positive bacteria.
- It is germicidal and anti-perspirant.

### **Organic solvents**

Alcohol e.g. Ethyl alcohol, Isopropyl alcohol

- . Disorganize cell membrane lipid structure.
- . Denatures protein.
- . Active against Gram-positive bacteria, Gram-negative bacteria and acid-fast bacilli.

Uses:

1. Potent skin disinfectants
2. Disinfects clinical thermometer

NB: Ethanol is potent at concentration of 70%.

### **Chemical agents that denature proteins**

E.g. Acids and alkalis, Quates, Alcohol

- . Causes conformational alteration of proteins (unfolding of polypeptide chain) resulting in irregular looping and coiling of polypeptide chain.

Acids like benzoic acid, citric acid and acetic acid are helpful as food preservatives: extending storage life of food products.

### **Chemical agents that modify functional groups of proteins and nucleic acids**

#### **Heavy metals**

1. Mercurials : mercuric chloride – limited use because of toxicity.  
Organic mercurials – less toxic than inorganic mercuric salts.

Used as antiseptics.

E.g. Merthiolate

Mercurochrome

2. Silver compounds

E.g. Silver nitrate, Silver sulfasalazine

Used as ophthalmic and wound (e.g. In burn patients) antiseptic.

**Oxidizing agents**

Converts functional –SH group into non-functional –S-S group.

1. Halogens e.g. Chlorine, Iodine

a. Chlorine: inactivated by organic materials.

**Preparations and uses:**

- Chlorine: water disinfectant; the dosage is 0.5-1.0 PPM as a disinfectant.
- Hypochlorite: sanitizing dairy and food processing industries, households and hospitals.
- Organic or inorganic chloramine : effective water disinfectant acting by liberating chlorine.

b. Iodine: effective skin disinfectant

**Preparations:**

- . Aqueous Iodine
- . Iodine Tincture: 2% iodine and 70% ethanol.
- . Iodophores (e.g. Betadine): Less toxic and less active than Aqueous iodine and iodine tincture.

2. Hydrogen peroxide (3%)

Used for cleansing of wound, disinfecting medical-surgical devices and plastic contact lenses.

### **Dyes**

E.g. Brilliant green

Malachite green

Crystal violet

. highly selective for Gram-positive bacteria.

Uses

. for treatment of dermatological lesions.

. for formulation of selective culture media.

### **Alkylating agents**

E.g. Formaldehyde

Glutaraldehyde

Ethylene oxide

### **Formaldehyde**

37% aqueous solution form is named as formalin.

Uses:

. Preservation of fresh tissues.

. Preparation of vaccines from bacterial surfaces, viruses and toxins.

. Bactericidal including spores.

### **Glutaraldehyde**

. 10 times more effective than formaldehyde.

. cold sterilizing for medical-surgical instruments.

### **Ethylene oxide**

. gaseous sterilizing chemical.

Use: sterilize medical-surgical devices that would be damaged by heat.

**Antiseptic agents:** Disinfectants that are applied on animate bodies.

Characteristics:

- . Never be toxic to cells
- . Never be corrosive
- . Should never change nature of skin

Eg. Savlon

Alcohol( 70%)

Iodine tincture

Iodophor

### 1.9.2. Physical methods of sterilization and disinfection

1. Heat: the most reliable and universally applicable method of sterilization.

#### **Mechanism of action**

- . Dry heat – denatures protein.
- . Moist heat – denatures and coagulates protein.

1.1. Dry heat : It is less efficient and requires high temperature and long period heating than moist heat.

Dry heat can be used by the following methods:

- a. Incineration : It is an efficient method of sterilization and disposal of contaminated needles, syringes and cover slips at high temperature
- b. Red heat : Inoculating wires, loops and points of forceps are sterilized by holding them in the flame of a Bunsen burner until they are red hot.

- c. Flaming: Scalpels and neck of flasks, bottles and tubes are exposed for a few seconds, but it is of uncertain efficacy.
- d. Hot Air Sterilizer (Oven): it is essential that hot air should circulate between the objects being sterilized and these must be loosely packed and adequate air space to ensure optimum heat transfer.

It is done by applying 160 °c for 1 hour.

Use: Sterilizes glassware, oils, greases, lubricants and powders.

- 1.2. Moist heat: It is preferred to dry heat due to more rapid killing.

Moist heat can be used by the following methods.

- a. Boiling: It is not reliable method of sterilization. It is done by applying 100 °c for 30 minutes.

Used for sterilizing catheters, dressing and fabrics.

- b. Tyndallization : Intermittent steaming (Fractional sterilization)

Steaming of the material is done at 100 °c for 30 minutes on three consecutive days.

The principle is that spores which survived the heating process would germinate before the next thermal exposure and then would be killed.

It is used for sterilizing heat sensitive culture media containing materials such as carbohydrates, egg or serum.

- c. Pasteurization: It is the process of application of heat at temperature of

62 °c for 30 minutes(Holder method) or 72 °c for 15 seconds (Flash method) followed by rapid cooling to discourage bacterial growth.

Uses:

- . Pasteurization of milk
- . Preparation of bacterial vaccines.

d. Autoclaving : Steam under pressure

It is based on the principle that when water is boiled at increased pressure, hot saturated steam will be formed which penetrates and gives up its latent heat when it condenses on cooler objects.

Hot saturated steam in autoclaving acts as an excellent agent for sterilization because of:

1. high temperature
2. High latent heat
3. ability to form water of condensation
4. contraction in volume that occurs during condensation

**Method of using an autoclave**

- Add the correct volume of water to the autoclave.
- Place the bottles and tubes of culture media with caps loosened in the inner chamber of the autoclave.
- Secure the lid i.e. Open the air-outlet and close the draw-off knob.
- Adjust the safety valve to the required pressure and temperature.
- When the required pressure and temperature has been reached, begin timing.



NB: Most culture media are sterilized at a pressure of 15 lb/in<sup>2</sup>, at a temperature of 121 °C for 15 minutes.

- At the end of sterilizing time, turn off the heat and allow the autoclave to cool naturally.

It destroys bacterial endospores and vegetative cells.

Uses: Sterilize solid and fluid culture media, gowns, medical and surgical equipment.

**Time –Temperature-Pressure level relationship in moist heat sterilization (autoclaving)**

<u>Temperature</u>	<u>Time</u>	<u>Pressure level</u>
121 °C	15 minutes	15 lb/inch <sup>2</sup>
126 °C	10 minutes	20 lb/inch <sup>2</sup>
134 °C	3 minutes	30 lb/inch <sup>2</sup>

**Methods of controlling sterilization**

1. Recording of temperature and time of each sterilizing cycle.
2. Heat-sensitive autoclave tape fixed to the outside of each pack.
  - . Color change of autoclave tape from blue to brown-black indicates complete sterilization.
3. Biological indicator : Use of paper strips impregnated with spores of *Bacillus stereothermophilus*
  - . Put the paper strip in the culture medium after autoclaving and observe for germinating bacteria to check for growth. In complete sterilization there should not be bacterial growth.

e. Freezing: Inactivation of living bacteria by cold.

It prevents active multiplication of bacteria by decreasing the metabolic activity of bacteria.

Lyophilization : Freeze-drying : Involves rapid freezing with subsequent drying.

Use:

- . Preservation of microbial cultures.
- . Preservation of vaccines.

f. Filtration : Mechanical sieving through membrane filters.

Uses:

. Sterilization of thermolabile parental and ophthalmic solutions, sera and plasma.

- . Microbial evaluation of water purity.
- . Viable counting procedures.
- . Determination of viral particle size

g. Radiation : Ionizing and ultra violet radiation

Ionizing radiation includes  $\alpha$  ray,  $\gamma$  ray and  $\beta$  ray.

These induce break down of single stranded or sometimes double stranded DNA.

Ultra violet radiation has less quantum energy with low penetrating power than ionic radiation.

Spore forming bacteria are more resistant to ionic and ultra violet radiation than vegetative bacteria because of:

1. The spore coat confers protection.
2. DNA is in different state in spores.

Use: Sterilize surgical sutures, catheters, petridishes, culture media while dispensing and pharmaceutical products like hormones, enzymes and antibiotics.

It also sterilize biological safety cabinet (Laboratory rooms).

### 1.9.3. Anti-Microbial agents and Sensitivity Testing

#### Anti- Microbial drugs

Anti-microbial drugs include

- . Antibiotics
- . Chemical anti-microbials

Antibiotics:

Definition: Antimicrobial substances produced by living micro-organisms.

Chemical anti-microbials

Definition: synthetically produced anti-microbial compounds.

Anti-microbial drugs show specific toxicity to microbial cells due to differences in cell envelope, protein and enzymes to host cells.

#### Mechanism of action of anti-microbial drugs

1. Those inhibiting cell wall synthesis, leading to cell lyses.
  - penicillin
  - cephalosporin
  - vancomycin

2. Those damaging cell membrane leading to loss of cell contents and then cell death.
  - polymyxin
  - Amphotericin B
3. Those inhibiting protein synthesis and then arresting bacterial growth
  - aminoglycosides
  - tetracycline
  - erythromycin
  - chloramphenicol
  - clindamycin
4. Those inhibiting nucleic acid synthesis
  - 4.1. Preventing DNA synthesis
    - Nalidixic acid
    - Quinolones
  - 4.2. Preventing RNA synthesis
    - rifampicin
5. Those inhibiting nucleotide synthesis
  - sulfonamide
  - trimethoprim

### **Resistance of bacteria to anti-microbial drugs**

Production of enzymes that destroy or inactivate anti-microbials

Eg. B.lactamase destroying B-lactam ring of penicillin

1. Altering permeability of bacterial cell membrane  
Eg. resistance to polymixin and tetracycline
2. Developing an altered structural target for the drug  
Eg. resistance to aminoglycosides and erythromycin
3. Developing an altered metabolic pathway that bypasses the reaction inhibited by the drugs  
Eg. resistance to sulfonamides
4. Developing an altered enzyme that can still perform its metabolic function but is much less affected by the drug  
Eg. resistance to trimethoprim

### **Dangers of indiscriminate use of antimicrobial drugs**

1. Wide spread sensitization resulting in hypersensitivity and anaphylactic reaction, and drug rashes.
2. Changing normal microbial flora leading to “super infection” due to over growth of drug-resistant micro-organism.
3. Direct drug toxicity  
Eg. renal and auditory nerve damage due to aminoglycosides toxicity.
4. Masking serious infection without eradicating it.
5. Development of drug resistance by micro-organisms.

## **Anti-microbial sensitivity testing**

Anti-microbine activity is measured in vitro in order to determine:

- the potency of an anti-microbial agent
- concentration of anti-microbial agent in body tissues or fluids
- the sensitivity of a given micro-organism to known concentrations of the drug

Measurement of anti-microbial activity

Techniques

- a diffusion technique
- a dilution technique

### **Diffusion Sensitivity Tests**

It is the routinely used sensitivity test by most microbiology laboratories.

A filter paper disk containing measured quantities of drug is placed on a solid medium that has been seeded with the test organisms. The drug diffuses from the disk into the medium.

Following over right incubation, the diameter of the clear zone of inhibition surrounding the deposit of drug is taken as a measure of the inhibitory power of the drug against the particular test organism.

Bacterial strains sensitive to the drug are inhibited at a distance from the disk whereas resistant strains grow up to the edge of the disk.

In the Kirby-Bauer technique, the zone of inhibition is measured and compared to a prepared scale, which correlates the zone of inhibition size with the minimum inhibition concentration (MIC).

NB: Minimal inhibitory concentration (MIC) is the lowest concentration of antimicrobial agent that is required to inhibit in vitro bacterial multiplication under specified conditions.

Minimal bactericidal concentration (MBC) is the least concentration of the anti-microbial required producing a sterile culture.

#### **Dilution Sensitivity Tests**

- Agar dilution tests
- Broth dilution tests

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) or to kill the test bacteria (MBC).

Nowadays the above tests are either time consuming or cumbersome, so the advent of microdilution both solution tests has simplified the method and permit a quantitative result to be reported, indicating the amount of a given drug necessary to inhibit or kill the test micro-organism.

### Factors affecting anti-microbial activity in vitro

1. P<sup>H</sup> of the environment
  - some drugs more active at acidic or alkaline P<sup>H</sup>
2. Components of medium
  - media composition components enhance or inhibit bacterial growth.
3. Stability of drug
4. Size of inoculum: the larger the bacterial inoculum, the lower the apparent sensitivity of the organisms.
5. Length of incubation: short exposure of moisture to the drug inhibits their growth but does not kill them; longer exposure of moisture to a drug gives a chance for resistant mutants to emerge.
6. Metabolic activity of moistures
  - actively and rapidly growing micro-organisms are more susceptible to drug action than those in the resting phase.

Techniques of routinely used antimicrobial sensitivity testing (disc diffusion tests)

Required:

- sensitivity testing media
- Anti-microbial discs
- Control strains
- Turbidity standard



Sensitivity testing media: The commonly used media is Mueller-Hinton agar.

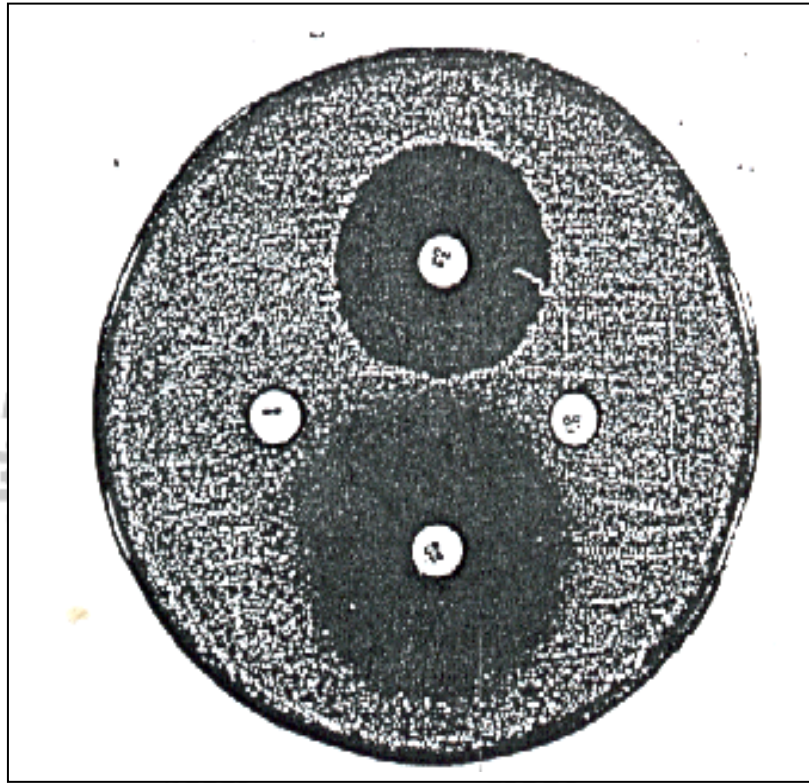
For pathogens requiring enriched media like *Neisseria gonorrhoea*, *Haemophilus influenzae* and *Streptococcus pneumoniae*, it is necessary to add blood to (heat it if needed) sensitivity testing agar.

Turbidity (Opacity) standard: This is a barium chloride standard against which the turbidity of the test inocula can be compared. The turbidity of the standard is equivalent to the turbidity of subcultured broth test micro-organism.

#### Method

- Emulsify several colonies of similar appearance of the test organism in a small volume of sterile nutrient broth.
- Match the turbidity of the subculture against the turbidity standard.
- Apply a loopful of the test organism subculture to the sensitivity testing plate using a sterile loop.
- Spread the inoculum evenly across the plate using a sterile dry cotton wool swab.
- Allow the inocula to dry for a few minutes with the petridish lid in place.
- Place the anti microbial discs into the test organism in petridish using a sterile forceps or dispenser.  
NB: each disc should be pressed down on the medium and should not be moved once in place.
- Incubate the plate aerobically at 35-37 °c over night after 30 minutes of applying the discs.

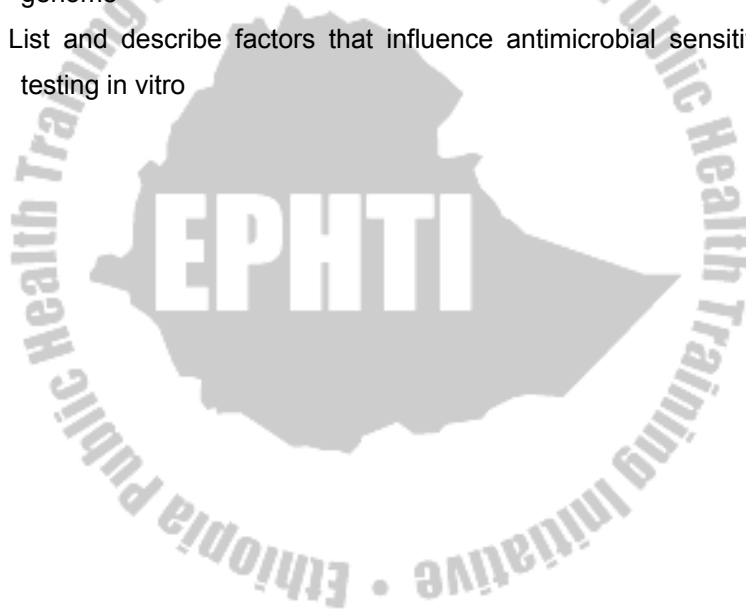
- Read the tests and interpret as 'sensitive (S)', " resistant (R) "or " intermediate (I)" comparing the chart of the sensitivity test.



**Fig. 1.16** Antimicrobial sensitivity test Media

## Review Questions

1. Mention the function of bacterial cell envelope
2. Mention the procedure of gram's and Ziehl-Neelson's staining method
3. Discuss the different types of culture media
4. Label and describe each bacterial growth phase
5. Describe the types of gene transfer that alter the bacterial genome
6. List and describe factors that influence antimicrobial sensitivity testing in vitro



## CHAPTER TWO

### COLLECTION, TRANSPORT AND EXAMINATION OF SPECIMEN

- ◆ If pathogens are to be isolated successfully, the type of specimen, its collection, time and method of its dispatch to the laboratory must be correct.
- ◆ Adequate information about the patient's condition and antimicrobial treatment must also be sent in the Specimen.

#### **Type of specimen**

The correct type of specimen to be collected will depend on the pathogens to be isolated.

For example: a cervical not a vaginal swab is required for the most successful isolation of *N.gonorrhoeae* from a woman.

:sputum not a saliva is essential for the isolation of respiratory pathogens.

#### *Time of collection*

- ❖ Specimens such as urine and sputum are best collected soon after a patient wakes when organisms have had the opportunity to multiply over several hours.
- ❖ Blood for culture is usually best collected when a patient's temperature begins to rise.

- ❖ The time of collection for most other specimens will depend on the condition of the patient, and the time agreed between medical, nursing and laboratory personnel for the delivery of the specimen to the laboratory.
- ❖ Every effort must be made to collect specimens for microbiological investigation before antimicrobial treatment is started.

### **Collection techniques**

- ◆ The laboratory should issue written instruction to all those responsible for collecting specimens including staff of wards, out patient clinics and health centres.
  - ◆ Precaution apply to the collection of most microbiological specimens:
    - 1) Use a collection technique that will ensure a specimen contains only those organisms from the site where it was collected.
      - If contaminating organisms are introduced in to a specimen during its collection or subsequent handling, this may lead to difficulties in the interpretation of cultures and delays in the issuing reports.
      - A strictly sterile (aseptic) procedure is essential when collecting from site that are normally sterile.

Example: collection of blood, CSF or effusions

This teaching is necessary not only to prevent contamination of the specimen but also protect the patient.

- 2) Avoid contaminating discharges or ulcer material with the skin commensals. The swabs used to collect the specimens must be sterile and the absorbent cotton wool from which the swabs are made must be free from antibacterial substances.
- 3) Collect specimens in sterile, leak-proof, dry containers, free from all traces of disinfectant.  
Container must be clean but need not be sterile for the collection of feces and sputum.
- 4) Those responsible for collecting specimens should report any abnormal features, such as coldness in a specimen which should appear clear, abnormal coloration or presence of pus, blood, mucus or parasites.

*Labelling of specimens and sending of a request form*

Each specimen must be accompanied by a request form which gives:

- The patient's name, age (whether an infant, child or adult), number, and ward or health center.
- Type of specimen and the date and time of its collection.
- Investigation required
- Clinical note giving details of the patient's illness, suspected disease, and any antimicrobial treatment that may have been started at home or in the hospital.

***Specimen containing dangerous pathogen***

- ❖ Those delivering, receiving and examining specimens must be informed if a specimen is likely to contain highly infection organisms. Such specimen should be labelled **HIGH RISK**, and whenever possible carry a warning symbols such as red dot, star or triangle
- ❖ Specimen which should be marked as **HIGH RISK** include:
  - Sputum likely to contain *M. tuberculosis*
  - Faecal specimen that may contain *V. cholerae* or *S.typhi*
  - Fluid from ulcers postules that may contain *anthrax bacilli* or treponemes
  - Specimens from patient with suspected HIV infection, hepatitis, viral haemorrhagic fever, or plague.

***Preservatives and transport media for microbiological specimen***

- ❖ In general, specimens for microbiological investigations should be delivered to the laboratory as soon as possible. This will help to ensure that pathogens are living when they reach the laboratory.
- ❖ When a delay in delivery is unavoidable, For example, when transporting a specimen from health center to a hospital laboratory, a suitable chemical preservative or transport culture medium must be used.

- ❖ This will help to prevent organisms from dying due to enzyme action, change of pH, or lack of essential nutrients.  
Example. Amies transport medium widely used and effective in ensuring the survival of pathogens like the more delicate organisms such as *Neisseria gonorrhoeae*.

Cary - Blair medium is used as transport medium for faeces that may contain *Salmonella*, *shigella*, *campylobacter* or *vibro species*.

Example of Preservatives include boric acid added to urine and cetylpyridinium- chloride sodium chloride (CPC-NaCl) added to sputum for the isolation of *M.tuberculosis*.

#### *Transport of microbiological specimens collected in a hospital*

- Specimen should reach to the laboratory as soon as possible or a suitable preservative or transport medium must be used.
  - Refrigeration at 4-10 °c can help to preserve cells and reduce the multiplication of commensals in unpreserved specimens.
  - However, specimens for isolation of *Haemophilus*, *S. pneumoniae* or *Neisseria species*, must never be refrigerated because cold kills these pathogens.
- ❖ If specimen are to be mailed, the regulations regarding the sending of “pathological specimens” through the post should be obtained from the post office and followed exactly.  
When dispatching microbiological specimens:



- 1) Keep a register of all specimens dispatched.

Record Name

Number

Ward or health centre of the patient.

Type of specimen

Investigation required

Date of dispatch

Method of sending the specimen (eg. Mailing, hand –delivery etc.)

- 2) Ensure that the specimen container is free from cracks, and the cap is leak proof. Seal round the container cap with adhesive tape to prevent loosening and leakage during transit.
- 3) If the container is glass tube or bottle, use sufficient packaging material to protect a specimen.

If the specimen is fluid, use sufficient absorbent material to absorb it.

- 4) Mark all specimen that may contain highly infectious organism “**HIGH RISK**”
- 5) Dispatch slides in a plastic slide container or use slide carrying box.
- 6) Label specimens dispatched by mail, “FRAGILE WITH CARE- PATHOLOGICAL SPECIMEN”.

*Collection, Transport and examination of sputum*

**Possible pathogens**

**Gram positive**

*Streptococcus pneumoniae*

*Staphylococcus aureus*

*Streptococcus pyogenes*

**GRAM NEGATIVE**

*Haemophilus influenzae*

*Klebsiella pneumoniae*

*Pseudomonas aeruginosa*

*Proteus species*

*Yersinia pestis*

**Sputum commensals**

- ❖ Sputum as it is being collected passes through the pharynx and the mouth. Therefore, it becomes contaminated in the small number of commensal organisms from the upper respiratory tract and mouth

**Gram positive**

*Staphylococcus aureus*

*Staphylococcus epidermidis*

*Streptococcus Viridans*

*Streptococcus pneumoniae*

*Enterocci*

*Diphtheroids*

*Yeast-like fungi*

**Gram negative**

*Neisseria*

*Branhamella catarrhac's*

*Haemophilus influenzae*

*Fusobacteria*

*Coliforms*

**In a hospital with a microbiology Laboratory.**

1. Give the patient a clean (need not be sterile), dry, wide-necked, leak- proof container and request him or her to cough deeply to produce a sputum specimen

Note: The specimen must be sputum, not saliva.

Sputum is best collected in the morning, soon after the patient wakes and before any mouth-wash is used.

If the patient is young child and it is not possible to obtain sputum, gastric washings can be used for the isolation of *M.tuberculosis* but not for other respiratory

2. Label the container and fill the request form.
3. If pneumonia or bronchopneumonia is suspected, deliver the sputum to the laboratory with as little delay as possible.
  - Because organisms such as *S.pneumoniae* and *H. influenza* require culturing as soon as possible.
  - If the specimen is for the isolation of *M.tuberculosis*, ensure it is delivered to the laboratory within 2 hours or kept at 4 °c until delivery is possible.
  - If pneumonia plague is suspected: Deliver the sputum to the laboratory as soon as possible. Make sure that the specimen is marked **HIGH RISK**.

### **For dispatching to a microbiology laboratory**

Collect the sputum in a container as usual.

Depending on whether the sputum is for the isolation of *pneumonia* and bronchopneumonia or *M.tuberculosis*, proceed as follows:

#### *Pneumonia and Bronchopneumonia pathogen*

- ❖ Collect a purulent part of the sputum on a cotton wool swab, and insert in a container of Amies transport medium. Label the container using a lead pencil
- ❖ Amies will help the pathogens to survive and from being overgrown by fast-multiplying commensals.
- ❖ Make a smear on slides for gram staining and fix using heat or alcohol and send the swab and the request form to reach to the laboratory.

#### ***M.tuberculosis***

- Make a smear of the sputum on slides for Ziel-Neelsen staining, from the most purulent materials.
- Fix with alcohol.

#### **If pneumonia plague is suspected:**

- Send the swab of the sputum in cary-Blair transport medium to reach the microbiology laboratory.

Caution: *Y. pestis* is highly infectious organism.

*Laboratory examination of sputum*

**1. Describe the appearance of the specimens**

- Purulent: Green- looking with pus and mucus.
- Mucopurulent: Green- looking with pus and Mucus
- Muroid: Mostly mucous
- Mucosalivary: Mucus with a small amount of saliva
- Bloody: should be reported.

**2. Examine the specimen microscopically.**

**Gram smear**

Look for pus cells and bacteria

- Gram positiveve diplococci that could be *S. pneumoniae*
- Gram positive cocci that could be *S. aureus*
- Gram negative rods that could be *H. influenzae*
- Gram negative capsulated rods that could be *K. pneumoniae*.

**Additional investigation**

Look for:

- **saline preparation**, if paragonimiasis is suspected
- **Eosin paration**, if asthma or other allergic condition is suspected.
- **Potassium hydroxide (KOH) preparation**, if fungal infection is suspected (yeast cells, Nocardia species, actinomycetes).

- **Giemsa smear**, if histoplasmosis or pneumonic plague is suspected.

### 3) Culture the specimen

To obtain as pure a culture as possible of a respiratory pathogen it is necessary to reduce the number of commensals inoculated.

Ways of reducing commensal numbers include washing the sputum free from saliva or liquefying and diluting it.

#### **Blood agar and chocolate agar**

- Wash a purulent part of the sputum in about 5ml of sterile physiological saline.
- Inoculate the washed sputum on plates of blood agar and chocolate (heated blood) agar.
- Add an optochin disc to the chocolate agar plate. This will help to identify *S. pneumonia*.
- Incubate the blood agar plate aerobically and the chocolate agar in a CO<sub>2</sub> enriched atmosphere at 35-37°C for up to 48 hours, examining for growth after overnight incubation.

Additional:

**Lowenstein Jensen medium, if pulmonary tuberculosis is suspected.**

- About 20 minutes before culturing, decontaminate the specimen by mixing equal volumes of sputum and

sodium hydroxide (NaOH) 40 g/l or ( 4% W/v ) solution.  
Shake at intervals to homogenize the sputum.

- Using a sterile pasteur pipette, inoculate 200 µl (0.2ml) of the well-mixed homogenized sputum on a slope of acid Lowenstein Jensen medium. Allow the specimen to run down the slope.
- Incubate at 35°C –37°C in a rack placed at an angle of about 45° to ensure that the specimen is in contact with the full length of the slope.
- After one week, place the slope in an upright position and continue to incubate, examining twice a week for growth.

#### 4. Examine and report the culture

Examine the blood agar & Chocolate agar culture for:

*S. pneumoniae*

*S. aureus*

*H. influenzae*

*K. pneumoniae*

*Pse. Aeruginosa*

Examine Lowenstein Jensen culture for:

M.. TUBERCULOSIS

## Collection, transport and examination of mouth and throat specimens

### Possible pathogens

#### Gram positive

*Streptococcus pyogenes*

Other *beta-haemolytic streptococci*

*Corynebacterium diphtheriae*

#### Gram negative

Vincent's organisms

**Virus:** Respiratory viruses, enteroviruses and herpes simplex virus type-I

**Fungi:** *Candida albicans*.

- Pathogens in the upper respiratory tract such as *Bordetella pertussis*, *Streptococcus pneumoniae*, and *Neisseria meningitidis*, are usually more successfully isolated from pernasal and nasopharyngeal specimens.

### Throat and mouth commensals

#### Gram positive

*Viridans streptococci*

Non-haemolytic streptococci

*Streptococcus pneumoniae*

*Staphylococcus epidermidis*

*Micrococci*

*Lactobacilli*

#### Gram negative

*Branhamella catarrhalis*

*Neisseria Pharyngitidis*

*Fusobacteria*

*Coliform*



### Collection and Transport of Throat and Mouth swabs

- Whenever possible it should be collected by a medical officer or experienced nurse.

#### In a hospital with a microbiology laboratory

1. In a good light and using the handle of a spoon to depress the tongue, examine the inside of the month.

Look for inflammation, and the presence of any membrane, exudate or pus.

- With diphtheria – a greyish- yellow membrane (later becoming grayish Green-black and smelly) can often be seen over the soft palate and backwards on to the pharyngeal wall.
  - With streptococcal sore throat, the tonsils are inflamed and often covered in a yellow spots.
  - With infectious mononucleosis, the tonsils may be covered with a white exudate
  - With *C. albicans* infection, patches of white exudate may be seen attached in places to the mucosal membrane of the month.
2. Swab the affected area using a sterile cotton. Taking care not to contaminate the swab with saliva, return it to its sterile container.
    - Before swabbing the patient must not be treated with antibiotics or antiseptic mouth-washes (gargles) for 8 hours.

- For children, swabbing may cause obstruction of the child's airway instead of blood for culture should be collected.
3. Within 2 hours of collection, deliver the swab with a request form to the laboratory

#### **Dispatching to the microbiology laboratory**

1. Using a sterile swab or silica gel collect a specimen from the infected area.
2. Taking care not to contaminate the swab, return it to its tube.  
Seal with adhesive tape and label using a lead pencil.
3. Send the swab with its request form to reach the microbiology laboratory within three days.

#### *Laboratory Examination of throat and mouth swabs*

#### **1. culture the specimen**

##### **Blood agar**

- Inoculate the swab on a plate of blood agar.
- If the swab is received in silica gel ( eg. from health centre), moisten it first with sterile nutrient broth and then inoculate the plate.
- Add a bacitracin disc. This will help in the identification of *S. pyogenes*.
- Incubate the plate preferably anaerobically or in a CO<sub>2</sub> enriched atmosphere overnight at 35-37°C

- Beta-haemolytic streptococci produce larger Zones of haemolysis when incubated anaerobically.
- A minority of Group A streptococcus strains will only grow if incubated anaerobically.

***Additional***

**Modified Tinsdale medium and tellurite blood agar if diphtheria is suspected.**

- If in an area where diphtheria occurs or the disease is suspected, inoculate the swab on modified Tinsdale medium and tellurite blood agar.
- Incubate the plates aerobically at 35-37<sup>0</sup>c for up to 48 hours, examining for growth after overnight incubation.

*Sabouraud agar if thrush is suspected*

- Inoculate the swab on sabourad agar
- Incubate at 35-37<sup>0</sup>c for up to 48hours checking for growth after overnight incubation

**2. Examine the specimen microscopically**

**Gram smear**

Examine the smear for pus cells and Vincent's organisms:

- Vincent's organism are seen as Gram negative spirochaetes ( B. vincenti)

And fusiform rods, (*L. buccalis*).

- If thrush is suspected, look for Gram positive yeast-like cells.
- If diphtheria is suspected, look for Gram positive pleomorphic rods
- Commensal diphtheroids, however, are strongly Gram positive and Unlike *C. diphtheriae*, they show little variation in size or shape.

**Additional**

**Albert stained smear**

Examine the smear for bacteria that that could be *C. diphtheriae*:

- Most strains of *C. diphtheriae* contain dark-staining volutin granules.
- The pleomorphic rods tend to join together at angles giving the appearance of Chinese letters.

**3. Examine and report the cultures.**

**Blood agar culture**

- look for beta-haemolytic colonies that could be group *Streptococcus* (*S. pyogenes*)
- or a beta-haemolytic streptococcus belonging to another lancefield group such as group C or G.
- Most group A strains show sensitivity to bacitacin.

**Reporting of the throat swab cultures:**

- If a B-haemolytic streptococcus sensitive to bacitracin is isolated, report the culture as "S. pyogenes presumptive group A isolated, Lancefield group to be confirmed.
- If a B-haemolytic streptococcus that is not sensitive to bacitracin is isolated (confirm that the colonies are streptococci), report the culture as "Beta-haemolytic streptococcus isolated, lancefield group to follow".

**Additional:**

**Modified Tinsdale medium (MTM) and tellurite blood agar (TBA) Cultures.**

- On MTM, c. diphtheriae produces grey-black raised colonies surrounded by a dark brown area.  
-If there is no growth, reincubate to the plate for further 24 hours.
- Examine the TBA plate for grey or grey-black colonies measuring 0.5-2 mm in diameter.

*Sabouraud agar culture* **Look for candida albicans**

**Collection transport and examination of Nasopharyngeal aspirates and Nasal swabs**

**Nasopharyngeal Aspirates and perinasal swabs**

Possible pathogens

**Gram positive**

*Streptococcus pneumoniae*

*Corynebacterium diphtheriae*

**Gram negative**

*Haemophilus influenzae*

*Neisseria meningitidis*

(carriers)

*Bordetella pertussis*

*Bordetella parapertussis*

*Klebsiella species*

**Also *M. leprae***

**Viruses:** Respiratory viruses and enteroviruses.

**Anterior Nasal Swabs**

**Possible pathogens**

- Most anterior nasal swabs are examined to detect carriers of pathogens

**Gram positive**

**Gram negative**

*S. aureus*

*N. meningitidis*

*S. pyogenes*

*H. influenzae* (mostly non capsulate)

**Nasopharyngeal Aspirates, perinasal and Anterior Nasal swabs  
Commensals**

**Gram positive**

**Gram negative**

*S. viridans*

*Neisseria species*

*S. pneumoniae*

*Haemophilus species*

*Enterococci*

*Staphylococcus species*

*Micrococci*

**Collection and Transport of upper Respiratory Tract specimens**

Nasopharyngeal aspirates: Gently pass a sterile catheter through one nostril as far as the nasopharynx.

: Attach a sterile syringe to the catheter, and aspirate a specimen of mucopus.

: Dispense the specimen into a small sterile container.

: Label and deliver with a completed request form to the laboratory as soon as possible.

**Pernasal swabs for the culture of *B. pertussis*:**

If whooping cough is suspected and the nasal passages are clear, collect a penasal swab as follows:

1. Using a sterile cotton or alginate wool swab attached to an easily bent pieces of wire, gently pass the swab along the floor of one nostril directing the swabdown wards and backward as far as the Nasopharynx.
2. Taking care not to contaminate the swab, replace it in its sterile container.
3. Label, and deliver immediately to the laboratory with a request form.

Note:- *B. pertussis* does not survive well on a swab

-It must be cultured as soon as possible.

-If plating cannot be performed at the bedside, the swab should be placed in special transport medium

-If it is not possible to collect a per nasal swab, a less satisfactory way of isolating *B. pertussis* is to hold the plate of culture medium in front of the child's mouth during a coughing attack.

**Anterior nasal swabs to detect carriers:**

1. Using a steile cotton wool swab moistened with sterile peptone water, gently swab the inside surface of the nose.
2. Taking care not to contaminate the swab, replace it in its sterile container.



3. Label, and within 2 hours deliver the swab with a request form to the laboratory.

*Laboratory examination of upper respiratory tract specimen*

**1. culture the specimen**

**Blood agar and chocolate agar**

- To detect *H. influenzae*, *N. meningitidis*, and *S. aureus* carrier:
  - Inoculate the swab on chocolate (heated blood) agar.
  - Incubate the plate in carbondioxide enriched atmosphere at at 35<sup>0</sup>-37<sup>0</sup>c for up to 48 hours., examinig for growth after overnight incubation.
- To detect *S. pyogenes* and *S. aureus* carriers:
  - Inoculate the swab on blood agar.
  - Incubate the plate preferably anaerobically at 35<sup>0</sup>-37<sup>0</sup>c overnight (if for the isolations of *S. aureus* only, incubate aerobically).

**Additional**

**Charcoal Cephalixin blood agar if whooping cough is suspected**

- Inoculate the swab over the entire surface of a plate of charcoal cephalixin blood agar (CCBA).

- Incubate the plate aerobically in a moist atmosphere (in a plastic bag or polythene container with a wet piece of cotton wool) at 35-37<sup>0</sup>c for up to 6 days,
- Examining for growth after about 48 hours incubation.

**Culture of swab received in bordetella transport medium:**

- Inoculate the swab on a plate of CCBA
  - Return the swab in its container and incubated it at 35-37<sup>0</sup>c
  - Examine the plate for growth after 48hours incubation.
- ❖ If no growth is seen, inoculate a second plats of CCBA with the incubated swab.
- Reincubate the first plats for a further four nights.
  - Examining for growth every 24 hours.
  - The second plate for up to 6 days.

**2. Examine and report the cultures**

**Blood agar and chocolates agar cultures(routine)**

Look for coloniess that could be

*H. influenzae*

*Neissena meningitidis*

*S. aureus*

*S. pyogenes (Group A)*

Additional

**Charcoal ceplea lecin blood agar (CCBA) culture**

The examination of a CCBA culture for *berdetela* species and the identification of *B. pertussis* and *B. parapertussis*.

*Collection, Transport and examination of Ear Discharges*

**Possible pathogens**

**Gram positive**

*S. aureus*

*S. pyogenes*

*Other beta-haemolytic streptococci*

*S. pneumoniae*

**Gram negative**

*P. aeruginosa*

*H. influenzae*

*Klebsiella species*

*proteus species*

*E.coli and other coliforms*

*Bacteriodes species*

**Fungi:** Aspergillus species especially *A. niger*, candida species, and occasionally various species of dermatophyte or phycomycete.

A fungal infection of the ear is called otomycosis

❖ External Ear infection are more commonly caused by:

*S. aureus*

*S. pyogenes*

*P. aeruginos.*

**Middle ear infection or otitis media are commonly caused by :**

*s. pneumoniae*  
*s. pyogenes*  
and other B- haemolytic streptococci  
*H. influenzae* (esp. in children)  
*coliforms*  
*Klebsiella spenil*  
*S. aureus*  
*P. aeruginosa*

- ❖ Chronic ear infection are often caused by Bacteroides species or fungi

**Commensals**

The middle ear is normally sterile. The following organisms may be found as commensals in the external ear:

**Gram positive**

*Viridans streptococci*  
*other coliforms S.epidermidis*  
*Bacillus species*  
*Corynebacterium species*

**Gram negative**

*Escherichia coli and*

*Collection and Transport of Ear Discharges*

1. Collect a specimen of the discharge on a sterile cotton.
2. Place it in container of Amies transport medium, breaking off the swab stick to allow the bottle top to be replaced tightly.
3. Make a smear of the discharge on a slide (for Gram staining).
4. Label the specimens and send them with its request form to the laboratory Within 6 hours.

**Laboratory examination of Ear Discharges**

1. culture the specimen

**Blood agar and Macconkey agar**

Inoculate the specimen on blood agar and macconkey agar

Incubate both plates aerobically at 35-37<sup>0</sup>c overnight.

Additional: Chocolate agar if the patient is a child: Inoculate the specimen on chocolate (heated blood) agar for the isolation of H. influenza.

Incubate the plate in a carbon dioxide enriched atmosphere at 35-37<sup>0</sup>c for up to 48 hours, examining for growth after overnight incubation.

*Blood agar (Kanamycin) for anaerobic incubation if the infection is chronic*

Inoculate the specimen on blood agar, preferably that which contains Kanamycin to inhibit the growth of commensals.

Incubate the plate anaerobically for up to 48hours, checking for growth after overnight incubation.

**Sabouraud agar if a fungal infection is suspected**

Inoculate the specimen on sabouraud agar, and incubate at room temperature for up to 6 days.

**2. Examine the specimen Microscopically**

**Gram smear**

- Make an evenly spread of the specimen on a slide.
  - Allow the smear to air-dry in a safe place.
  - Fix and stain by Gram technique.
  - Look for pus cells and bacteria
- 
- Gram positive cocci that could be *S.aureus*
  - Gram positive streptococci or diplococi that could by streptococci pathogens

- Gram negative rods that could be H.influenzae, p. aeruginosa, klebsiella specis, proteus species, E.coli or other s.
- Gram positive yeast cells that could be candida species.
- Small numbers of Gram positive cocci, streptococci, rods and also Gram negative rods may be seen in smears of ear discharges because these organisms form part of the normal microbial flora of the external ear.

**Additional:**

**Potassium hydroxide preparation if a fungal infection is suspected**

- Mix a small amount of the specimen with a drop of potassium hydroxide, 200g/l (20%W/v) on a slide, and cover with a coverglass.
- After 10 minutes, or when the preparation has cleared sufficiently, examine microscopically using 10x or 40x objective.

**Look for:**

- Brnaching septate hyphae with small round spores, that could be Aspergillns speies
- Pseudohyphae with yeast cells, that could be candida specis (Gram positive)

- Branching septate hyphae, that could be a species of dermatophyte
- Branching aseptate hyphae, that could be a species of phycomycete.

### 3. Examine and Report the culture

**Blood agar and MacConkey**

**Examine chocolate agar**

*S. aureus*

*H. influenza*

*B-haemolytic streptococcus*

*E. Coli*

*Profeus spese*

*P. aeruginosa*

*S. pneumonia*

**examine sabouroud agar for:**

**examine the anaerobic blood**

**fungi elements**

**agar mete for:**

Bacterioid species



## Collection, transport and examination of eye specimens

### Possible pathogens

#### Gram positive

*Staphylococcus aureus*

*Streptococcus pneumoniae*

*Streptococcus agalactiae* (Group – B)

*Streptococcus Pyogenes* (Group – A)

Other B- hemolytic streptococci

#### Gram negative

*Nesseria gonorrhoeae*

*Haemophilus influenzae*

*Haemophilus aegyptius*

*Pseudomonas aeruginosa*

*Moraxella lacunata*

*Erterobacteria*

**Chlamydiae:** *C trachomatis*

**Viruses:** Adenoviruse, herpesviruses and enteroviruses

#### Note:

1. Inflammation of the the delicate membrane lining the eyelid and covering the eyeball conjunctiva is called conjunctivitis. Bacterial conjunctivitis is mainly caused by *S. aureus*, *H. influenzae*, *H.aegyptius*, *S. Pneumoniae* and occasionally by *Moraxella lacunata*.
2. Eye infection of new born infants can be caused by:
  - *N. gonorrhoeae*, transmitted as the infant passes down the birth canal. It causes a severe purulent conjunctivitis that can lead to blindness if not treated. Infection usually develops within 72hours of birth.

- *Streptococcus Group B (S.agalactiae)* and other *B-hemolytic streptococci* that can be transmitted during birth.
  - *C. trachomatis*, that is transmitted during birth and causes conjunctivitis 5-12 days after birth.
  - *S. aureus* that is acquired after birth (commonly referred to as “sticky eyes”)
3. *C. trachomatis* serotype A, B and C cause endemic trachoma, a major cause of blindness especially in children and also in adults in rural areas of developing countries.
  4. *P. aerogunisa* eye infections are frequently caused by the use of contaminated eye drops.
  5. Herpes simplex virus can cause severe inflammation of the cornea (Keratitis)

#### **Commensals**

- That may be found in the eye discharges:

#### **Gram positive**

*Viridans streptococci*  
*Staphylococci*

#### **Gram negative**

*Non-pathogenic neisseriae*  
*Moraxella speires*

#### **Collection and transport of eye specimen**

- Eye specimen should be collected by medical officer or experienced nurses. Conjunctival scrapings to detect *C. trachomatis* must be collected by medical person.

- Specimen from the eye must be cultured as soon as possible after collection because the natural secretions of the eye contain antibacterial enzymes.

### **Procedure**

1. Using a dry sterile cotton wool swab, collect a specimen of discharge (if an infant, swab the lower conjunctival surface).
2. Inoculate the discharge on the following culture media:
  - Blood agar
  - Chocolate (heated blood) agar
  - MNYC *N. gonorrhoeae* selective medium, if the patient is a newborn infant.
3. Make a smear of the discharge on slide (frosted-ended) for staining by the Gram technique. If *C. trachomatis* infection of the newborn is suspected, make a second smear for staining by the Giemsa technique and label the plate and slide.
4. As soon as possible, deliver the inoculated plates and smear(s) with request form to the laboratory.

### **Laboratory examination of the eye specimen**

#### **1. Culture the specimen**

Routine:

#### **Blood agar and chocolate agar**

- Inoculate the eye discharge on blood agar and chocolate (heated blood) agar.

- Incubate the blood agar aerobically at 35-37°C overnight.
- Incubate the chocolate agar plate (CAP) in a CO<sub>2</sub> enriched atmosphere for 48hours, checking for growth after overnight incubation.

**Additional**

**MNYC selective medium if gonococcal conjunctivitis is suspected (infant less than 3 weeks old).**

- Inoculate the discharge on the plate
- Incubate at 35-37°C in a CO<sub>2</sub> enriched atmosphere overnight.

**Loeffler serum slope if Moraxella infection is suspected:**

- Inoculate the eye discharge on a loeffler serum slope.
- Incubate at 35-37°C overnight.

**2. Microscopically examination**

Routine:

**Gram smear**

Look for:-

- Gram negative intracellular diplococci that could be *N. Gonorrhoeae*. If found, a presumptive diagnosis of gonococcal conjunctivitis can be made. A cervical swab from the mother should also be cultured for the isolation of *N.gonorrhoeae*.

- Gram positive streptococci or diplococci that could be streptococci pathogens.
- Gram positive cocci that could be *S.aureus*
- Gram negative rods that could be Haemophilus species.

Additional

**Giemsa smear if *C. trachomatis* infection is suspected:**

- 1) Fix the air-dried smear by covering it with methanol for 3 minutes.
- 2) Dilute the Giemsa stain in the buffered water  
*C. trachomatis*, dilute the stain 1 in 40:
  - Fill a small cylinder to the 19.5 ml (mark with buffered water)
  - Add 0.5ml Giemsa stain to 20ml mark.  
Eg. 0.5ml + 19.5ml  
For Other organisms, 1ml + 19ml
- 3) Stain the smear with diluted Giemsa in dish  
*C. trachomatis*, stain 1 ½ - 2 hours  
For other organisms, stain 25-30 minutes
- 4) Wash the slide from the dish and rise the smear with buffered water. And let the smear to air-dry and examine with oil immersion objective.

Result

- ✓ trachomatis inclusion bodies ----- Blue-mauve to dark purple. Depending on the stage of development; If the inclusion body is more mature, it will contain ---- red-mauve staining elementary particles.

- ✓ Chlamydia bodies must be differentiated from bacteria that may infect the eye such as neisseriae, Hemophilus species, staphylococci, streptococci, and moraxella species.
- ✓ All these bacteria, with the exception of Hemophilus species, stain dark blue in Giemsa stained smear, but Haemophilus rods stain pale blue.
- ✓ Report the smear as “chlamydial inclusion bodies present” or “No chlamydial inclusion bodies seen”.

### **Examine and Report the cultures**

#### **Blood agar and chocolate agar**

Look for colonies that could be:

*Haemophilus Influenzae*

*Staphylococcus aureus*

*Beta- hemolytic streptococci*

*Streptococcus Pneumoniae*

*Pseudomonas aeruginosa*

#### **Loeffler serum slope culture**

Look for *Moraxella lacunata*

Additional

#### **MNYC Medium culture**

- Look for *N. gonorrhoeae*
- ✓ . oxidase positive.

- ✓ . CHO utilization test can also confirm the organism
- ✓ . The organism should also be tested for B-lactamase production.

	Glu	lact	mal	suc
N.gonorrhoeae	A	-	-	-
N.meningtidis	A	-	A	-

Glu =glucose, Lac= lactose, mal=maltose, suc,= sucrose, A= acid produced  
 Acid produced (yellow or orange yellow)

**Collection, transport and examination of skin specimen**

**Possible pathogens**

**Gram positive**

- S. aureus*
- S. pyogenes*
- Enterococci*
- Anaerobic streptococci*
- Bacillus anthracis*
- Corynebacterium ulcerans*

**Gram negative**

- Escherichia coli*
- Proteus*
- Pseudomonas aeruginosa*
- yersinia pestis*
- Vincent's organisms*

Also *M. leprae*

*M. ulcerans*

*T. Pertenuae*

*T. Carateum*

Virus: pox viruses and herpesviruses

Fungi: Ringworm

parasite: Leishmania spp

: onchocerca volvulus

:D. medinensis

## Commensales

### Gram positive

*Staphylococci*

*Micrococci*

*Anaerobic cocci*

*Viridans streptococci*

*Enterococci*

*Diphtheroids*

### Gram negative

*Escherichia Coli* and other coliforms

## Collection of skin specimens and ulcer materials

1. Using a sterile dry cotton wool swab, collect a sample of discharge from the infected tissue.

If there is no discharge, use swabmoistened with sterile physiological saline to collect a specimen.

Insert the swab in a sterile tube.

- If the tissue is deeply ulcerated and necrotic (full of dead cells); Aspirate a sample of infected material from the side wall of the ulcer using a sterile needle and syringe.
- Fluid from pustules and blisters: Aspirates a specimen using a sterile needle and syringe.
- Serous fluid from skin ulcers, papillomas or papules, that may contain treponemes:
  - Collect a drop of the exudates directly on a clean cover glass and invert on a clean slide.



- Delivery immediately the specimen to the laboratory for examination by dark-field microscopy.
2. If the specimen has been aspirated, transport the needle and syring in a sealed water proof container immediately to the laboratory.

#### **Laboratory examination of skin specimens**

- 1) Culture the specimen

#### **Blood agar and MacConkey**

- Inoculate the specimen
- Incubate both plate aerobically at 35-37<sup>0</sup>C overnight.

Additional:

#### **Sabourand agar if a fungal infection is suspected**

- Inoculate to agar plate
- Send to a Mycology Reference laboratory.

Modified Tinsdale Medium (MTM), if cutaneous diphtheria is suspected:

- Inoculate the sample for isolation of *C. Ulcerans*
- Incubate aerobically at 35-37<sup>0</sup>C for up to 48hours, examining the growth after overnight incubation.

Blood agar and MacConkey agar at room temperature, if bubonic plague is suspected:

- Inoculate the specimen
- Incubate both pletes aerobically at room temperature far up to 48hours.

- Examination for growth after overnight incubation.
  - ✓ *Y. pestis* is a highly infectious organism.Maximum care should be taken

**Lownstein Jensen (LJ) Medium if Buruli ulcer is suspected:**

- Decontaminate the swab by immersing it in sodium hydroxide 40g/l (4% W/v) solution for 10 minutes.
- Inoculate the decontaminated specimen on two slopes of acid LJ medium.
- Incubate one slope at 35-37<sup>0</sup>C as described for culture of *M. tuberculosis* and incubate the other slope at 32<sup>0</sup>C for up to 8 weeks.

**2.) Microscopical examination**

Routine:

Gram Smear:

Look for:

- Gram positive cocci that could be *S. aureus*
- Gram positive streptococci that could be *S. pyogenes* or other streptococci.
- Gram negative rods that could be *p. aeruginosa*, *proteus speices*, *E.coli* or other coliforms.
  - ✓ **If tropical ulcer is suspected**, look for vincent's organisms
  - ✓ **If cutaneous anthrax is suspected**, look for large Gram variable rods lying in chains that could be *B. anthracis*.

- Examine also a polychrome methylene blue stained smear.
- ✓ **If bubonic plague is suspected**, look for Gram negative coccobacilli that could be *Y. pestis*.

Additional:

**Potassium hydroxide preparation, if ringworm or other superficial fungi infection is suspected.**

For detection of ringworm:

**Giemsa techniques or wayson's techniques,if bubonic plague is suspected.**

**Polychrome Loeffler methylene blue smear if cutaneous anthrax is suspected.**

- Fix with potassium permanganates 40g/l solutions for 10 minutes.
- Look for chain of large blue-stained capsules characteristic of *B. anthracis*.

**Ziel-Neelsenstained smear if buruli ulcer is suspected**

examine for acid fast bacilli.

**Dark-field microscope to detect treponemes**

- look for motile treponeme if yaws or pinta is suspected

**Examine and report the culture**

**Blood agar and MacConkey agar cultures**

Look for: *S. aureus*

Additional investigations

*S. pyogenes*

**sabouraud agar**

*P. aeniginosa*

-if fungal infection is suspected

*Enterococci*

**Lowenstein Jensen**

*Proteus species*

-if Buruli ulcer is suspected

*Escherichia coli*

**MTM**

- if cutaneous diphtheria is suspected

**Collection, Transport and Examination of urogenital specimens  
possible pathogens**

**Urethral swabs**

- *N. gonorrhoeae*
- *S. Pyogenes*
- *Ureaplasma urealyticum*
- *Chlamydia trachomatis* and
- Occasionally *Trichomonas vaginalis*

**Cervical swabs from non-puerperal women:**

- *N. gonorrhoeae*
- *S. pyogenes*
- Other *B.hemolytic streptococci*
- *Chlamydia trachomatis* and
- *herpes simplex virus*

**Cervical swabs from women with puerperal sepsis or septic  
abortion:**

- *S. pyogenes*
- other *B – haemolytic*
- *streptococci*

- *anaerobic streptococci*
- *enterococci*
- *S. aureus*
- *clostridium perfringes*
- *Listeria monocytogenes*
- *Bacterioles species*
- *protens species*
- *E. Coli & other coliform*

**Vaginal Swabs:**

*T. vaginalis*

*candida species*

*Gardnerella vaginalis* (*Haemophilus vaginalis*)

*Neisseria gonorrhoeae*.

**Fluid and pus from genital ulcers**

*T. pallidium*

*C. trachomatis*

*Calymmatobacterium granulomatis* (*Donovania granulomatis*)

*H. ducreyi*.

**Collection and transport of urogenital specimen**

- Amies medium is the most efficient medium for transporting urethral, cervical and vaginal swabs.

### **Specimen required for diagnosis of gonorrhoea**

Male patients:

- . Smears of urethral discharge
- . Rectal swab from homosexual patient

Female patients

- . Smears of mucopus from the cervix and urethra

Note: *N. gonorrhoeae* infects the mucous membranes of the cervix, not the vagina.

The pathogen is, therefore, more likely to be isolated from a cervical swab than from a vaginal swab.

### **Collection of Urethral specimens**

1. cleans the urethral opening using a swab moistened with sterile physiological saline.
2. Gently massage the urethra from above downwards, and collect a sample of pus on a sterile cotton wool swab.
  - The patient should not have passed urine preferably for 2 hours before the specimen is collected.
3. Make a smear of the discharge on a slide for staining by the Gram technique and label the specimen.

### **Collection of Cervical specimen**

1. Moisten a vaginal specimen with sterile warm water and insert into the vagina.
2. Cleanse the cervix using a swab moistened with sterile physiological saline.
3. Pass a sterile cotton wool swab into the endocervical canal and gently rotate the swab to obtain a specimen.

- Amies transport
- Make a smear of the cervical mucopus for Gram staining and label the specimen

#### Collection of vaginal specimen

- Using sterile swab, collect a sample of vaginal discharge.
  - Amies
  - Smear and label the sample

#### Laboratory examination of urogenital specimen

##### Routine culture

- **MNYC medium**

Incubate in moist CO<sub>2</sub> atmosphere

⇒ *Neisseria gonorrhoeae*

##### Microscopy

⇒ Gram smear:

Look for pus cells and bacteria

##### Suspected gonorrhoeae:

Look for intracellular gram negative diplococci

##### vaginitis:

Look for yeast cells, and epithelial cells in the gram variable coccobacilli

##### Suspected puerperal sepsis or septic abortion

Look for gram positive rods, streptococci, cocci and gram negative rods.

##### Suspected chancroid

Look for Gram negative coccobacilli showing bipolar staining

**Additional culture**

**Blood agar (aerobic and anaerobic), macCokey agar, and cooked meat medium, if puerperal sepsis or septic abortion is suspected**

Sabourand medium, if vaginal candidiasis is suspected and yeast cell not detected microscopically

Serum culture, if chancroid is suspected

⇒ *H. ducreyi*

**Microscopy**

Sahlie preparation, if trichomoniasis is suspected.

Gemsa stained smear: If donovanosis is suspected

Dark field preparation, if syphilis is suspected.

**Colleciton, transport and examination of cerebrospinal fluid**

Possible pathogens

Gram positive

*S. pneumonia*

*S. aureus*

*S. agalactiae (Group B)*

*Listeria monocytogenes*

*Bacillus anthracis*

Gram negative

*Neisseria meningitides*

*H.influenzae type b*



*Escherichia coli*

*Pseudomonas aeruginosa*

*Proteus species*

*Salmonella species*

⇒Also M. tuberculosis

**Viruses:**

Enteroviruses, especially echoviruses and coxsackieviruses.

Rarely polioviruses may also be isolated from CSf.

**Fungi:** *Cryptococcus neoformans*

**Parasites:** Trypanosoma species

Naegleria fowleri

Acanthamoeba species and rarely the larvae of  
Angiostrongylus cantonensis and Dirofilaria immitis

**Note:**

1. Inflammation of the meninges (membranes that cover the brain and spinal cord) is called meningitis.

Pathogens reach the meninges in the blood stream or occasionally by spreading from nearby sites such as the middle ear or nasal sinuses.

Meningitis is described as:

- Pyogenic (purulent), when the C.S.f contains mainly polymorphonuclear neutrophils (pus cell), as in acute meningitis caused by *N. meningitidis*, *H. influenzae* and *S. pneumoniae*.

- Pus cells are also found in the C.S.f in acute amoebic meningoencephalitis.
- Lymphocytic, when the C.S.f contains mainly lymphocytes, as in meningitis caused by viruses, *M. tuberculosis* and *C. neoformans*.
- Lymphocytes are also found in the C.S.f in trypanosomiasis meningoencephalitis and neurosyphilis.
- Eosinophilic, when the CSf contain mainly eosinophilus. This rare form of meningitis is caused by helminthes larvae such as *Angiostrongylus cantonensis* and *Dirofilaria immitis*
- Meningitis of the newborn (neonatal meningitis) is caused mainly by *E.coli*, *S. agalactiae* (Group B), *S. avreus*, *L. Monocytogenes*.
- Haemophilus meningitis occurs mainly in infants and young children.

#### **Commensals**

No normal microbial flora

#### **Collection of Csf**

- It should be collected by medical officer in aseptically procedure
- The fluid is usually collected from the arachnoid space. A sterile wide-bore needle is inserted between the 4<sup>th</sup> and 5<sup>th</sup> lumbar vertebrae and

C.s.f. is allowed to drip into a dry sterile container.

- A delay in examining C.S.f reduces the chances of isolating a pathogen.
- It will also lead to a falsely low glucose value due to glycolysis. If typanosomes are present, they will not be found because they are rapidly lysed once the C.S.f has been withdrawn.

- 1) Take two sterile, dry, screw-cap containers and label one No 1 (first sample collected, to be used for culture), and the other No 2 (second sample collected, to be used for other investigations).
- 2) Collect about 1ml of C.S.f in container No 1 and about 2-3ml in container No2
- 3) Deliver immediately the samples with a request form to the laboratory.

**Note:** It is necessary to culture the C.S.f in the health center because pathogens such as *N.meningitidis*, *H. influenzae* and *S. pneumoniae*, are unlikely to survive transport to the bacteriology laboratory.

- If unable to perform a cell count and estimate the protein and glucose, transfer C.S.f sample No 2 to a screw-cap bottle containing sodium fluoride oxalate and mix

This will preserve the cells and prevent the breakdown of glucose. Protein can also be estimated from a fluoride oxalated specimen.

### **Laboratory examination of C.S.f**

- It should be performed without delay and should be reported micro-organism especially Gram smear. The fluid should be handled with special care because it is collected by lumbar puncture and only a small amount can be withdrawn.

#### **Two samples of C.S.f**

- It is usual to use sample No 1 for the culture, and sample No 2 for the cell count, microscopy and biochemistry.

This is because sample No 1 may contain blood (due to a traumatic lumbar puncture) which will affect the accuracy of the cell count and biochemical estimations.

### **I. Describe the appearance of the specimens**

#### **Report:**

- Whether the C.S.f is clear, slightly cloudy, cloudy or definitely purulent (looking like pus).
- If the C.S.f is purulent or markedly cloudy, make immediately a smear for gram staining, and report it as soon as possible.
- Whether it contain blood and if so whether sample No 2 contains as much blood as sample no 1.

If blood is present in the C.S.f due to a traumatic lumbar puncture, sample No 1 will usually contain more blood than sample No 2.

If the blood is due to haemorrhage in the CNS, the two samples will probably appear equally blood-stained.

Following a subarachnoid haemorrhage the fluid may appear xanthochromic, i.e. Yellow-red (after centrifuging)

The fluid may also appear xanthromic if the patient is jaundiced or when there is spinal constriction.

**Whether it contains clots**

- Clotted C.S.f indicate an increase in fibrinogen.
- It occurs when there is spinal constriction
- Clots can also be found in C.S.f. from patients with pyogenic meningitis (sterile beads to break the clot)
- In tuberculosis meningitis, if the C.S.f is allowed to stand for several hours; skin (spider web clot) may form on the surface of the fluid.

This should be transferred to a slide, pressed out, alcohol-fixed, and stained by the Ziel-Neelsen method I.

**2. Perform a cell count (1:2) value  $5 \times 10^6$  cell/l (sample No 2)**

**3. Test the specimen biochemically**

- Glucose estimation  $\frac{1}{2}$  -  $\frac{2}{3}$  of that found in blood, i.e 2.5 – 4.0 mmol/l (45-72mg%)
- Low Glucos → pyogenic meningitidis
- Normal → viral miningitis.

-High glucose → hyperglycemia

- Total protein estimation and globulin test

Value: 0.15 – 0.40g/l (15-40mg%)

- A positive pandy's test for increase in total C.S.f protein in all form of meningitis.
- When the total protein exceeds 2.8/l (200mg%), the fibrinogen level in usually increased sufficiently to cause the C.S.f to clot. (sever pyogenic meningitis)

#### 4. Culture the specimen (sample No 1)

It is necessary, if the fluid contains cells and, or, the protein concentration is abnormal.

**Note:** C.S.f should be cultured as soon as possible after collection.

If a delay is unavoidable, the fluid should be kept at 35-37<sup>0</sup>C (never refrigerated).

If the C.S.f appears only slightly cloudy, centrifuge it in a sterile tube for 15-20minute and use the sediment for inoculating the plates.

Routine

**Chocolate (heated blood) agar**

Inoculate        -*N. meningitides*  
                      -*S. Pneumonia*  
                      -*H. influenza*

Incubate in a CO<sub>2</sub> enriched atmosphere at 35-37<sup>0</sup>C for 48hours, checking for growth after overnight incubation.

Additional

**MacConkey and blood agar** if the patient is a newborn infant incubate both plate at 35-37<sup>0</sup>C overnight

- *E.coli* or other coliform
- *S. agalactae* (Group B)
- *Lesteriae monocytognes*
- *S. aureus*

**Lowenstein Jensen medium** if tuberculous meningitis is suspected

**Sabouraud agar** if cryptococcal meningitis is suspected.

If capsulated yeast cells are seen in the microscopical preparations, inoculate a plate of sabouraud agar. Incubate at 35-37<sup>0</sup>C for up to 72hours, checking for growth after overnight incubation.

**5. Microscopy**

The microscopical examination of C.S.f is required if the specimen appears abnormal, contains cells and, or, the total protein is raised with a positive pandy's test.

Routine:

Gram smear

- Gram negative intracellular diplococci that could be *N. meningitidis*.
- Gram positive diplococci or short streptococci that could be *S. pneumoniae*. It is often possible to see the capsules as unstained areas around the bacteria.
- Gram negative rods, possibly *H. influenzae*
- Gram negative rods, could also be *E. coli* or other coliforms, especially if the C.S.f is from a newborn infant.
- Gram positive cocci in groups and singly, possible *S. aureus*.
- Gram positive streptococci, possibly *S. agalactiae* (G -B)
- Gram positive yeast cells, *C. neoformans*.

Additional

**Ziel-Neelsen – smear**

*M. tuberculosis*

**Indian ink preparation** if *cryptococcal meningitis* is suspected:

**Wet preparation** to detect amoebae or Trypanosome

**Giemsa stain** to detect morulla cells or Burkett's lymphoma cells

These can be found when trypanosomes have invaded the CNS.



- They are larger than most lymphocytes, stain dark red mauve and contain vacuoles.
- Morula cells contain Igm and are thought to be degenerate plasma cells.

**Collection, transport and Examination of Blood AND Bone marrow**

**Possible pathogens**

**Gram positive**

*S. aureus*  
*Viridans streptococci*  
*S. Pneumoniae*  
*S. pyogenes*  
*Enterococci*  
*Anaerobic streptococci*  
*Clostridium prefringes*

**Gram negative**

*Salmonella typhi*  
*Other salmonella*  
*Brucella species*  
*H. influenzae*  
*P. aeruginosa*  
*Klebsiella strains*  
*E. coli*  
*Proteus species*  
*Bacteriodes species*  
*Neisseria meningitidis*  
*Yersinia pestis*

**Fungi:** *Candida albicans* and other yeast Cells and other systemic mycosis

**Parasites:** *Plasmodium* species (malaria parasites)

*Trypanosoma* species  
*Wuchereria bancrofti*  
*Brugia* species

Loa loa

Leishmania donovani

**Note:**

1. The presence of bacteria in the blood is called bacteraemia. The term septicaemia refers to a severe and often fatal infection of the blood in which bacteria multiply and release toxins into the blood stream.
2. Bacteria that are associated with neonatal septicaemia include E.coli and other coliforms, staphylococci and B.haemolytic Group B streptococci.
3. In typhoid, salmonella typhi can be detected in the blood of 75-90% of patients during the first 10 days of infection and in about 30% of patients during the third week.
  - In chronic salmonellosis, bacteria are often more rapidly and successfully isolated from bone marrow, especially once antimicrobial treatment has been started.
4. Y. pestis can be isolated from the blood in septicaemia plague. The organism is highly infectious.

**Commensal**

Neither blood nor bone has a normal microbial flora.

**Collection and culture of Blood and Bone marrow**

- ✓ Blood and bone marrow require culturing immediately after collection, before clotting occurs.

Choice of culture media

- ✓ Because septicaemia is such a serious condition, it is essential to use media that will provide the fastest growth and isolation of as wide a range of pathogens as possible.
- ✓ The following media are suitable for routine culture of blood and bone marrow:
  - Tryptone soya (tryptic soy) diphasic medium
  - Thioglycollate broth medium

### **Tryptone soya (tryptic soy) diphasic medium**

- A diphasic (two phase) medium is one that combines an agar slope with a broth medium.

Because the bacteria can be seen growing on the slope, the need to subculture on a solid medium every few days is avoided, thus reducing the risk of contamination.

- Tryptone soya diphasic medium consists of a tryptone soya agar slope and a tryptone soya broth to which is added Liquoid and P-aminobenzoic acid.

**Liquoid:** It is the commercial name for sodium polyanethol sulphonate. It prevents clotting of the blood and neutralize the natural bactericidal substances in fresh blood.

**P. Aminobenzoic acid:** This neutralizes the action of sulphonamides should these be present in the blood.

- Tryptone soya diphasic medium is suitable for the growth of a wide range of pathogens.

Incubation in CO<sub>2</sub> is required for the culture of brucella species.  
Strict anaerobes will not grow in this medium.

### **Thioglycollate broth**

- This consists of a nutrient broth to which is added thioglycollate to provide the condition necessary for the growth of anaerobes. Most aerobic bacteria will also grow in thioglycollates broth.
- Because liquid is not added to this medium, a sufficient volume of broth must be used to prevent the blood from clotting and to dilute out the blood's natural bactericidal substances.

The blood should be diluted at least 1 in 10 in the broth.

### **Examination of Blood and Bone marrow**

1. Collect and culture the specimen

#### **Blood**

- It should be collected before antimicrobial treatment has been started and at the time the patient's temperature is beginning to rise.
- To increase the chance of isolating a pathogen, it is usually recommended that at least two specimens (collected at different times) should be cultured.
- Blood for culture must be collected as aseptically as possible.

2. Insert the needle through the rubber line of the bottle cap and dispense 5ml of blood into each culture bottle.

3. Gently mix the blood with the brush.

- The blood must not be allowed to clot in the culture media because any bacteria will become trapped in the Culture medium.

Incubate the inoculated media:

#### **Thioglycollate broth**

At 35-37<sup>0</sup>C for up to 2 weeks, examining and sub-culturing

- Look for visible signs of bacterial growth such as turbidity above the red cell layer, colonies growing on top of the red cells ("cotton balls"), haemolysis, gas bubbles and clots.
- A sterile culture usually remains clear
- If there are signs of bacterial growth, subculture the broth and examine a gram stained smear for bacteria

#### **Tryptone soya diphasic medium**

At 35-37<sup>0</sup>C for up to 4 weeks.

- Look for colonies on the agar slope (preferably using a hand lense), and signs of bacterial growth in the broth.
- Colonies of staphylococ, s .typhi, brucellosis and most coliforms can usually be seen easily. Where as colonies of pneumococci, S. pyogens, and Y.pestis are not easily seen.

- If growth is present subculture to blood agar plate, Chocolate agar plate, MacConkey

**Collection, transport and examination of effusions (synovial, pleural, pericardial, ascitic and hydroceles fluids)**

- An effusion is fluid which collects in a body cavities

**Fluid** which collects due to an inflammatory process is referred to as an **exudates** and that which forms due to a non-inflammatory condition is referred to as a **transudates**.

- If the effusion is all exudates, it is important to investigate whether the inflammatory process is all infective one.
- Effusions sent to the laboratory for investigation include:

Fluid	Origin
Synovial	From joint
Pleural	From the pleural cavity (space between the lungs and the inner chest wall)
Pericardial	From the pericardial sac (membranous sac surrounding the heart)
Ascitic (peritoneae)	From the peritoneal (abdominal) cavity
Hydrocele	Usually from the sacs surrounding the tests.

1. **Synovitis** means inflammation of the synovial membrane (lining of a joint capsule). It can be caused by bacteria, rheumatic disorder or injury.
2. Inflammation of a joint is called **arthritis**.  
The term polyarthritis is used when many joints are affected. Arthritis may be caused by bacteria (infective arthritis), rheumatoid arthritis, gout and pseudogout, osteoarthritis
3. The term pleural effusion is used to describe a non-purulent serous effusion which sometimes forms in pneumonia, tuberculosis, malignant disease etc  
**Empyema** is used to describe a purulent pleural effusion when pus is found in the pleural space.
4. Peritonitis means inflammation of the peritoneum, which is the serous membrane that lines the peritoneal cavity. Ascites refers to the accumulation of fluid in the peritoneal cavity causing abdominal swelling.

### **Commensals**

No microbial flora

Collection is carried out by a medical officer

- 2-3ml without anticoagulant, to see whether clotting occurs.
- 9ml which contain 1ml sterile sodium citrate (3g/l (3% w/v) solution).
  - do the csf:
    - cell count
    - protein estimate
    - microscopy
    - culture

## CHAPTER THREE

### Learning Objective

1. At the end of the lesson, the student shall be able to:
  - List the antigenic structure of bacteria
  - Apply the different chemical laboratory methods to identify the pathogenic bacteria
  - Develop the major classification scheme for gram-positive and gram-negative bacteria

### GRAM POSITIVE COCCI

Genus Staphylococci

Genus Streptococci

#### 2.1.1. GENUS: STAPHYLOCOCCI

Characteristics:

- Gram positive non spore-forming non-motile, spherical cells, usually arranged in grape-like clusters
- Single cocci , pairs, tetrads and chains are seen in liquid cultures
- Young cocci stain strongly gram-positive, on aging many cells become gram-negative
- The three main species of clinical importance
  - . Staphylococcus aureus
  - . Staphylococcus epidermidis



. Staphylococcus saprophyticus

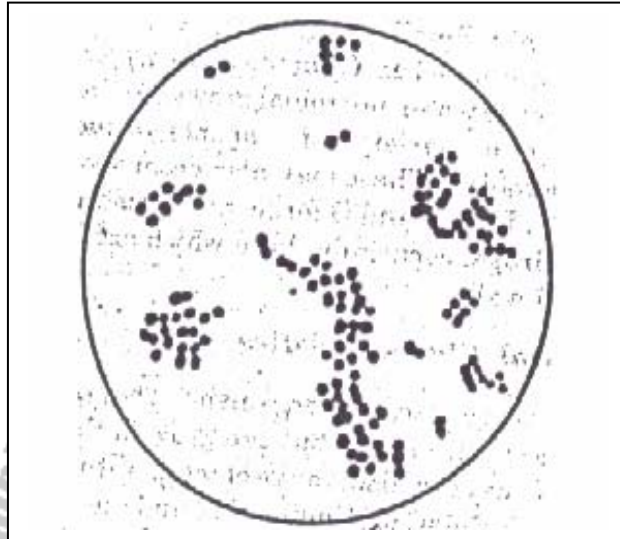
Less common staphylococcal species

. Staphylococcus lugdenensis

. Staphylococcus hominis

. Staphylococcus warneri

- Can readily grow in ordinary media under aerobic and micro-aerophilic conditions
- grow most rapidly at 37 0c but form pigment best at room temperature of 20-25 oc
- Colonies in solid media are round, smooth, raised and glistening.
- Some of them are normal flora of the skin and mucus membrane of human, others cause suppuration abscess formation and fatal septicemia
- Produce catalase, which differentiate them from the streptococci.
- relatively resistant to drying , heat, and 9% NaCl, but readily inhibited by 3 % hexachlorophene



**Fig 3.1** Staphylococci

Antigenic structure:

1. Peptidoglycan( Mucopeptide): Polysaccharide polymer which provide the rigid exoskeleton of the cell wall. It is important in the pathogenesis of infection like eliciting production of cytokines and opsonic antibodies; chemoattractant for polymorphs;and activate complement
2. Teichoic acid: Polymer of glycerol or ribitol phosphate
3. Protein A: Important in immunologic diagnostic test (coagglutination test).
4. Capsule: Anti-phagositic property
5. Enzymes
  - . Catalase- Produced by staphylococci  
Converts  $H_2O_2$  into  $H_2O$  and  $O_2$

Catalase test differentiates staphylococci(catalase-positive) from streptococci(catalase-negative)

- . Coagulase and clumping factor
- . Coagulase clots oxidated or citrated plasma. Coagulase may deposit fibrin on the surface of organism and alter ingestion by phagocytic cells.
- . Clumping factor: A surface compound that is responsible for adherence of the organism to fibrinogen and fibrin

Produced by Staphylococcus aureus

Determines Invasive potential of the organism.

Coagulase test differentiates S.aureus(coagulase-positive) from S.epidermidis (coagulase-negative)

- . Hyaluronidase-Spreading factor
- . Proteinases and lipases
- . Staphylokinase- Fibrinolysin
- .  $\beta$ -lactamase-Provides resistance of staphylococcus to  $\beta$ -lactam antibiotic like penicillin.
- . Dnase: Deoxyribonucleotidase
- . Nuclease

#### 6. Toxins

- . Exotoxins( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ )
- . Enterotoxin-Produced by S.aureus when grown in carbohydrate and protein foods.  
Multiple (A-E, G-I, K-M) soluble heat-stable, gut enzyme resistant toxins which act on neural receptors

in the gut to stimulate vomiting center in the central nervous system. It is superantigen causing staphylococcal food poisoning

. Toxic shock syndrome toxin- Superantigen desquamative toxin Produced by S.aureus and Causes fever, shock, multiple-organ failure and skin rash.

. Exfoliative toxin-Epidermolytic superantigen produced by S.aureus and causes generalized desquamation of the skin (staphylococcal scalded skin syndrome).

Epidermolytic toxin A: Chromosomal gene product and heat stable

Epidermolytic toxin B: Plasmid mediated and heat labile

. Leukocidin: S aureus toxin which kills WBCs by forming pores and increasing cation permeability

Clinical features:

- . Folliculitis: Infection of one hair follicle.
- . Carbuncle: Infection of multiple hair follicle and surrounding skin.
- . Cellulitis: Infection of skin and subcutaneous tissue.
- . Abscess formation: focal suppuration
- . Mastitis: Infection of breast, especially in lactating mother
- . Bullous impetigo: Crusted superficial skin lesion
- . Pneumonia: Infection of lung parenchyma.
- . Empyema: Accumulation of pus in pleural space
- . Osteomyelitis: Infection of bone
- . Endocarditis and meningitis: Infection of heart tissue and leptomeninges respectively.
- . Food poisoning: Caused by enterotoxin produced by S.aureus

- . Characterized by violent nausea, vomiting, and diarrhea
- . Toxic shock syndrome: Caused by toxic shock syndrome toxin-1 produced by *S.aureus*
- . Characterized by abrupt onset of high fever, vomiting, diarrhea, myalgia, scarlatiform rash, and hypotension with cardiac and renal failure in the most severe disease
- . Occurs within 5 days after the onset of menses in young women who use tampons
- . Staphylococcal scalded skin syndrome: Caused by exfoliative toxin produced by *S.aureus*.

*S. saprophyticus*: Relatively common cause of urinary tract infections in young women

*S. epidermidis*: occasional cause of infection often associated with implanted appliances and devices

Laboratory Diagnosis:

Specimen: Surface swabs, pus, blood, sputum, cerebrospinal fluid

Smear: Gram positive cocci in clusters, singly or in pairs.

Culture: Grow well aerobically and in a CO<sub>2</sub> enriched ordinary media at an optimal temperature of 35<sup>0</sup>c-37<sup>0</sup>c.

Colony appearance:

*S.aureus*: characteristically golden colonies.

frequently non-pigmented after over-night incubation.

hemolytic on blood agar plate.

7.5% NaCl containing media is used for mixed flora contaminated specimen

Mannitol slt agar is used to screen for nasal carriers of *S. aureus*

*S. epidermidis*: white colonies, non-hemolytic

*S. saprophyticus*: may be white or yellow, non-hemolytic.

Biochemical reaction

1. Catalase test

Active bubbling.....Catalaseproducing Bacteria (Staphlococci)

No active bubbling.....Non-catalase producing bacteria (streptococci)

2. Coagulase test

a. Slide test: To detect bound coagulase

Clumping with in 10 seconds..... *S. aureus*

No clumping with in 10 seconds.....CONS(Coagulase negative staphylococci)

c. Tube test: To detect free coagulase

Fibrin clot.....*S. aureus*

No fibrin clot.....CONS

Sensitivity testing:

Novobiocin sensitive..... *S. aureus* and *S. epidermidis*

Novobiocin resistant.....*S. saprophyticus*

**Table 2.1 DIFFERENTIATION OF SPECIES**

<u>Organism</u> <u>appearance</u>	<u>Colony</u>	<u>Catalase</u> <u>Production</u>	<u>Coagulase</u> <u>Production</u>	<u>Novobiocin</u> <u>sensitivity</u>	<u>Hemolysion</u> <u>Blood agar</u>
S. aureus	Golden	Positive	Positive	Sensitive	Positive
S. epidermidis	White-Gray	Positive	Negative	Sensitive	Negative
S. saprophyticus	White-Gray	Positive	Negative	Resistant	Negative

**Treatment**

Penicillin sensitive staphylococci.....penicillin, ampicillin

Penicillin resistant staphylococci.....cloxacillin, Nafcillin

Methicillin resistant staphylococci..... Vancomycin

**Prevention and control**

Source of infection is shedding human lesions, the human respiratory tract and skin

Contact spread of infection occur in hospitals

Treatment of nasal carriers with topical antiseptics or rifampin and anti-staphylococcal drug

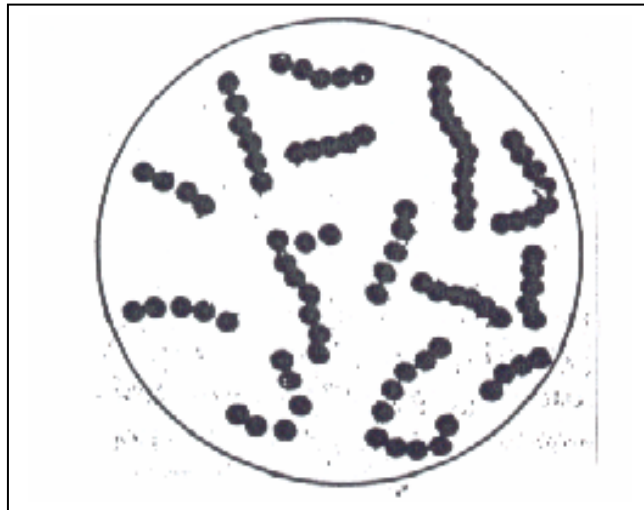
**2.1.2. GENUS: STREPTOCOCCI**

Characteristics:

- They are non-motile, non-sporulating, gram- positive facultative anaerobes
- Spherical or oval cells characteristically forming pairs or chains during growth

- Grow well on ordinary solid media enriched with blood, serum or glucose.
- Most streptococci grow in solid media as discoid colonies
- Capsular streptococcal strains give rise to mucoid colonies
- They are aerobic bacteria in which growth is enhanced with 10% carbondioxide.
- They are catalase-negative.
- They are widely distributed in nature and are found in upper respiratory tract, gastrointestinal tract and genitourinary tract as normal microbial flora.
- They are heterogeneous group of bacteria, and no one system suffices to classify them.
- The currently used classification is based on colony growth characteristics, pattern on blood agar, antigenic composition of group specific cell wall substance and biochemical reaction





**Fig. 3.2** Streptococci

Classification of streptococci: Based on

1. Colony morphology and hemolytic reaction on blood agar
2. Serologic specificity of the cell wall group specific substance and other cellwall capsular antigens
3. Biochemical reactions and resistnace to physical and chemical factors
4. Ecologic features

**Table 2.2 Hemolytic reaction of streptococci:**

<u>Hemolysis</u>	<u>appearance</u>	<u>Designation</u>	<u>Example</u>
.Complete	colorless, clear, Sharply defined zone	Beta( $\beta$ )	S. pyogenes
.Partial streptococci	Greenish discoloration (reduced hemoglobin)	Alpha( $\alpha$ )	Viridans
.None	No change	Gamma( $\delta$ )	Enterococci

Lancefield grouping of streptococci:

Streptococci produce group specific carbohydrates (C carbohydrates) identified using group specific antiserum.

It is designated A-H and K-V.

The clinically important streptococci are grouped under A,B,C,D,F and G.

The main species and groups of medical importance

S. pyogenes..... Lancefield group A

S. agalactiae..... Lancefield group B

Enterococci.....Lancefield group D

NB: Viridans streptococci and anaerobic streptococci are not grouped under Lancefield Classification.

Antigenic structure:

1. Group-specific cell wall antigen

Streptococcal cell wall obtained carbohydrate is the basis for serologic grouping of streptococci (Lancefield groups A-H, K-U)

## 2. M protein

They are found in hair-like projections of the streptococcal surface and determine virulence

Major virulent factor for group A streptococci.

There are two major structural classes of M protein (class I & class II) and

More than eighty serotypes of M protein of group A streptococci

The class I M protein may be a virulence determinant for rheumatic fever

Conserved antigenic domains on the class I M protein induce antibodies that cross react with cardiac muscle tissue

3. T substance: Acid and heat labile unlike M protein, and has no relation to virulence of streptococci.

## 4. R protein

*Streptococcus pyogenes* (Group A  $\beta$ -hemolytic streptococci)

The most pathogenic member of the genus

It is present as a commensal in the nasopharynx in a variable proportion of healthy individuals.

It produces different types of enzymes and exotoxins.

These are:

1. Streptokinase: Fibrinolysin.

It is an active proteolytic enzyme which lyses fibrin by catalytic conversion of plasminogen to plasmin.

Has been given intravenously for the treatment of pulmonary edema and of arterial and venous thrombosis

2. Streptodornase: Streptococcal deoxyribonuclease

Mixtures of streptokinase and streptodornase are used in “enzymatic debridement”

3. NADase: Nicotinamide adenine dinucleotidase

4. Hyaluronidase: Spreading factor

It degrades the ground substance of connective tissue (hyaluronic acid) and aids in spreading infectious micro-organism

5. Hemolysins: Two types

Streptolysin O and Streptolysin S

Antistreptolysin O antibody titer > 1:200 todd:

Supportive evidence for Acute rheumatic fever

### 2.3 Comparison of the streptolysins

Hemolysin	stability of oxygen	antigenic
Streptolysin O	No	Yes
Streptolysin S	Yes	No

6. Erythrogenic toxin: Pyrogenic exotoxins

It is responsible for the erythematous rash in scarlet fever.

Clinical features:

- . Skin infection: Impetigo, cellulitis, erysipelas
- . Scarlet fever
- . Acute streptococcal sorethroat
- . Ear infection: Acute otitis media and mastoiditis
- . Puerperal fever: septicemia originating in the infected uterus
- . Post-streptococcal diseases: Immunological diseases
  1. Acute rheumatic fever

Immunological damage to the heart valves and muscle following

Streptococcal upper respiratory tract infection

It clinically presents with fever, malaise, migratory non-purpurative polyarthritits, carditis, erythema marginatum and subcutaneous nodules

2. Post streptococcal acute glomerulonephritis

Immunological damage to the kidney following infection of skin with streptococci

It clinically manifests with generalized body edema, elevated blood pressure, protein and blood in the urine, blood urea nitrogen retention and low complement level

. Necrotizing fasciitis(Streptococcal gangrene): Extensive and rapidly spreading necrosis of skin and subcutaneous tissue

**S. agalactiae**

Clinical features

- . Neonatal sepsis, pneumonia, and meningitis
- . Septic abortion
- . Puerperal sepsis

**Enterococci**

Clinical features

- . Frequent cause of nosocomial infection
- . Abdominal abscess
- . Sub acute bacterial endocarditis

### **Viridans streptococci**

Eg. Streptococcus mitis

Streptococcus mutans

Streptococcus salivarius

Streptococcus sanguis

Clinical features

. Sub acute bacterial endocarditis

Laboratory Diagnosis:

Specimen:

S. pyogenes- Throat swab, pus, blood

S. agalactiae- High vaginal swab of women; blood and cerebrospinal fluid of new born

Enterococci- Blood, pus

Viridans streptococci- Blood

Smear: Non-motile gram-positive cocci in chains

Culture, biochemical tests and sensitivity testing:

Grow in aerobic and anaerobic environment at a temperature of 35-37 °c.

Grow in ordinary media with shiny or dry colonies with grey-white or colorless appearance.

S. pyogenes- Shows clear zone of hemolysis in blood agar plate.

Does not grow in mac Conkey agar plate.

Bacitracin sensitive.

PYR-Positive

*S. agalactiae*- Shows clear zone of hemolysis in blood agar plate.

May show double zone of hemolysis when incubated anaerobically

Active in hippurate hydrolysis

CAMP test is used to identify the bacteria.

Viridans streptococci- Show greenish discoloration of blood agar plate.

Optochin resistant.

Not soluble in bile salts.

Do not ferment inulin.

Enterococci- Non-hemolytic or  $\alpha$  hemolytic changes in blood agar plate.

Grow in the presence of 6.5% NaCl.

Grows in mac conkey agar

Identified by litmus milk reduction test.

PYR-Positive

Bile esculin-positive

Nutritionally Variant Streptococci (Nutritionally deficient streptococci)

Eg. *Abiotrophia adjacens*

*Abiotrophia defectiva*

. Require pyridoxal or cysteine for growth in blood agar

. Usually  $\alpha$  hemolytic or non-hemolytic

. cause infective endocarditis

Treatment:

Streptococci other than enterococci ..... Penicillin/ Erythromycin

Enterococci ..... Penicillin + Gentamicin

### **Streptococcus pneumoniae**

- Fastidious, lancet-shaped gram positive diplococci.
- Possess a capsule of polysaccharide that permits typing with specific antisera.
- Found as a normal flora in the upper respiratory tract.

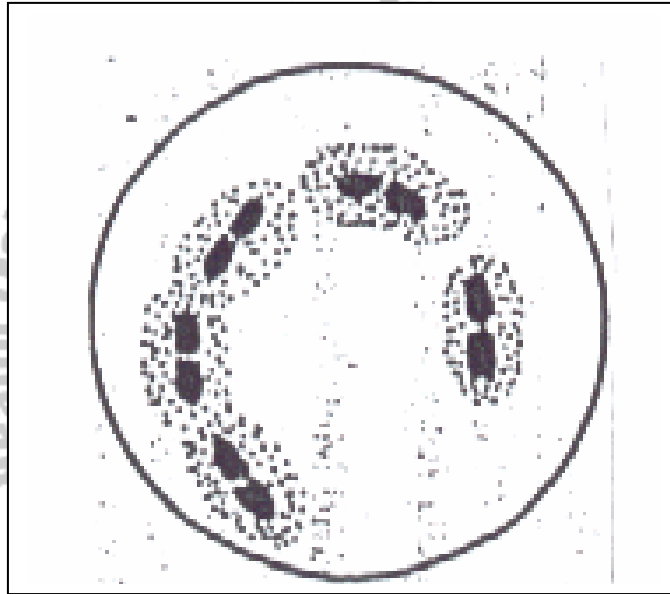


Fig. 3.3 Streptococcus pneumoniae

#### Antigenic structure:

- . Capsular polysaccharide: Pathogenicity determinant with anti-phagocytic property. There are more than 80 serotypes of the bacteria based on capsular typing.



Identified by capsule swelling test  
(quellung reaction).

- . C substance: Cell wall associated antigen
- . Protein M antigen
- . IgA<sub>1</sub> protease: Enzyme which cleaves IgA<sub>1</sub>

Clinical features:

- . Lobar Pneumonia
- . Otitis media
- . Sinusitis
- . Bacteremia.....Meningitis
  - .Endocarditis
  - . Septic arthritis

Laboratory Diagnosis:

Specimen: Sputum, blood, cerebrospinal fluid, ear discharge and sinus drainage.

Smears: Lancet-shaped gram positive diplococci

Culture: Grow best in chocolate agar media in CO<sub>2</sub> enriched atmosphere.

- Shows  $\alpha$ -hemolytic, draughts man colony appearance: Sunken centre colony due to spontaneous autolysis of older bacteria.
- Young colonies resemble dew-drops due to capsule.
- Bile soluble, ferment inulin.
- Optochin sensitive.

Serology: Quellung reaction

.Good for rapid identification of *S.pneumoniae* in fresh specimen.

Principle:

.Mix specific serotype of *S.pneumoniae* with specific anti-polysaccharide serum of the same serotype or with polyvalent anti-serum on a slide.

. Look for the appearance of capsule swelling under the 100X objective microscope

Treatment: Amoxicillin

Chloramphenicol

Third generation Cephalosporins

Prevention and control:

Pneumococcal conjugate vaccine: Immunization of individuals with type specific polysaccharide vaccine

Biochemical reaction to diagnose streptococci

- . Bile solubility test
- . Litmus milk reduction test
- . CAMP test
- . Bacitracin test
- . **Optochin test**

**Table 2.4 Differentiation of streptococcus species**

Species	Catalase test	Bacitracin test	.Optochin test Bile solubility test	Litmus milk reduction test	CAMP test
S. pyogenes	-ve	+ve	-ve	-ve	-ve
S. agalaciae	-ve	-ve	-ve	-ve	+ve
Enterococci	-ve	-ve	-ve	+ve	-ve
Viridans streptococci	-ve	-ve	-ve	-ve	-ve
.S.pneumoniae	-ve	-ve	+ve	-ve	-ve

**GRAM POSITIVE SPORE FORMING RODS**

Genus Bacillus

Genus Clostridium

**2.2.1. GENUS: BACILLUS**

**Characteristics:**

- Aerobic, non-motile, spore-forming, gram-positive chain forming rods.
- Bacillus species are ubiquitous saprophytes  
Important human pathogen
  - B. anthracis
  - B. cereus

### **Bacillus anthracis**

- Major agent of bioterrorism and biological warfare
- Major pathogen of domestic herbivores that come in contact with humans

Antigenic structure:

- Capsule: Polypeptide of D-glutamic acid: Enables the organism to evade phagocytosis
- Polysaccharide somatic antigen
- Protein somatic antigen
- Anthrax toxin- Protein toxin: Complex of 3 protein factors  
Edema factor + Protective antigen = Edema toxin  
Lethal factor + Protective antigen = Lethal toxin

Pathogenesis and Clinical feature:

Acquired by the entry of spores through injured skin in cutaneous anthrax, or mucus membrane in intestinal anthrax, or inhalation of spores in the lung while handling skin and hides

There are four forms of anthrax.

1. Cutaneous anthrax (Malignant pustule): 95 % of anthrax presentation  
Characterized by a black necrotic lesion with a definite edematous margin on hands, arms, face or neck with regional lymphadenitis associated systemic symptoms.
2. Pulmonary anthrax (Wool sorter's disease): 5% of anthrax presentation

Presents with substernal pain, cough with haemorrhagic mediastinitis and CXR-revealing mediastinal widening; and fatal if not treated early

**3. Bacteremic anthrax: presents with clinical features of sepsis**

4. Intestinal anthrax: Presents with abdominal pain, vomiting, and bloody diarrhea

Bacteremic and intestinal anthrax are rare to occur

Laboratory diagnosis:

Specimen: Fluid or pus from skin lesion, Blood, sputum

Smear: Non-capsulated gram-positive rods with centrally located spores from culture

Large capsulated gram-positive rods with out spores from primary specimen.

Culture: Grows aerobically in ordinary media over wide range of temperature.

Non-hemolytic, large, dense, grey-white irregular colonies with colony margin of “Medussa Head” or “curled-hair lock” appearance due to composition of parallel chaining of cells.

Biochemical reaction:

Gelatin-stab culture: Gelatin liquefaction

Growth along the track of the wire with lateral spikes longest near the surface Providing “inverted fur tree” appearance.

Serology: ELISA has been developed to measure antibodies to edema toxin and lethal toxin

Positive result: 4-fold change or single titer > 1:32

Treatment:

Ciprofloxacin

Penicillin+ gentamicin or streptomycin

Prevention and control:

- Disposal of animal carcasses by deep burial or burning
- Decontamination (autoclaving) of animal product
- Protective clothing and gloves for handling potentially infected materials
- Active immunization of domestic animals with live attenuated vaccine
- Immunize high occupation risk persons with anthrax toxoid

### **Bacillus cereus**

General characteristics:

Exhibit motility by swarming in semisolid media

Produce  $\beta$  lactamase, so not sensitive to penicillin

Clinical features

#### 1. Food poisoning

Pathogenicity determinant: Exotoxin

##### a. Emetic type food poisoning

IP is 1-5 hrs after ingestion of preformed toxin contaminating rice and pasta dishes

Characterized by nausea, vomiting, abdominal cramps, and self-limited within 24 hrs

##### b. Diarrheal type food poisoning

IP is 1-24 hrs after ingestion of contaminated meat dishes with sporulating or preformed toxin

Characterized by profuse diarrhea and abdominal cramps.

Fever and vomiting is uncommon

Lab. Diagnosis

>10<sup>5</sup> org/gm of food

Isolation of *B.cereus* in stool is not diagnostic since it is present in normal stool specimen

Treatment: Fluid replacement

Antibiotics not required

#### 2. Ocular infection

Ocular disease following trauma from non-surgical penetrating objects

Manifests with keratitis, endophthalmitis, and panophthalmitis

Treatment: Clindamycin + Aminoglycosides

### 2.2.2. Genus: Clostridium

Characteristics:

- Clostridia are anaerobic, spore-forming motile, gram-positive rods.
- Most species are soil saprophytes but a few are pathogens to human.
- They inhabit human and animal intestine, soil, water, decaying animal and plant matter
- Spores of clostridia are wider than the diameter of organism and located centrally, subterminally and terminally
- Species of medical importance:
  - C. perfringens
  - C. tetani
  - C. botulinum
  - C. difficile

#### **Clostridium perfringens**

Characteristics:

- Capsulated, non-motile, short gram-positive rods in which spores are hardly seen.
  - there are five toxigenic groups : A-E
  - Human disease is caused by C. perfringens type A and C
- Pathogenicity determinant:



1. Enzymes: Digest collagen of subcutaneous tissue and muscle.

- . Collagenase
- . Proteinase
- . Hyaluronidase
- . Dnase

2. Toxins

- . PhospholipaseC ( $\alpha$  toxin)

It has lethal, necrotizing and hemolytic effect on tissue.

It causes cell lysis due to lecithinase action on the lecithin which is found in mammalian cell membrane.

- . Theta toxin

It has hemolytic and necrotic effect on tissue.

- . Enterotoxin

Clinical manifestation:

1. Clostridial myonecrosis: Gas gangrene

- . IP(Incubation period) =1-3 days

Colonization of devitalized traumatized wound by *C.perfringens* spores, and organism germination and release of toxins

Presentation: Muscle and subcutaneous tissue necrosis and crepitation

Foul smelling wound discharge

Fever, toxemia, hemolytic anemia, Shock

## 2. Clostridial food poisoning

It causes secretory diarrhea due to release of enterotoxin in the intestine

Self-limiting diarrhea similar to that produced by *B. cereus*

Laboratory diagnosis:

Specimen: Infected tissue, pus, vomitus, left over food, serum

Smear: Non-motile, capsulated, thick brick-shaped gram-positive rods in smears from tissue; spores are rarely seen.

Culture:

### 1. Blood agar medium

.  $\beta$ -hemolytic colonies are seen in blood agar in anaerobic atmosphere.

. Some strains produce double zone of hemolysis.

### 2. Cooked meat medium (Chopped meat-glucose medium)

Thioglycolate medium

. Saccharolytic property showing reddening of the meat with a rancid smell due to carbohydrate decomposition.

. Proteolytic property showing blackening of the meat with unpleasant smell due to protein decomposition.

. Formation of gas

Biochemical reaction:

. Nagler reaction: Lecithinase C activity- Opacity in the egg-yolk medium due to lecithin break down

Procedure:

1. Streak colonies of *C. perfringens* on egg-yolk agar.
2. Cover half of the medium with *C. perfringens* antitoxin.
3. Look for dense opacity production by the growth of *C. perfringens*; but no opacity on the area with antitoxin.

. Lactose fermentation: Reddening of the medium; red colonies when exposed to air.

. Litmus milk medium: "stormy- clot" formation due to acid and gas formation.

Identification of *C. perfringens* rests on colony form, hemolysis pattern, biochemical reaction, and toxin production and neutralization by specific antisera.

Treatment: Penicillin

Prompt and extensive wound debridement

Polyvalent antitoxin

Prevention and control

Early adequate contaminated wound cleansing and debridement

### **Clostridium difficile**

General characteristics:

- . Not frequently found in the healthy adult, but is found often in the hospital environment
- . Produce cytotoxins ( A and B)
- . Human feces are the expected source of the organism

Pathogenesis and clinical features:

Administration of antibiotics like ampicillin, clindamycin and cephalosporins results in killing of colonic normal flora and proliferation of drug resistant C.difficile and release of cytotoxins

Clinically presents with pseudomembranous colitis and manifests with fever, abdominal cramps, watery or bloody diarrhea leading to dehydration, septicemia and shock

Lab. Dignosis: Identification of toxin A and B in feces by latex agglutination test

Treatment:

Discontinuation of offending drugs

Administration of metronidazole or vancomycin

## **Clostridium tetani**

General characteristics:

- World wide in distribution in the soil and in animal feces
- Longer and thinner gram-positive rods with round terminal spores giving characteristic “drum-stick” appearance.
- There are ten antigenic types of *C. tetani* but all produce the same neurotoxin.
- The toxin has two components:
  1. Tetanospasmin: Neurotoxic property
  2. Tetanolysin: Hemolytic property

Pathogenesis and Clinical manifestation:

Infection of devitalized tissue (wound, burn, injury, umbilical stump, surgical suture) by spores of *C. tetani* → Germination of the spore and development of vegetative organism → Neurotoxin release from vegetative cells → The toxin binds to receptors on the presynaptic membrane of motor neuron the retrograde axonal transport to the spinal cord and brain stem → Inhibition of inhibitory glycinergic and GABAergic secreting neurons → Spastic paralysis, muscle spasms and hyperreflexia

IP= 4-5 days to several wks

Tetanus classical presentation:

- . Lock jaw or trismus
- . Arched back or opisthotonus
- . Arm flexion and of leg extension

- . Fever and sweating
- . Muscle spasm and rigidity

Laboratory diagnosis: The bacteria can be cultured in a media with anaerobic atmosphere. Proof of isolation of *C.tetani* must rest on production of toxin and its neutralization by specific antitoxin

Diagnosis is exclusively by clinical picture and history of injury

Treatment:

- Administration of penicillin
- Proper wound debridement
- Provision of tetanus antitoxin (TAT)

Prevention and control:

- Avoid traditional application of mud or ash over the umbilical stump
- Proper wound handling
- Immunization with tetanus toxoid

NB: Since treatment of tetanus is not satisfactory, prevention is all important

### **Clostridium botulinum**

General characteristics:

- Spores of *C. botulinum* are widely distributed in soil, they often contaminate vegetables, fruits and other materials.

- Produce a neurotoxin which is the most active known poison, and considered to be the major agent of bioterrorism and biologic warfare
- There are seven serotypes(A-G) of which A,B and E are the principal causes of human illness.

Pathogenesis and Clinical manifestation:

1. Food botulism

. IP = 18-24 hrs

. Route of entry is under cooked consumption of C. botulinum toxin contaminated spiced, smoked, vacuum-packed or canned food

.The toxin is absorbed from the gut and acts by blocking the release of acetylcholine at synapses and neuromuscular junction and manifests with flaccid paralysis and visual disturbance, inability to swallow, and speech difficulty

Death is secondary to respiratory failure or cardiac arrest

2. Infantile botulism

C.botulinum type A or B is usually implicated and affects infants when mixed feeding starts (after fourth month of life). Ingestion and colonisation of the gut with C.botulinum, and production of toxin and adsorption of toxin leads to poor feeding, paralysis (floppy baby), and cranial nerve palsy.

Diagnosed by demonstration of the organism or toxin from the stool

### 3. Wound botulism

C.botulinum type A is usually implicated and caused by the production of toxin by C.botulinum in wounds.The symptoms are the same as those in food poisonig.

#### Laboratory diagnosis:

- .Demonstration of toxin in patient's serum and left over food.
- . Death of mice after intra-peritoneal injection of toxin.

#### Treatment:

- Administration of intravenous trivalent antitoxin ( A,B,E)
- Mechanical ventilator for respiratory support

#### Prevention and control:

- . Sufficient heating of cannaed foods before consumption
- . Strict regularion of commercial canning
- . Proper home canning methods

## **GRAM POSITIVE NON-SPORE FORMING RODS**

### **GENUS CORYNEBACTERIA**

Medical important species: Corynebacteria diphteriae

#### **C. diphteriae**

##### Characteristics:

Non-spore forming, non-capsulated, non-motile aerobe or facultative anaerobe gram-positive rods

Possess irregular swelling at one end that give them the “club-shaped” appearance



Possess granules (metachromatic granules) near the poles that give the rod a beaded appearance

It tends to lie in parallel (pallisades) or at acute angles to one another in stained smears, forming V, L, W shapes, so called Chinese-character arrangement

It has four biotypes named as gravis, mitis, intermedius and balfanti based on growth characteristics and severity of disease produced

Pathogenesis and clinical features:

Found in nature in the respiratory tract, in wounds, or on the skin of infected person or normal carriers

Spread by droplets or by direct contact

The organism colonize the mucus membrane or skin abrasions and toxigenic *C.diphtheriae* start producing exotoxin, possessing two components, fragment A and B. Fragment B transpots fragment A into the cell and fragment A inhibit polypeptide chain elongation by inactivating the elongation factor EF-2, required for translocation of polypeptidyl-tRNA from the acceptor to the donor site on the eukaryotic ribosome, leading to abrupt arrest of protein synthesis and result in cell necrosis and neurotoxic effect.

Diphtheria toxin causes respiratory tract epithelial destruction resulting in formation of necrotic epithelium with pseudomembrane formation over the tonsils, pharynx, and larynx. Distant toxic damage includes parenchymal degeneration and necrosis in heart muscle, liver, kidney, adrenal glands and peripheral and cranial nerves.

Wound/skin diphtheria occurs chiefly in the tropics and forms membrane-covered wound that fails to heal. Systemic effect is negligible.

It clinically manifests with fever, sorethroat, suffocation (due to obstruction by upper respiratory tract pseudomembrane formation), arrhythmia, and difficulty of vision, swallowing and paralysis of upper and lower extremities.

NB: *C.diphtheriae* var *gravis* tends to produce more severe disease than var *mitis*.

Laboratory diagnosis:

Specimen: Swabs from the nose, throat, or suspected lesion

Smears: Beaded rods in typical arrangement when stained with alkaline methylene blue or gram's stain

Culture: Small, granular, and gray, with irregular edges with small zone of hemolysis on blood agar

Selective media are necessary for isolation from clinical specimens

Selective media

1. Loeffler's serum media: Grows rapidly within 8 hrs. after inoculation and show typical appearance
2. Blood tellurite agar: Produce characteristic grey-black colonies due to their ability to reduce potassium tellurite to tellurium

### Characteristics of *C.diphtheriae* strains

<i>C.diphtheriae</i>	Appearance in Loeffler's medium	Colonial type on Tellurite medium
Gravis strains	Club-shaped, few granules	Flat, grey with raised centre and irregular edge
Intermedius strains	Short irregularly staining rods	small, smooth colonies;

	with out granules but in Chinese character arrangement	grey-black with paler periphery
Mitis strains	Classic morphology with numerous granules and typical arrangement	Medium-sized,circular convex,glistening and black

Biochemical reaction: Acid production from a range of carbohydrate fermentation

Typing: Serotyping by agglutination tests, phage typing and bacteriocin typing have been used to subdivide strains of *C.diphtheriae* for epidemiologic studies

Toxin production: responsible for virulence; can be demonstrated by guinea pig inoculation or by gel precipitation test

1. Guinea pig inoculation: Inject suspension of the isolated strain of *C.diphtheriae* into two guinea pig, one protected with diphtheria antitoxin.

Death in 2-3 days of the unprotected animal

2. Gel-precipitation (Elek) test: a filter paper strip previously immersed in diphtheria antitoxin is incorporated into serum agar; the strain of *C.diphtheriae* under investigation is then streaked onto the agar at right angles to the filter paper strip. Incubate at 37 °c for 1-2 days, and observe for lines of precipitation in the agar indicating toxin-antitoxin interaction.

Schick test: a skin test to demonstrate immunity due to immunization or natural infection

Method: Intradermal injection of toxin into the anterior aspect of one forearm and heat-inactivated toxin into the other.

Objective: to detect susceptibility and hypersensitivity to diphteria toxin

Interpretation: Observe for erythema at the injection site at 36 hrs and 120 hrs. Reactions due to the toxin are slower and longer lasting than those resulting from hypersensitivity.

**The schick test**

Result	Test arm (toxin)		Control arm (Inactivated arm)		Interpretation	Immunization
	36hrs	120hrs	36hrs	120hrs		
-ve	-	-	-	-	Immune, not hypersensitive	Not required
+ve	+/-	+	-	-	Non-immune, Required	Not hypersensitive
-ve/Pseudo	+	-	+	-	Immune, Hypersensitive	Not required
+ve/Pseudo	+	+	+	-	Non-immune, Hypersensitive	Single injection of low dose vaccine

Treatment: Penicillin/Erythromycin  
Administration of diphteria antitoxin

Prevention and control:  
Administration of Diphtheria toxoid

## GENUS: LISTERIA

Most important species: *Listeria monocytogenes*

### **L. monocytogenes**

General characteristics:

- . Widely present in plants, soil and surface water
- . Zoonotic pathogen of domestic animals
- . Non-sporulating, facultative anaerobe, intracellular. Gram positive rods

Antigenic structure:

- . Listeriolysin( hemolysin)

Pathogenesis and clinical features:

Transmitted to humans through ingestion of poorly cooked meat and unpasteurized milk and milk products

1. Perinatal human listeriosis: Granulomatous infantisepticum
  - . Early onset syndrome: Intrauterine sepsis
  - . Late onset syndrome: Neonatal meningitis
2. Adult human listeriosis
  - . Meningoencephalitis
  - . Bacteremia

Lab. Diagnosis:

Specimen: Blood/ CSF

Culture: Grow in blood agar and demonstrate narrow zone of  $\beta$ -hemolysis

Produce “umbrella” growth pattern below the motility media surface at room T<sup>0</sup>, demonstrating motility at room temperature

Biochemical reaction: Catalase positive

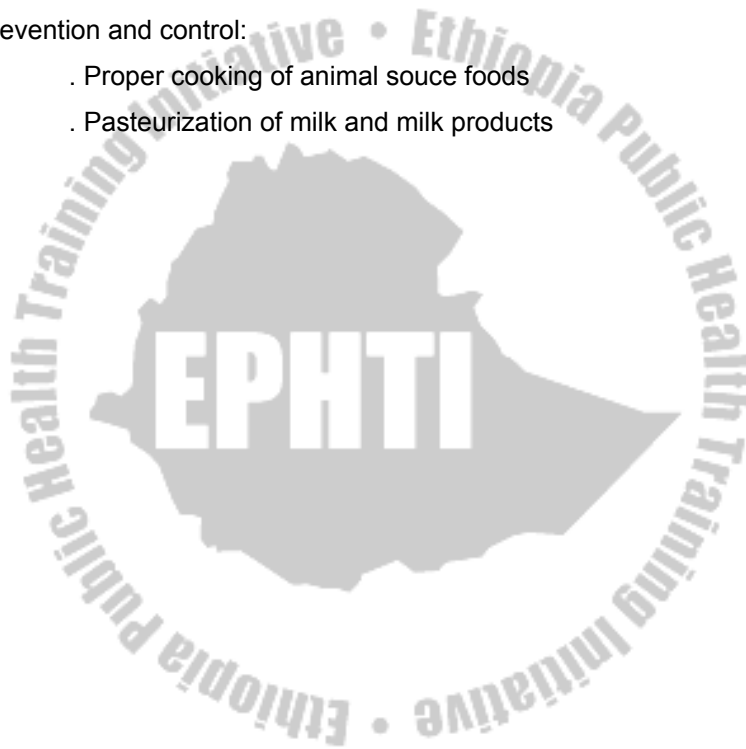
Oxidase negative

Treatment:

- . Ampicillin
- . Erythromycin
- . Cotrimaxazole

Prevention and control:

- . Proper cooking of animal source foods
- . Pasteurization of milk and milk products



## GENUS : ERYSIPELOTHRIX

*Erysipelothrix rhusiopathiae*

General characteristics:

. Slender, non-motile, Non-sporulating, gram-positive, facultative anaerobic rods

. Swine is major reservoir

Pathogenicity and clinical features:

Most human cases of disease are related to occupational exposure, i.e. direct inoculation from animals and animal products, like in fish handlers, fishers, butchers

1. Mild cutaneous form: Erysipeloid (Whale finger, seal finger)
2. Diffuse cutaneous form with systemic disease
3. Septic form: Bacteremia and endocarditis

Lab. Diagnosis:

Specimen: Blood

Culture: Shows  $\alpha$ -hemolysis on Blood agar

Biochemical reaction:

. Catalase negative

. Produce acid from sugar fermentation

Treatment: Penicillin G

## 2.2. 2.3. GRAM NEGATIVE DIPLOCOCCI

### 2.3.1. GENUS: NEISSERIA

Characteristics:

- . They are non-motile, gram-negative intracellular diplococci
- . Rapidly killed by drying, sunlight, heat, and disinfectants
- . Ferment carbohydrate producing acid but not gas
- . Each cocci is kidney-shaped with adjacent concave sides
- . Grow best on complex media under aerobic conditions containing 5%CO<sub>2</sub>

- . Oxidase positive.

- . The main species of medical importance are:

N. meningitidis

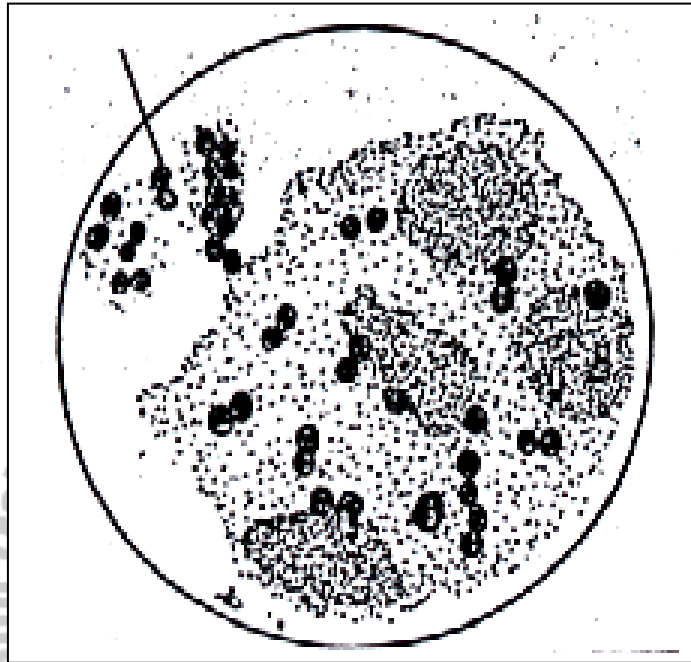
N.gonorrhoea.

#### **N. gonorrhoea**

Characteristics:

An obligate parasite of the human urogenital tract.





**Fig. 3.4.** *Neisseria gonorrhoea*

Antigenic structure: antigenically heterogeneous and capable of changing its surface structures.

1. Pili: Hair-like appendages extending from bacterial surface and enhance attachment to host cells and evade human defense.
  - . The pilus of almost all strains of *N. gonorrhoea* are antigenically different, and a single strain can make many antigenically distinct forms of pilin.
2. Por (Protein I)
  - . Pores on the surface of bacteria through which nutrients enter the cell.

3. Opa (Protein II)
  - . Important for attachment of bacteria to host cells.
4. RMP (Protein III): Reduction-modifiable protein
  - . It is associated with por in the formation of pores in the cell.
5. Lipooligosaccharide(LOS): Responsible to damage epithelial cells

Toxicity in gonococcal infection is largely due to the endotoxic effects of LOS

6. LiP(H8): Gonococcal surface exposed Heat-Modifiable like Opa
7. Fbp(iron binding protein): Expressed when there is limited available iron supply
8. IgA<sub>1</sub> protease: Splits and inactivates major mucosal IgA(IgA1)

Clinical manifestation:

Route of infection: Sexual contact

Male:

- . Gonococcal urethritis
  - If complicated: Urethral stricture
  - Gonococcal epididymitis
  - Gonococcal epididymo-orchitis
  - Infertility
- . Gonococcal suppurative arthritis

Female:

- . Gonococcal cervicitis
- . Gonococcal salpingitis
- If complicated: Gonococcal tubo-ovarian abscess

Pelvic peritonitis

Infertility

Infant (When delivered through the infected birth canal)

. Gonococcal ophthalmia neonatorum

If untreated and complicated leads to blindness

Laboratory diagnosis:

Specimen: Urethral swab, cervical swab, eye swab

Smear: Gram-negative intracellular diplococci

More than five polymorphs per high power field.

Culture: Requires an enriched media like chocolate agar  
or thayer-martin agar.

. Grows best in CO<sub>2</sub> enriched aerobic atmosphere with  
optimal temperature of 35-37<sup>o</sup>c.

.Fastidious- Dies with exposure to sunlight, room  
temperature and drying.

. Small glistening colonies.

.Culture of urethral exudate from men are not  
necessary when the gram stain is positive but  
culture should be done for women

Biochemical reaction: .Oxidase positive.

Ferment only glucose in carbohydrate utilization test

Serology: Antibodies to gonococcal pili and Omps can be

Detected by immunoblotting, RIA or ELISA tests

Treatment: Gonorrhoea is difficult to treat because of resistance to  
lots of antibiotics, especially in developing countries.

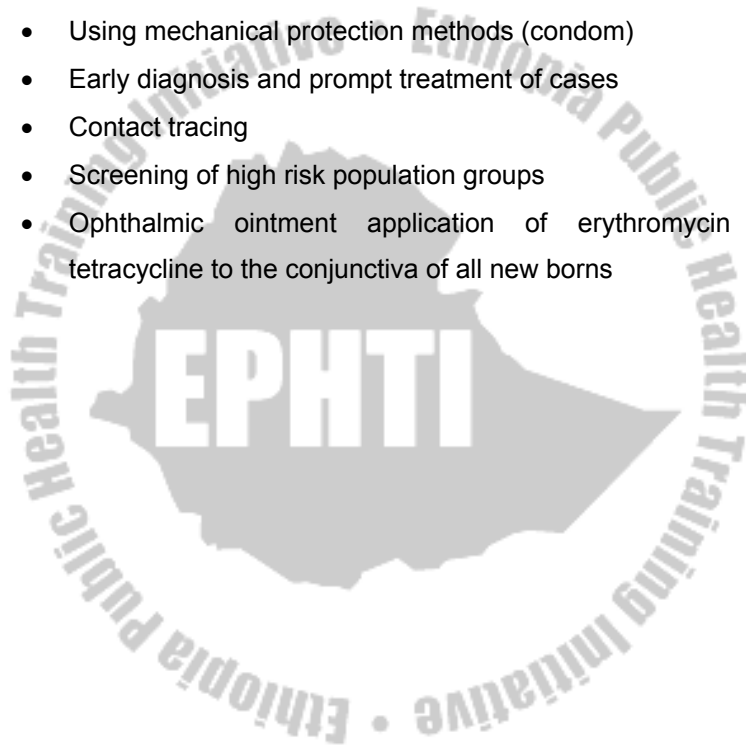
Penicillinase-producing *Neisseria gonorrhoea* (PPNG) strains are resistant to penicillin.

Drug of choice: Ceftriaxone

Ciprofloxacin

Prevention and control

- Avoid multiple sexual partner
- Using mechanical protection methods (condom)
- Early diagnosis and prompt treatment of cases
- Contact tracing
- Screening of high risk population groups
- Ophthalmic ointment application of erythromycin or tetracycline to the conjunctiva of all new borns



### **Neisseria meningitidis**

Characteristics:

- Gram-negative intra cellular diplococci.
- Present in the nasopharynx in 5-10% of healthy people.

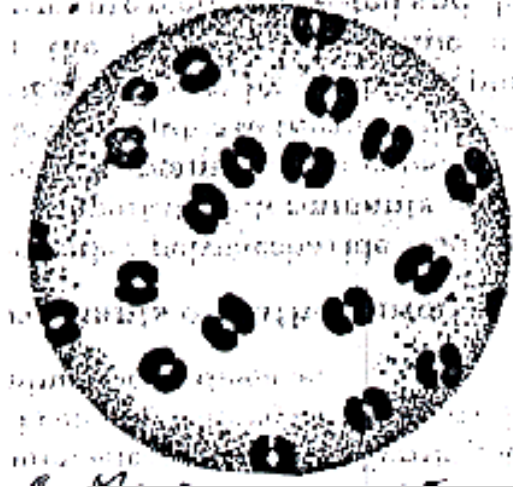


Fig. 3.5 *Neisseria meningitidis*

Antigenic structure:

- . Capsular carbohydrate

It is important for serogrouping of meningococci and there are 13 serogroups. The most important serogroups associated with disease in humans are A, B, C, Y and W135.

. Outer membrane protein

Analogous to por protein of gonococci and responsible for the formation of por in the meningococcal cellwall

20 known serotypes

It is responsible for serotype specificity of meningococci.

. Lipopolysaccharide

Responsible for the toxic effects found in meningococcal disease

Clinical manifestation:

. Meningococcal meningitis

. Meningococemia: Meningococcal septicemia

**Table 2.5 Comparison features of meningococcal meningitis and meningococemia**

Features	Meningococcal meningitis	Meningococemia
. Symptom/sign	Fever, headache, neck stiffness.	Fever, petechial rash,
low blood pressure		
. CSF appearance.	Cloudy.	Cloudy or clear
. Response to drugs.	Satisfactory.	Poor
. % during epidemics.	80-90% .	10-20%

Laboratory diagnosis:

Specimen: Cerebrospinal fluid, blood

Smear: Gram-negative intracellular diplococci

Culture: Transparent or grey, shiny, mucoid colonies in chocolate agar after incubation at 35-37<sup>o</sup>c in a CO<sub>2</sub> enriched atmosphere.

Biochemical reaction: Oxidase positive.

Ferment glucose and maltose in carbohydrate utilization test.

Serology: Latex agglutination test/ Hemmagglutination test

Treatment: Penicillin

Penicillin-allergic patients are treated with third-generation cephalosporins or chloramphenicol.

Prevention and control

- . Chemoprophylaxis( Rifampin or minocycline) for household or close contacts
- . Avoidance of over crowding
- . Vaccination with polyvalent conjugate vaccine to high risk groups

NB: Meningococcal meningitis occurs in epidemics in Africa and named as Meningitis belt.

N. meningitidis serogroup A is the cause of African meningitis epidemic.

During epidemics, the carrier state rises from 5-10% to 70-80%.

Rifampicin is used as prophylactic drug to reduce the carrier state during epidemics and given to household and other close contacts.

**2.6. Comparison features of N.gonorrhoea and N.meningitidis**

Features	N.gonorrhoea	N.meningitidis
. Site of infection	. Urethra/cervix	. Meninges
. Route of infection	. Sexual	. Inhalation
. Disease meningitis	Gonococcal urethritis /cervicitis	.Meningococcal
/Meningococemia		
. Specimen of choice	. Urethral/Cervical swab	. Cerebrospinal fluid
. Biochemical reaction	. Oxidase positive . Ferment glucose only	. Oxidase positive . Ferment glucose and maltose

**2.3.2. GRAM NEGATIVE COCCOBACILLI**

**GENUS: HAEMOPHILUS**

Characteristics:

- This is a group of small gram-negative, non-spore forming, non-motile, pleomorphic bacteria that require enriched media for growth.
- Growth is enhanced in CO<sub>2</sub> enriched atmosphere.
- Present in upper respiratory tract as a normal microbial flora in healthy people.
- The group is fastidious requiring growth factors for isolation.



- The growth factors are X-factor(Hematin) and V-factor (Diphosphopyridine nucleotide).

. Requirement for growth factor helps for differentiation of species.

Growth factor required    Haemophilus species

X and V factor                H. influenzae, H. aegyptius, H. hemolyticus

X factor                        H. ducreyji

V factor                        H. parainfluenzae, H. parahemolyticus

. The main species of medical importance are:

    H. influenzae

    H. ducreyji

    H. aegyptius

**Haemophilus influenzae**

Characteristics:

. Gram-negative cocobacilli.

. Fastidious bacteria requiring growth factors for isolation.

. Found in upper respiratory tract as normal flora in healthy people.

Antigenic structure

    . Capsular polysaccharide

        . There are six serotypes of H. influenzae, A-F.

        . Capsular antigen type b is composed of polyribose ribitol phosphate.

            . H. influenzae type b is the most common cause of disease in humans.

            . It is the main virulence factor which provides anti-phagocytic property.

    . Outer membrane protein

    . Lipo-oligosaccharide

Clinical features: The bacteria causes disease most commonly in young children.

- . Acute pyogenic meningitis
- . Acute epiglottitis
- . Pneumonia
- . Otitis media
- . Siusitis
- . Cellulitis
- . Acute pyogenic arthritis

Laboratory diagnosis:

Specimen: Cerebrospinal fluid, sputum, blood, pus

Smear: Gram-negative short rods.

Culture: Chocolate agar contain both X and V factor; blood agar contain only X factor.

Satellitism test is used to identify H. influenzae in blood agar.

Methods:

- . Mix a loopful of haemophilus growth in 2ml of sterile saline.
- . Inoculate the bacteria suspension on a plate of blood agar using a sterile swab.
- . Streak a pure culture of S. aureus across the inoculated plate which provides V-factor for H. influenzae.
- . incubate the plate over night in a CO<sub>2</sub> -enriched environment at 35-37 °c.
- . Look for growth and satellite colonies in next morning.

NB: Colonies are largest nearest to the S. aureus column of growth.

Serology: Quellung reaction (using specific antisera)

Immunofluorescence stain

Treatment:

- Ampicillin
- Chloramphenicol
- Cotrimoxazole
- Third generation cephalosporins

#### **H. ducreyii**

- Slender, gram-negative, ovoid bacilli, slightly larger than *H. influenzae*.
- Bacteria enmass have configuration of ‘shoals of fish’.
- It causes chancroid (tender genital ulcer).
- Cultured in special enriched media (20-30% rabbit blood agar) with colonic morphology of small grey glistening colonies surrounded by zone of hemolysis.
- It is treated by erythromycin, cotrimoxazole and third generation cephalosporins.

#### **H. aegyptius**

It causes contagious conjunctivitis.

#### **2.3.3. GENUS: BORDETELLA**

Characteristics:

. Minute strictly aerobic non-motile gram-negative rods.

Bordetella species of medical importance:

- B. pertussis

Antigenic structure:

- . Pili: Adheres to ciliated epithelial cells of respiratory tract.
- . Filamentous haemagglutinin: Adheres to ciliated respiratory tract.
- . Pertussis toxin:
  - . Lymphocytosis promoting factor
  - . Histamine sensitizing factor
  - . Insulin secretion enhancing factor
- . Adenylyl cyclase toxin
- . Dermonecrotic toxin
- . Hemolysin
- . Tracheal cytotoxin: Inhibits DNA synthesis in ciliated respiratory epithelial cells.
- . Lipopolysaccharide: Damages respiratory epithelial cells.

Clinical features:

Incubation period: 2 weeks

Route of transmission is respiratory from early cases and possibly carries.

It has three stages:

1. Catarrhal stage
2. Paroxysmal stage
3. Convalescence stage

During catarrhal stage, the patient is highly infectious but not very ill manifesting with mild coughing and sneezing.

During paroxysmal stage, the patient presents with explosive repetitive cough with characteristic 'whoop' upon inhalation leading to exhaustion, vomiting, cyanosis and convulsion.

During convalescence stage, the patient presents with prolonged cough.

Laboratory diagnosis:

Specimen: Saline nasal wash (Preferred specimen)

Nasopharyngeal swab or cough droplets on cough plate

Smear: Small, non-motile, capsulated, gram-negative coccobacilli singly or in pair, and may show bipolar staining.

Culture: Inoculate the primary specimen on Bordet-Gengue agar medium and incubate for 2-6 days at 37 °c in a moist aerobic atmosphere which produces small, raised, shiny, mucoid colonies.

Biochemical reaction:

- . No growth on blood agar
- . Oxidase positive
- . Most of them are catalase positive

Serology: Direct fluorescent antibody test is most helpful, in identifying *B. pertussis* after culture on solid media.

Treatment: Erythromycin

Administration of erythromycin during the catarrhal stage of disease promotes elimination of the organism and limits rate of transmission.

Treatment after the onset of paroxysmal stage does not alter the clinical course of the disease.

## GENUS BRUCELLA

General characteristics:

- Gram-negative, non-motile, non-sporulating, zoonotic, obligate intracellular aerobic coccobacilli
- 3 major human pathogenic species

Species	Primary animal host
---------	---------------------

B.abortus	Cattle
B. melitensis	Goat / Sheep
B. suis	Swine
B.canis	Dogs

Antigenic structure:

- . Lipopolysaccharide
- . Superficial L antigen

Pathogenesis and clinical features:

Brucellosis is a zoonotic disease primarily affecting goat, sheep, cattle, buffalo, pigs and other animals, and transmitted to man by direct contact with infected tissue via skin and mucus membrane, and ingestion of infected milk and milk products via intestinal tract

IP=1-6 wks

Brucellosis/ Undulant fever

2 stages of illness

1. Acute stage: Fever, malaise, sweating, hepatosplenomegally, lymphadenopathy

Associated with 80% spontaneous recovery

2. Chronic stage: Generally associated with hypersensitivity manifestations like fever, chest pain, and arthritis  
    . High agglutinin titer

Complication: Brucella spondylitis( Vertebral brucellosis)

Lab. Diagnosis:

Specimen: Blood, Biopsy material (Bone marrow, Lymphnodes),

serum Culture: Grow in blood agar, chocolate agar, or brucella agar incubated in 10% CO<sub>2</sub> at 35-37 °C for 3 wks

Biochemical reaction:

Non-hemolytic

Catalase positive

Oxidase positive

Urease test positive

Dye inhibition test positive

Serology: Agglutination test

IgG agglutination titer >1:80 indicate active infection

Treatment:

Doxycycline + rifampin

Tetracycline + streptomycin

Prevention and control:

Pasteurization of milk and milk products

Reduction of occupational hazards

Slaughter of all infected animals in dairy herds

Vaccination of cattle with live attenuated strain of B.

abortus

## GENUS FRANCISELLA

Francisella tularensis

General characteristics:

Small, facultative intracellular, gram negative, non-motile pleomorphic rods

Antigenic structure:

Polysaccharide antigens

Protein antigens

2 major biogroups (Jellison type A and B)

Type A produce lethal disease in humans unlike type B

Pathogenesis and clinical features:

It is primarily a zoonotic disease and transmitted to human by biting arthropods, direct contact with infected animal tissue, inhalation of aerosols, or ingestion of contaminated food and water

Tularemia: 4 types

1. Ulceroglandular tularemia: Ulceration of arms and hands with lymphadenitis after tick bite or direct contact of broken skin with infected tissue or blood
3. Oculoglandular tularemia: Accidental contamination of the conjunctiva with infected droplets/aerosols
4. Pneumonic tularemia: Contracted through contaminated aerosols
5. Typhoidal tulsremia: Following ingestion of inadequately cooked food



Lab. Dignosis:

Specimen: Skin lesion, lymphnodes, sputum, conjunctival scrapings

Culture: grow in blood-cysteine-gextrose agar incubated at 37 0c under aerobic condition

Serology: Agglutination test

Single titer of  $\geq 1:160$  is highly suggestive of tularemia

Paired serum samples collected two weeks apart can show a rise in agglutination titer

Treatment:

Streptomycin or gentamicin

Tetracycline

Prevention and control:

Immunization of high risk persons (eg. Lab.personel handling the specimen) with live attenuated vaccine

## **GENUS PASTEURELLA**

General characteristics:

- Gram-negative, non-motile, aerobic or facultative anaerobic coccobacilli with bipolar staining
- Grow in ordinary media at 37 0c
- Catalase positive
- Oxidase positive

*Pasteurella multocida*

- Occur in gasrointestinal and respiratory tract of many domestic and wild animals

- Most common organism in human wounds inflicted by bites from cats and dogs
- Treatment: Penicillin  
Tetracycline + flouoroquinolones

#### 2.4. GRAM NEGATIVE RODS

It comprises the following bacterial groups

##### 1. Oxidase negative

###### . Enterobacteriaceae

###### a. Lactose-fermenters

- . Escherichia spp.
- . Klebsiella spp.
- . Enterobacter spp.
- . Citrobacter spp.

###### b. Non-lactose fermenters

- . Salmonella spp.
- . Shigella spp.
- . Proteus spp.

##### 2. Oxidase Positive

- . Pseudomoas
- . Vibrio
- . Campylobacter
- . Helicobacter

### 2.4.1. ENTEROBACTERIACEAE

#### Characteristics

Named, as well coliforms or enterobacilli:

- . Found as normal flora in intestinal tract of humans and animals.
- . Gram-negative, non-spore forming, aerobic and facultative-anaerobic bacteria.
- . Most are motile.
- . Grow over a wide range of temperature in ordinary media.
- . All ferment glucose with acid production.
- . Oxidase negative.
- . Release endotoxin from their cell wall.
- . Some release exotoxin.

Most of them have possessed three types of antigenes.

These are :

- . H antigen- . Flagellar protein
  - . Found in the flagella.
  - . Possessed by motile enterobacteriaceae.
  - . Heat labile and sensitive to alcohol
  - May interfere with agglutination by O

antisera

- . K antigen- .Capsular polysaccharide or protein
  - . Surroundes the cell wall.
  - . Heat labile and may be associated with virulence
  - May interfere with agglutination by O antisera
- . O antigen- .Outer membrane lipopolysaccharide.
- . Found in the cell wall of enterobacteriaceae.

- . Resistant to heat and alcohol, and usually detected by bacterial agglutination
- . Antibodies to O ags are usually IgM

### **GENUS:ESCHERICHIA**

- . Main species of medical importance is Escherichia coli.

#### **Escherichia coli**

##### Characteristics:

- . Normal flora in human and animal gastrointestinal tract.
- . Found in soil, water and vegetation.
- . Most are motile; some are capsulated.

##### Clinical features:

- . Urinary tract infection- cystitis, pyelonephritis
- . Wound infection- appendicitis, peritonitis
- . Neonatal septicemia and meningitis
- . E.coli-associated diarrheal disease
  1. Enteropathogenic E.coli(EPEC)
    - . causes outbreaks of self-limiting infantile diarrhea
    - . they also cause severe diarrhea in adults
    - . antibiotic treatment shorten the duration of illness and cure diarrhea
  2. Enteroinvasive E.coli(EIEC)
    - . Non-motile, non-lactose fermenting E.coli invade the mucosa of the ileum and colon, and causes shigellosis-like dysentery in children in developing countries and travellers to these countries

3. Enterotoxigenic E.coli(ETEC)

. Colonization factor of the organism promote adherence to epithelial cells of small intestine followed by release of enterotoxin which causes toxin-mediated watery diarrhea in infants and young adults.

. It is an important cause of traveller's diarrhea

. Antibiotic prophylaxis can be effective but may increase drug resistance (Should not be uniformly recommended)

4. Entero haemorrhagic E.coli( EHEC)

Cytotoxic verotoxin producing E.coli serotype O157:H7 causes haemorrhagic colitis (severe form of diarrhea), and hemolytic uremic syndrome characterized by acute renal failure, hemolytic anemia and low platelet count

5. Enteroaggressive E.coli( EAEC)

.Adhere to human intestinal mucosal cells and produce ST-like toxin and hemolysin, and causes acute and chronic diarrhea in persons in developing countries

. Produce food-borne illness in developed countries

Laboratory diagnosis:

Specimen: Urine, pus, blood, stool, body fluid

Smear: Gram-negative rods

Culture: Lactose-fermenting mucoid colonies on mac conkey agar and some strains are hemolytic on blood agar .

Biochemical reaction: Produce indole from tryptophan-containing peptone water.

Reduce nitrate to nitrite.

Serology: For serotyping (Epidemiologic information)

Treatment: Base on antibiotic sensitivity pattern

**Genus: Klebsiella**

Characteristics:

Non-motile, lactose-fermenting, capsulated, gram-negative rods.

Main species of medical importance:

K. pneumoniae

K. rhinoscleromatis

K. ozenae

**K.pneumoniae**

It is found as a commensal in the intestinal tract, and also found in moist environment in hospitals.

It is an important nosocomial pathogen.

It causes:

- . Pneumonia
- . Urinary tract infection
- . Septicaemia and meningitis (especially in neonates)
- . Wound infection and peritonitis

**K. rhinoscleromatis**

It causes rhinoscleroma of nose and pharynx to extensive destruction of nasopharynx (hebra nose).

**K. ozaenae**

It causes ozena manifesting with foul smelling nasal discharge leading to chronic atrophic rhinitis.

Laboratory diagnosis of klebsiella species:

Specimen: Sputum, urine, pus, CSF, body fluid

Smear: Gram-negative rods

Culture: Large, mucoid, lactose-fermenting colonies on mac conkey agar, and shows stringy type growth when cultured in broth medium.

Serology: Capsular polysaccharide serotyping

More than 80 serotypes of *K. pneumoniae* recognized.

Treatment: Based on sensitivity testing

**GENUS: ENTEROBACTER**

It is gram-negative lactose fermenting motile rods, and found as a commensal in the intestinal tract of humans and animals and moist environments.

Medical important species is *Enterobacter aerogenes*.

It produces mucoid colony resembling *klebsiella* on Mac Conkey agar.

*Enterobacter aerogenes* is associated with urinary tract infection, wound infection and septicaemia in immunocompromised and chronically debilitated patients.

**GENUS: CITROBACTER**

It is gram-negative lactose fermenting motile rods, and opportunistic pathogen.

Medical important species is *Citrobacter freundii*.

*Citrobacter freundii* is associated with urinary tract infection, wound infection and septicaemia in immunocompromised and chronically debilitated patients.

**GENUS: SALMONELLA**

Most isolates of salmonellae are motile

It grows readily on simple media

It never ferment sucrose or sucrose

Form acid +/- acid from glucose or mannose

Species of medical importance are:

*S. typhi*

*S. paratyphi*

*S. enteritidis*

Clinical features:

1. Enteric fever

It is caused by *S.typhi* and *S.paratyphi*, and transmitted by fecal-oral route via contaminated food and drinks

Incubation period: 10-14 days

Predisposing factors:

.Reduced gastric acidity

.Disrupted intestinal microbial flora

.Compromised local intestinal immunity



Both manifest with persistent fever, headache, malaise, chills, enlargement of liver and spleen, and skin rashes.

Paratyphoid fever is milder than typhoid fever

Complications:

Intestinal perforation

Lower gastrointestinal bleeding

Disssemination to different body organs including meninges and brain

Mortality rate

Untreated cases: 10-15%

Treated cases: < 1%

## 2. Bacteremia with focal lesions

Causative agent: *S. choleraesuis*

Manifests with blood stream invasion with focal lesions in lungs, bones and meninges

Intestinal manifestation are often absent

## 3. Gastroenteritis

It is caused by *S. enteritidis*

*S. typhimurium*

IP= 8-48 hrs

It manifests with initial watery diarrhea, and later bloody mucoid diarrhea associated with crampy abdominal pain and tenesmus.

Bacteremia is rare (2-3 % of cases)

It usually resolves in 2-3 days

Variables	Enteric fever	Septicemia	Enterocolitis
Ip	7-20 days	Variable	8-48 hrs
Onset	Insidious	Abrupt	Abrupt
Fever	Gradual rise	Rapid rise	usually low
Disease Duration	Several wks	Variable	2-5 days
GIT symptoms	Early Constipation /later diarrhea	Often none	Diarrhea at onset
Blood culture	+ve in 1 <sup>st</sup> and 2 <sup>nd</sup> wks	+ve during high fever	Negative
Stool culture	+ve in 2 <sup>nd</sup> wk on	S/times +ve	+ve since onset

Laboratory diagnosis:

- Specimen: 1. Blood, Bone marrow, stool, urine and serum for enteric fever.
- . Blood – 80% positive in the first week.
  - . Stool- 70-80% positive in the second and third week.
  - . Urine- 20% positive in the third and fourth week.
  - . Serum for widal test- positive after the second week of illness.

2. Stool for gastroenteritis.

Gram reaction: Gram-negative rods

Culture: Bacteriologic methods for salmonella isolation

1. Differential medium
- . For rapid isolation of lactose non-fermenters

Egs. EMB agar  
Mac Conkey agar  
Deoxycholate agar

2. Selective medium

. favor growth of salmonella and shigella over other enterobacteriaceae

Egs. SS agar  
Hekton Enteric agar  
XLD agar  
Deoxycholate-Citrate agar

3. Enrichment cultures

. Inhibit replication of normal intestinal flora and permit replication of salmonella

Egs. Selenite F broth  
Tetrathionate broth

Non-lactose fermenting, H<sub>2</sub>S producing colonies in Mac conkey agar.

Biochemical reaction: Generally produce gas and acid from carbohydrate; except S.typhi which does not produce gas.

Serology: (wiedal test)

a. Tube dilution agglutination test

Used to determine antibody titers in patients with unknown illness

Method:

- Serial dilutions of unknown serum are tested against antigens from representative salmonella species.
- The highest diluted serum with positive result is taken as a tite

**Interpretation of result**

- High or rising titer to O antigen ( $\geq 1:160$ ) suggests active infection.
- High or rising titer to H antigen ( $\geq 1:160$ ) suggests past infection or immunization.
- High titer to the Vi antigen occurs in some cases

**Causes of false positive Widal test**

- Malaria infection
- Other acute febrile illness
- Poor quality reagent

**Causes of false negative widal test**

- Specimen collted after antibiotic adminastartion
- Specimen collted at early stage of diseases
- Technical errors

- a. Slide agglutination test/ Kauffman-White system  
Used to identify unknown cultures with known sera



S. boydii	C
S. sonnei	D

In developing countries, shigellosis (bacillary dysentery) is caused by *S. flexneri* and *S. dysenteriae*.

It is found in human intestinal tract as pathogen.

Pathogenesis and Clinical features:

Route of infection is fecal-oral route

Inoculum dose: 10<sup>3</sup> organisms

Pathogenicity determinant:

Toxins:

Endotoxin: irritate the bowel wall

Exotoxin: Enterotoxin and neurotoxin

*S. dysenteriae* type 1 (shiga bacillus) produce heat labile exotoxin-mediated diarrhea

IP: 1-2 days

It causes shigellosis (bacillary dysentery) characterized by sudden onset of bloody mucoid diarrhea, abdominal cramp, tenesmus, fever, generalized muscle ache and weakness.

Complication: Dehydration

Electrolyte and acid-base disturbance

High prevalence: Poor sanitation

Poor personal hygiene

Polluted water supply

Young children are frequently affected.

Laboratory diagnosis:

Specimen: Stool, serum

Gram reaction: Gram-negative non-motile rods.

Culture: Non-lactose fermenting colonies on Mac conkey agar and SS agar.

Biochemical reaction: It produces acid but not gas from carbohydrate.

Serology: For serogrouping and serotyping.

It is not used to diagnose shigella infection

Treatment: Ciprofloxacin

Cotrimaxazole

Suppress acute clinical attacks of dysentery

Shorten the duration of symptoms

Prevention and control:

Sanitary control of water, food and milk, sewage disposal and fly control

Antibiotic treatment of infected individuals

### **GENUS: PROTEUS**

Proteus species are found in the intestinal tract of humans and animals, soil, sewage and water.

They are gram-negative, motile, non-capsulated, pleomorphic rods.

Species of medical importance:

P. mirabilis

P. vulgaris

Clinical features:

P. mirabilis

. Urinary tract infection

. Septicemia

. Abdominal and wound infection

- . Secondary invader of ulcer, burn, pressure sores and chronic discharging ear.

*P. vulgaris*

- . Important nosocomial pathogen.
- . Isolated in wound infection and urinary tract infection.

Laboratory diagnosis:

Specimen: Urine, pus, blood, ear discharge

Smear: Gram-negative rods

Culture: Produce characteristic swarming growth over the surface of blood agar.

Ditching of culture media prevents spread of proteus species.

Non-lactose fermenting colonies in mac conkey agar.

Proteus species have a characteristic smell.

Biochemical reaction:

*Proteus* spp..... Urease positive

*P. vulgaris*..... Indole positive

*P. mirabilis*..... Indole negative

Serology: Cross react with Weil-felix test

Treatment: Based on sensitivity testing.

**GENUS YERSINIA**

General characteristics:

- Animals are natural hosts of yersinia, and humans are accidental hosts of yersinia infection
- Short, pleomorphic microaerophilic or facultatively anaerobic gram negative rods exhibiting bipolar staining with special stains



Important human pathogens

*Y. pestis*

*Y. pseudotuberculosis*

*Y. enterocolitica*

### **Yersinia pestis**

. Plague bacillus with gram negative, non-motile, facultatively anaerobe possessing bipolar granules

Antigenic structure:

LPS: Endotoxic effect

Envelope protein (Fraction I): Antiphagocytic property

V-W antigens: Plasmid gene-encoded virulence factor

Coagulase (produced at 28 °C; mice body T°)

Exotoxin (lethal for mice/unknown role in humans)

Bacteriocin (pesticin)

Pathogenesis and clinical features:

Rat flea (*Xenopsylla cheopis*) gets infected by biting an infected rodent → infected rat flea bites human (accidental host) → organism migrate to regional lymphnodes from the site of bite (bubonic plaque) and gets into the blood via lymphatics (septicemic plaque), or Primary pneumonic plaque results from inhalation of infective droplets, usually from an infected coughing person

IP=2-6 days

Human Plaque: 3 types

1. Bubonic plague: Fever, vomiting, painful lymphadenitis(buboes) in the groin or axillae
2. Pneumonic plague: Ip is 1-3 days  
Profuse mucoid or bloody expectoration with signs of pneumonia
3. Septicemic plague  
Fever, vomiting, diarrhea, hypotension, altered mentation, renal and heart failure, intra vascular coagulopathy

Lab. Diagnosis:

Specimen: Lymphnode aspirate, CSF, blood

Smears: Wright's stain, immunofluorescence stain, methylene blue stains, basic fuchsin stain

Wayson's stain to demonstrate bipolar granules

Culture: Grow in blood agar or mac conkey agar

NB: All cultures are highly infectious and must be handled with extreme caution

Biochemical reaction:

Catalase positive

Oxidase negative

Serology: Fluorescent antibody technique using Y. pestis antisera

Prognosis: Mortality rate is 50% (100% for pneumonic plague)

Treatment:

Streptomycin

Tetracycline

Streptomycin + tetracycline or chloramphenicol

Prevention and control:

. Chemoprophylaxis for contacts of patients

- . Formalin-killed vaccine for travellers to hyperendemic areas and high risk persons

*Yersinia enterocolitica* and *Yersinia pseudotuberculosis*

Non-lactose fermenting gram negative rods

Urease positive

Oxidase negative

*Y. enterocolitica*

- . > 50 serotypes

. *Y. enterocolitica* Serotype 03, 08, and 09 cause human disease

- . Human infection occurs by contaminated food and drinks from domestic animals or rodents

*Y. pseudotuberculosis*

- . Six serotypes

. *Y. pseudotuberculosis* serotype 01 accounts for most human infection

- . Human infection results from ingestion of food and drinks contaminated by animal feces

Antigenic structure

- . Inv (invasion) locus
- . AIL (attachment invasion locus)

Pathogenesis and clinical feature:

Route of transmission: Contaminated food and drinks

Inoculum dose: 10<sup>8</sup>-10<sup>9</sup> org

IP=5-10 days

Yersinosis: Enterocolitis

- . Fever, abdominal pain, toxin and invasion-mediated diarrhea
- . Usually self-limited disease
- . Post-diarrheal diseases
  - . Arthritis
  - . skin rash/nodules
- . Complication: Sepsis/ Meningitis

Lab. Diagnosis:

Specimen: Stool, blood, rectal swab

Culture: Grow in routine enteric media

Biochemical tests for species identification

Treatment:

Fluid replacement for enterocolitis (Antibiotics not required)

Cephalosporin (3<sup>rd</sup> generation) + Aminoglycosides for sepsis/ meningitis

Prevention and control:

Conventional sanitary precautions

#### 2.4.2. GENUS: PSEUDOMONAS

General characteristics:

- . Gram-negative motile aerobic rods having very simple growth requirement.
- . Can be found in water, soil, sewage, vegetation, human and animal intestine.
- . Species of medical importance:
  - P. aeruginosa
  - P. pseudomallei

### **Pseudomonas aeruginosa**

. Found in human and animal intestine, water, soil and moist environment

in hospitals.

. Primarily a nosocomial pathogen.

. Invasive and toxigenic, produces infections in patients with abnormal host defenses

Antigenic characteristic:

. Pili: Adhere to epithelial cells

. Exopolysaccharide: Anti-phagocytic property/ inhibit pulmonary clearance

. Lipopolysaccharide: Endotoxic effect

. Enzymes

.Elastases: Digests protein (elastin, collagen, IgG)

.Proteases

.Hemolysins

.Phospholipases C (heat labile): Degrade cytoplasmic membrane components

. Exotoxin A: Cytotoxic by blocking protein synthesis

Clinical features:

Pathogenic only when introduced into areas devoid of normal defenses eg. Breached mucus membrane or skin, use of IV line or urinary catheterization, neutropenia of any cause

- . Urinary tract infection- chronic, complicated Urinary tract infection and associated with indwelling catheter.
- . Wound infection of burn sites, pressure sores and ulcers.
- .Septicaemia- “Ecthyma gangrenosum” skin lesion (haemorrhagic skin necrosis)
- . Otitis externa- Malignant external ear infection in poorly treated diabetic patients.
- . Pneumonia- Infection of the lung in patients with cystic fibrosis.
- . Eye infection- Secondary to trauma or surgery.

Laboratory diagnosis:

Specimen: pus, urine, sputum, blood, eye swabs, surface swabs

Smear: Gram-negative rods

Culture:

.Obligate aerobe, grows readily on all routine media over wide range of temperature(5-42 °C).

. Bluish-green pigmented large colonies with characteristic “fruity” odor on culture media.

. Biochemical reaction:

. Oxidase positive

. Catalase positive

. Citrate positive

. Indole negative

. Produce acid from carbohydrate by oxidation, not by fermentation.

NB: identification of the bacteria is based on colony morphology, oxidase-positivity, characteristic pigment production and growth at 42 °C

Treatment: Ticarcillin or piperacillin and aminoglycosides

Aztreonam

Imipenem

Ceftazidime

Cefoperazone

Fluoroquinolones

Prevention and control:

Special attention to sinks, water baths, showers and hot tubs

Polyvalent vaccine to high risk groups.

#### 2.4.3. GENUS: VIBRIOS

- . Actively motile, gram-negative curved rods.
- . Species of medical importance: *Vibrio cholerae*-01

*Vibrio cholerae*

Characteristics:

- . Found in fresh water, shellfish and other sea food.
- . Man is the major reservoir of *V. cholerae*-01, which causes epidemic cholera.
- . Readily killed by heat and drying; dies in polluted water but may survive in clean stagnant water, esp. if alkaline, or sea water for 1-2 weeks.

Antigenic structure:

. O antigen

- . Six major subgroups.
- . All strains possess a distinctive O antigen and belong to subgroup I with subdivision into three serotypes; Ogawa, Inaba, Hikojima.
- . Any serotype can be either Classical or EITor biotype.
- . EITor biotype is more resistant to adverse conditions than Classical diotype of *V. cholerae*.

H antigen

- . Little value in identification

Clinical features:

Route of infection is fecal-oral route.

After ingestion of the *V. cholerae*-01, the bacteria adheres to the intestinal wall with out invasion then produces an exotoxin causing excessive fluid secretion and diminished fluid absorption resulting in diarrhea (rice water stool) which is characterized by passage of voluminous watery diarrhea containing vibrios, epithelial cells and mucus; and result in severe dehydration.

Laboratory diagnosis:

Specimen: Stool flecks

Smear: Gram-negative motile curved rods

Motility of vibrios is best seen using dark-field microscopy.

Presumptive diagnosis: Inactivation of vibrios in a wet preparation after adding vibrio antiserum.

Culture:



1. TCBS (thiosulphate citrate bile salt sucrose agar) media Selectivemedia for primary isolation of V.cholerae.

. Observe for large yellow sucrose-fermenting colonies after 18-24 hrs of incubation.

2. Alkaline peptone water: Enrichment media for V.cholerae-01

Growth on and just below the surface of peptone water with in 4-6 hours at room temperature as well as 37 °c.

Biochemical Reaction:

- . Oxidase-positive.
- . Ferment sucrose and maltose(acid; no gas).
- . Do not ferment L-arabinose.

Treatment: Sensitive to tetracycline and chloramphenicol.

Fluid and electrolyte replacement are the first line of management for cholera.

#### 2.4.4. GENUS: CAMPYLOBACTER

Characteristics:

- . Small, delicate, spirally curved gram-negative bacteria.
- . Motile bacteria with single polar flagellum.
- . Stricly microaerophilic bactria requiring 5-10% o<sub>2</sub> and 10% co<sub>2</sub> enriched environment.
- . Oxidase and catalase positive.

Species of medical importance:

Campylobacter jejuni

Campylobacter coli

*Campylobacter jejuni and Campylobacter coli*

Characteristics:

- Gram-negative non-spore forming motile rods with comma, S or 'gull-wing' shapes.
- Requires selective media like skirrow's and Butzler's media for isolation of the bacteria from faecal specimen.

Antigenic structure:

- . Lipopolysaccharide
- . Cytopathic extracellular toxin
- . Enterotoxin

Clinical features:

Inoculum dose:  $10^4$  organisms

Source of infection is contaminated food, drinks, and unpasteurized milk

The organism multiply in small intestine, invade the epithellium and produce inflammation

Campylobacter enteritis manifests with fever, headache, malaise, crampy abdominal pain and bloody mucoid diarrhea, and usually self-limited enteritis in a week period

Laboratory diagnosis:

Specimen: Stool

Microscopy: Typical 'gull-wing' shaped gram-negative rods.

Typical darting motility of the bacteria under dark field microscopy or phase contrast microscopy

Culture: Grow best at 42°C on selective media but can be cultured at 37°C.

Watery and spreading or round and convex colonies on solid media at low oxygen tension.

Biochemical reaction:

*C. jejuni* ..... hydrolyzes hippurate.

*C. coli* ..... does not hydrolyze hippurate.

Treatment: Erythromycin

Shorten the duration of fecal shedding of bacteria

### **Helicobacter pylori**

General characteristics:

- . Spiral-shaped gram negative, microaerophilic, motile rods with polar flagella

Antigenic structure:

Pili

Protease

Urease

Pathogenesis and clinical features:

Route of entry: Ingestion of contaminated food and drinks

Familial clustering of *H. pylori* infection occurs

. Type B chronic antral gastritis

. Peptic ulcer disease (gastric and duodenal ulcer)

- . Gastric carcinoma
- . Gastric lymphoma

Lab. Diagnosis:

Specimen: Gastric biopsy, serum

Smear: Giemsa or silver stain

Culture: Skirrow's media

Translucent colonies after 7 days of incubation

Biochemical reaction:

- . Catalase positive
- . Oxidase positive
- . Urease positive

Serology:

- . Detection of antibodies in the serum specific for *H. pylori*
- . Detection of *H. pylori* antigen in stool specimen

Special tests:

- . Urea breath test

Treatment:

Triple or quadruple therapy:

. Amoxicillin + clarithromycin/ metronidazole +  
Proton pump inhibitors (PPI (Omeprazole or  
lansoprazole))

or

. Metronidazole + Bismuth subsalicylate/ Bismuth  
subcitrate + Amoxicillin / Tetracycline + PPI

Prevention and control:

- . Improving sanitary hygiene

**GENUS: LEGIONELLA**

**L. pneumophila**

General characteristics:

Fastidious, aerobic, gram negative intracellular rods  
Ubiquitous in warm moist environment

Antigenic structure:

Complex surface antigens  
>10 serogroups  
L.pneumophila serogroup 1 is the most common  
serogroup isolated in humans

Proteases

Phosphatases

Lipases

DNase

RNase

Major secretory protein (Metalloprotease): Possess cytotoxic  
and hemolytic property

Pathogenesis and clinical features:

Route of transmission: Inhalation of aerosols generated from  
contaminated cooling towers, heat exchange apparatus, shower  
water, tap water, and potable water following chlorination

1. Legionnaires disease: Pneumonic presentation with high  
fever, chills, dry cough, hypoxia, diarrhea, and altered  
mentation
2. Pontial fever: Fever, chills, malaise, headache, malaise,  
altered mentation

Laboratory diagnosis:

Specimen: Bronchial washing, Lung biopsy, Blood

Smears: DFA (direct fluorescent antibody) staining

Silver staining

Culture: Grow in BCYE (buffered charcoal-yeast extract)

agar media

Biochemical tests:

Catalase positive

Oxidase positive

Hydrolyse hippurate

Serologic testing: Useful in the diagnosis of retrospective outbreaks of legionella infection

Treatment:

Erythromycin

Rifampin

Prevention and control

Hyperchlorination

Super heating of water

**Infection caused by anaerobic bacteria**

Anaerobic bacteria are found through out human body (Skin, mucous membrane, and gastrointestinal tract) as part of resident flora, and cause infection when contaminate normally sterile body sites

- . Grow at low or negative oxidation-reduction potential
- . Do not have cytochrome systems for oxygen metabolism

- . Lack superoxide dismutase and catalase, and susceptible to the lethal effects of oxygen and oxygen radicals.
- . Most anaerobic infections are caused by “moderately obligate anaerobes”, and polymicrobial in nature caused by combination of anaerobes, facultative anaerobes and aerobes.

### Anaerobic bacteria found in human infections

1. Bacilli	Anatomic site
a. Gram negative	
Bacteroides fragilis group	colon
Prevotella melaninogenica	Mouth
Fusobacterium	Mouth/Colon
b. Gram positive	
Actinomyces	Mouth
Lactobacilli	Vagina
Propionibacterium	Skin
Clostridium	Colon
2. Cocci	
a. Gramnegative	
Veillonella	Mouth/Colon
b. Gram positive	
Peptostreptococci	Colon
clinical features:	

<b>Representative anaerobic infections</b>	<b>Commonly isolated anaerobic bacteria</b>
Brain abscess	Peptostreptococci
Oropharyngeal infection	Actinomyces P. melaninogenica Fusobacterium
Pleuropulmonary infection	Peptostreptococci Fusobacterium P. melaninogenica B. fragilis
Intra-abdominal infection	
Liver abscess	Mixed anaerobes
Abdominal abscess	B. fragilis
Genital tract infection	
Pelvic abscess	P. bivia P. disiens Peptostreptococci
Skin, soft tissue, and bone infection	Mixed anaerobic flora
Bacteremia	B. fragilis Clostridium



Peptostreptococci

Propionibacterium

Endocarditis

B. fragilis

Diagnosis of anaerobic infection

1. Clinical

- . Foul smelling discharge due to short chain fatty acid products of anaerobic metabolism
- . Proximity to a mucosal surface
- . Gas in tissue due to production of CO<sub>2</sub> and H<sub>2</sub>

2. Lab. diagnosis

- . Grow most readily in complex media (Trypticase soy agar, Schaedler blood agar, Brucella agar, Brain-heart infusion agar) incubated at 35-37 °C in anaerobic atmosphere enriched with CO<sub>2</sub>

Identification of anaerobes is based on:

- . Colony morphology
- . Pigmentation
- . Fluorescence
- . Biochemical reaction
- . Fatty acid production

Treatment:

Antimicrobials + Surgery

Clindamycin

Metronidazole

Cefotetan

Cefoxitin

Piperacillin

Penicillin

## 2.5. GENUS: MYCOBACTERIA

Characteristics:

- Non-spore forming, non-motile, aerobic, Acid-fast bacilli.
- Acid-fastness depends on the waxy envelope-mycolic acid of cell wall.
- More resistant to chemical agents than other bacteria.
- Once stained with primary stain, they resist decolorization by acid-alcohol.
- All bacteria are decolorized by acid-alcohol except Mycobacteria.

. Mycobacteria of medical importance:

M. tuberculosis

M. leprae

### **Mycobacterium tuberculosis**

Characteristics:

- . Strictly aerobic acid-fast bacilli.
- . The main reservoir is an infected human.

Antigenic structure:

1. Lipids: Mycolic acid, waxes, phosphatides  
.responsible for acid-fastness, granuloma formation and caseation necrosis.
2. Proteins

- . Elicits the tuberculin reaction and antibody production.

### 3. Polysaccharides

- . Induce the immediate type of hypersensitivity.

Clinical manifestation:

Incubation period: 4-6 weeks.

Source of infection: Tuberculous patients

Route of infection: Respiratory- Inhalation of droplet nuclei

Ingestion of infected milk

Disease: Pulmonary and extrapulmonary tuberculosis

The disease generally manifests with low-grade persistent fever, night sweating, significant weight loss, fatigue and generalized weakness.

Laboratory diagnosis: Identification of *M. tuberculosis*

Specimen: Sputum; pleural, peritoneal and cerebrospinal fluid

Smear: Acid fast bacilli from primary specimen.

Cord forming acid-fast bacilli from culture.

Culture:

#### 1. Semisynthetic agar media

- . Middle brook 7H10 and 7H11

- . Used for observing colony morphology, susceptibility testing, and as selective media

#### 2. Inspissated egg media

- . Lowenstein-Jensen medium

It is the ordinary selective media for tubercle bacilli

Raised, dry, cream colored colonies of tubercle bacilli after 3-6 wks of incubation

### 3. Broth media

Middle brook 7H9 and 7H12

- . Support the proliferation of small inocula

Pigment production test:

- . Leave the culture in the light for 2 hrs.
- . Reincubate it at 35-37 °c over night.
- . Reexamine the colonies for pigment production

- . M. tuberculosis does not produce pigment in dark or light.

Incubation of a subculture at 25°c

- . M. tuberculosis will not grow at 25 °c.

Growth of the bacteria on Lowenstein-Jensen medium containing

4(P)- nitrobenzoic acid(PNB).

- . No growth of M. tuberculosis on PNB media.

Biochemical reaction:

- .Niacin test is Positive.

New techniques:

- . Molecular probes (DNA probes)- It detects Mycobacterial RNA sequence.
- . High-performance liquid chromatography
- . Polymerase chain reaction
- . Enzyme immunoassay

Treatment:

Anti-tuberculosis drugs

#### 1. First-line drugs

- . Isoniazid
- . Ethambutol
- . Rifampin
- . Pyrazinamide
- . Streptomycin

2. Second-line drugs

- . Kanamycin . Cycloserine
- . Capreomycin . Ofloxacin
- . Ethionamide . Para-aminosalicylic acid (PAS)

Prevention and control:

- . Prompt and effective treatment of patients with active tuberculosis and careful follow up of their contacts with tuberculin test and CXR
- . Immunization with BCG (Bacillus-Calmette-Guerin) vaccine
- . Pasteurization of milk and milk products

**Mycobacterium leprae**

Characteristics:

- Typical acid-fast bacilli, arranged in singly, parallel bundles or in globular masses.
- Not grown in non-living bacteriologic media.
- Characteristic lesions are grown in laboratory animals.

Eg. Foot pads of mice

Armadillos

Clinical features:

Incubation period is months to years.

Route of infection is through nasal mucus secretion.

Disease: Hansen's disease or leprosy.

The lesion involves the cooler parts of the body, Eg. Ear lobes.

Clinical triads: Anaesthetic skin patches

Peripheral neuritis

Presence of acid-fast bacilli from skin lesion

Two major types of leprosy

1. Lepromatous leprosy
2. Tuberculoid leprosy

**Table 2.7. Comparison of the two types of leprosy**

Characteristics	Lepromatous leprosy	Tuberculoid leprosy
1. Course	. Progressive	. Benign and non-progressive
2. Manifestation	. Nodular skin lesion	. Macular skin lesion
3. Involvement of nerve	. Slow and symmetrical	. Severe and asymmetrical
4. Cell mediated immunity	. Weak	. Strong
5. AFB from skin lesion	. Abundant	. Scanty
6. Lepromin skin test	. usually negative	. Usually positive

Laboratory diagnosis:

Specimen: Skin scrapings from the ear lobe.

Smear: Acid fast bacilli from the primary specimen.

**Bacterial index (BI)** indicates number of organisms present in a smear.

Number of *M. leprae* bacilli found in smears are related to type of leprosy and effect of drug therapy.

**GRADING:**

- 0.....No bacilli per field, count  $\geq 100$  fields
- 1+.....Average 1-10 bacilli per smear, count  $\geq 100$  fields
- 2+.....Average 1-10 bacilli per 10 fields, count  $\geq 100$  fields
- 3+.....Average 1-10 bacilli per 10 fields, count 25 fields
- 4+.....Average 10-100 bacilli per fields, count 25 fields
- 5+..... Average 100-1000 bacilli per fields, count 10 fields
- 6+..... Average  $>1000$  bacilli per fields, count 10 fields

**NB: Count BI for each smear and calculate the average to give an over all BI.**

**Morphologic index** indicates percentage of viable bacteria in a smear.

It is used to judge the response of a patient to anti-leprosy drugs.

Viable bacilli stain clearly and evenly as solid red bacilli.

Non-viable bacilli stain poorly and unevenly as fragmented, beaded and granular red bacilli.

**Methods of collecting skin smears and staining of *M. leprae***

**Procedure:**

- . Explain to the patient the procedure you are going to do.
- . Label the slide with the date, name and number.
- . Fit the sterile scalpel blade in its scalpel holder.
- . Cleanse the area from where the smear is to be taken using alcohol swab.

- . When dry, hold fold of skin tightly between the thumb and forefinger until it becomes pale.
  - . Using the sterile blade, make a small cut through the skin surface, 5mm long and 2-3mm deep, where the bacteria is be found.
  - . Turn the scalpel blade until it is at a right angle to the cut.
  - . Make a small circular smear of the tissue juice  
(Cover the cut with a small dressing)
  - . When the smears are dry, gently fix with heat.
  - . Cover the smear with the filtered carbol-fuchsin stain.
    - . Heat the stain until vapor just begins to rise; Don't over heat.
    - . Allow the smear heated stain to remain on the slide for 15 min.
  - . Wash off the stain with clean water.
  - . Decolorize the smear with 1% V/V acid-alcohol for 10 min.
  - . Wash off with clean water.
  - . Cover the smear with malachite green stain for 1-2 min.
  - . Wash off the stain with clean water.
  - . Wipe the back of the slide clean, and place in a draining rack for the smears to air-dry (protect from direct sun light).
  - . Examine the smear under microscope with 100X objective.
- NB: The bacteria is not cultured in artificial media; but can be cultivated in vivo by inoculation into the foot pads of mice and Armadillos.
- The animal develops slow-growing granulomas at the site of injection.



Treatment: Anti-leprosy drugs

- . Dapsone
- . Rifampicin
- . Clofazimine

### **ACTINOMYCETES**

- . Most are soil saprophytes, but some are human pathogens responsible to cause actinomycosis nocardiosis and actinomycetoma
- . Large group of gram positive bacilli with a tendency to form chains and filaments
- . Related to mycobacteria and corynebacteria
- . Endogenous members of the bacterial flora in the mouth and lower gastrointestinal tract

Actinomycosis

Chronic suppurative and granulomatous infection with interconnecting sinus tracts that contain sulfur granules

Etiology:

*Actinomyces israeli*

*Actinomyces naeslundii*

Characteristics:

- . Gram positive, facultative anaerobe substrate filaments that grow in CO<sub>2</sub> enriched condition

Pathogenesis and clinical features:

- . Infection is initiated by trauma that introduces these endogenous bacteria into the mucosa

1. Cervico facial actinomycosis

Fluctuant mass with draining fistula in jaw area, and may extend to involve bone and lymphnodes in the head and neck

2. Thoracic actinomycosis

Resemble subacute pulmonary infection with extension to chest wall and ribs

3. Abdominal actinomycosis

May be secondary to ruptured appendix or ulcer with extensive involvement of abdominal organs

Lab. Diagnosis:

Specimen: Tissue, pus, sputum

Smear: Gram-positive filaments with lobulated sulfur granules

Culture: Thioglycolate broth or blood agar incubated anaerobically or CO<sub>2</sub> enriched condition

Biochemical reaction: Catalase positive/negative

Treatment: Penicillin

Clindamycin + Surgery

Erythromycin

Nocardiosis

Etiology: Nocardia asteroides complex

N. abscessus

N. farcinia

N. nova

Nocardia brasiliensis

Nocardia otitidiscaviarum

Characteristics:

- . Found world wide in soil and water, and opportunistic pathogen
- . Aerobic gram positive, partially acid fast bacilli

Pathogenesis and clinical features:

Route of transmission: Inhalation

Usual presentation is subacute or chronic pulmonary infection with dissemination to the brain and skin

Lab. diagnosis

Specimen: Sputum, pus, CSF, biopsy material

Smear: Gram positive, partially acid fast bacilli

Treatment: Cotrimoxazole + Surgery

Non-responders to cotrimoxazole

Amikacin

Imipenem

Cefotaxime

Actinomycetoma

Slowly progressive, painless, destructive subcutaneous tissue infection

Etiology: *Nocardia brasiliensis*

*Streptomyces somaliensis*

*Actinomadura madurae*

Treatment: Combination of streptomycin, cotrimoxazole and dapsone

## 6. SPIROCHETES

### Characteristics:

- . Long, slender, helically coiled, spiral or cork-screw-shaped gram-negative rods.
- . Move by bending and rotating body movements.
- . Spirochete consist of protoplasmic cylinder bounded by a cell wall and outer membrane. There is an axial filament or endoflagella between the cell wall and outer membrane.

### Spirochetes of medical importance:

- . Treponema
- . Borellia
- . Leptospira

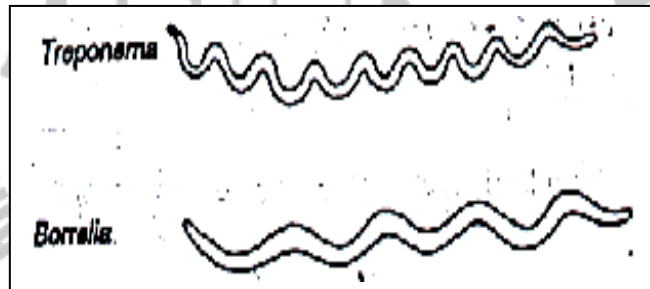


Fig. 3.6. Spirochetes

### 2.6.1. GENUS: TREPONEMA

#### Species of medical importance:

- T. pallidum causes syphilis
- T. pertenuis causes yaws
- T. carateum causes pinta
- T. endemicus causes bejel

### **Treponema pallidum**

#### Characteristics:

- . Slender spiral, microaerophilic gram-negative rods.
- . Not cultured in artificial media, in fertilized eggs and tissue culture, but the saprophytic Reiter strain grows in anaerobic culture
- . Actively motile, rotating steadily around their endoflagella
- . Remain viable in the blood or plasma store at 4 °c at least for 24 hrs (transmitted via blood transfusion)

#### Antigenic structure:

- . Membrane proteins
- . Outer sheath proteins
- . Endoflagellar core proteins
- . Cardiolipin
- . Hyaluronidase

#### Pathogenesis and Clinical features:

Natural infection with *T. pallidum* is limited to the human host

Incubation period is 3-4 weeks.

Route of transmission is sexual contact.

A. Acquired syphilis

It has four stages.

1. Primary stage: Hard chancre: Clean-based, non-tender, indurated genital ulcer with inguinal lymphadenopathy.
1. Secondary stage: Manifests with generalized maculopapular rash condylomata lata and white patches

in the mouth. There may be syphilitic meningitis, nephritis, periostitis, hepatitis and retinitis.

Primary and secondary syphilis are rich in spirochete from the site of the lesion and patients are highly infectious.

2. Latent stage: Patients are symptom-free but relapse can occur.

Diagnosis is by serological test.

Early latent stage: Relapse of symptoms and signs occur, and patients are infectious. It occurs within 2 years of developing primary syphilis.

Late latent stage: There is no relapse of symptoms and signs.

Patients are not infectious. It occurs after 2 years of developing primary syphilis.

3. Tertiary stage: Manifesting with gumma (granulomatous lesion) in bone, skin and liver; meningovascular syphilis, syphilitic paresis, tabes dorsalis, syphilitic aortitis and aortic aneurysm.

#### **Out come of acquired syphilis in untreated cases**

1. One third of cases seem spontaneously cured during primary and secondary syphilis but no clear evidence
2. One third of cases become positive for serological tests of syphilis.
3. One third of cases develop tertiary syphilis.

## B. Congenital syphilis

Route of transmission: Mother-to-child during gestation.

Out come: Abortion

Fetal death

Still birth

Early neonatal death

Organ damage: Congenital syphilis triad

- . Interstitial keratitis
- . Hutchison's teeth
- . Deafness

Laboratory diagnosis:

Specimen: Tissue from skin lesion

1. Dark field microscopy

. Motile spirochetes in dark field illumination are observed.

2. Immunofluorescence stain

Procedure:

- . Put tissue fluid on a glass slide.
- . Fix and stain with fluorescein-labeled anti-treponeme serum.
- . Observe fluorescent spirochetes in Immuno-fluorescence microscopy.

3. Serological tests for syphilis (STS)

Specimen: Serum

a. Non-treponemal antigen tests

Antigen- Cardiolipin from beef heart

1. Flocculation test –VDRL , RPR

. Positive after 2-3 wks of untreated syphilitic infection

. Positive result revert to negative with in 6-18 months of effective therapy of syphilitic infection

Principle: Antigen and antibody (Reagin) reaction results in clumping after aggitation

. It can give quantitative results, and valuable in establishing a diagnosis and in evaluating effect of treatment

2. Complement fixation test: Wasserman test; Kolmer test

Principle: Reagin-containing sera (mixture of IgM and IgA) fix complement in the presence of “cardiolipin-cholesterol-lecithin complex” antigen.

NB: Flocculation and Complement fixation tests are valuable in establishing diagnosis and in evaluation of chemotherapy effectiveness.

False-positive results in both tests can occur since the tests are non-specific.

b. Treponemal antibody tests

. Fluorescent treponemal antibody-absorption test (FTA-abs)

. Treponema pallidum- particle agglutination test (TP-PA)



- . Treponema pallidum immobilization test (TPI)

#### FTA-Abs test

Principle: The test employ indirect immunofluorescence, i.e combination of killed T.pallidum + patient's serum + Labelled antihuman gammaglobulin

- . The first to become positive in early syphilis, and remains positive for several months after effective therapy

#### TP-PA test

Procedure:

- . Sensitize T.pallidum antigens with gelatin particles
- . Add diluted serum containing antibody to the sensitized gelatin particle in a microdilution tray
- . Positive result when agglutination occurs

Treatment: Penicillin  
Tetracycline  
Erythromycin

Control measures: Treatment of cases and screen contacts

Practice safe sex with condoms

Health education

### 2.6.2. GENUS: BORELLIA

Borellia recurrentis: Causative agent of epidemic relapsing fever

Borellia duttoni: Causative agent of endemic relapsing fever

General characteristics:

- . Highly flexible irregular spiral organism, and move by rotation and twisting
- . Cultured in complex serum-rich artificial media and embryonated eggs.
- . Famous in antigenic variation.
- . Stain readily with bacterioicic dyes and blood stains

Pathogenesis and Clinical Features:

Disease	Reservoir	Vector
Epidemic RF	Human	Pediculus humanus (body louse)
Endemic RF moubata (Ticks)	Rodents	Ornithodoros

In epidemic RF, infection due to B.recurrentis occurs when abraded skin of the host is contaminated with coelomic fluid of the lice which has been crushed on

In endemic RF, infection due to B. duttoni occurs by bite or by crushing the tick on the abraded skin, and occasionally by contact with the blood or tissue of infected rodents

Incubation period: 3-10 days

Clinical features of both types of relapsing fever is almost similar but epidemic relapsing fever is more severe and associated with high mortality rate,

Manifestation: Sudden onset of fever, headache, malaise for 3-5 days followed by an non-febrile period of one week. 3-5 relapses can occur with diminishing severity.

Conditions favoring disease transmission

- . Overcrowding/ Poverty/ Famine/drought for epidemic RF
- . High prevalence of tick in the locality for endemic RF

Laboratory diagnosis:

Specimen: Blood

Smear: Giemsa's stain / wright's stain

Seen as large, loosely coiled spirochetes

Culture: Cultured in serum-rich complex medium

Animal inoculation: Intraperitoneal inoculation rat with spirochete-containing blood, and examine the rat tail blood for spirochetes after 2-4 days

Treatment: Penicillin

Tetracycline

Control measures:

For epidemic RF

Delousing with insecticides

Improve personal and family hygiene

For endemic RF

Avoidance of exposure to ticks

## **GENUS LEPTOSPIRA**

### **L. interrogans**

More than 200 serovars

General characteristics:

- . Tightly coiled, thin, flexible spiraled spirochetes forming one polar hooked ends
- . Grow best in semisolid (Fletcher's or Stuart's) media under aerobic condition at 28-30 °C
- . Can survive for weeks in alkaline PH water
- . Fatty acid oxidation is major source of energy

Antigenic structure:

Lipopolysaccharide: Determine the specificity of human immune response to the organism and serologic classification of leptospirae

Pathogenesis and clinical features:

Essentially zoonotic infection and humans are accidental host

Source of infection is contaminated food and water with leptospira spp.

IP=1-2 weeks

Leptospirosis is characterized by biphasic illness initially presenting with fever, prostration, jaundice, hemorrhage and nephritis followed by aseptic meningitis

Lab. Diagnosis

Specimen: Blood, CSF, urine, tissue, serum

Smears: Dark field examination

Fluorescein-conjugated antibodies staining

Immunohistochemical staining

Giemsa staining

Culture: Cultured in fletcher's semisolid media

Growth in the media is slow, requiring incubation for at least 8 wks

Animal inoculation: Intraperitoneal inoculation of young hamsters with spirochetal contaminated fresh plasma or urine

Demonstration of spirochetes after few days in peritoneal cavity

Serology: EIA/ Agglutination tests

High titers of agglutinating antibodies after 5-8 wks of leptospiral infection

Treatment:

Doxycycline

Ampicillin/Amoxicillin

Prevention and control:

- . Preventing exposure to potentially contaminated water
- . Reducing contamination by rodent control
- . Chemoprophylaxis: Using doxycycline during heavy exposure

## 2.7. GENUS: RICKETTSIAE

Characteristics:

- . Obligate intracellular pleomorphic gram-negative coccobacilli occurring in single, pairs, short rods and filaments.
- . Poorly stained in gram reaction.
- . The organism stains red in macchiavello's stain.

- . The organism stains blue in giemsa's stain.
- . Grow in yolk sac of embryonated eggs, cell culture and laboratory animals.
- . Destroyed by heat, drying and bactericidal chemicals.

Antigenic structure:

Group-specific antigens

Species-specific antigens

Clinical Features: Clinical illness is due to the invasion and multiplication of rickettsiae in the endothelial cells of small blood vessels. It manifests with fever, headache, malaise, skin rash and enlargement of liver and spleen.

The genus rickettsiae has three main groups based on their antigenic structure.

These are: Typhus group

Scrub typhus group

Spotted fever group

**Table 2.8. Hosts and vectors of the medically important rickettsiae**

<u>Organism</u>	<u>Disease</u>	<u>Hosts</u>	<u>Vectors</u>
1. Typhus group			
R. prowazekii	Louse-borne typhus	Man	Body louse
R. typhi	Murine typhus	Rat	Rat flea
2. Scrub typhus			
R. tsutsugamushi	scrub typhus	Rodents	Mite
3. Spotted fever group			
R. conorii	African tick typhus	rodents, dogs	tick

R. rickettsi	Rocky mountain Spotted fever	Rodents, dogs	Tick
R. akari	Rickettsial pox	Mice	Mite

Species of medical importance in our country

R. prowazekii

R. typhi

### **Rickettsia prowazekii**

. It causes epidemic or louse-borne typhus and the milder recrudescence form,

Brill-Zinzer disease.

Clinical Features: It is transmitted by self-inoculation of the organism by scratching after bite by infected louse (*Pediculus humanus corporis* and *pediculus humanus capitis*). The illness manifests with sudden onset of fever, headache, malaise, prostration and skin rash. Epidemics of the disease are associated with overcrowding, cold weather, lack of washing facilities and fuel, famine and war.

Brill-Zinzer disease (BZD) is recrudescence of infection in persons who have had classical typhus in the past.

Early IgG antibodies response rather than IgM antibodies and milder course of the disease is characteristic of BZD due to development of partial immunity

### **Rickettsia typhi**

. It causes endemic or flea-borne typhus.

Clinical Features: It is transmitted to man when bitten by an infected rat-flea.

The disease is milder than louse-borne typhus and occurs in those individuals living or working in highly rat-infested area.

Laboratory diagnosis:

Specimen: Serum for serological tests

The serological tests to diagnose typhus are:

1. Indirect fluorescent antibody test
2. Complement fixation test
3. Weil –felix reaction: Some of the antigen of Proteus strain (OX-19, OX-2,OX-K) agglutinates with sera from patients with rickettsial diseases.

Treatment:

Tetracycline

Chloramphenicol

### **2.8. GENUS: MYCOPLASMA**

Characteristics:



- . Part of normal flora of human genital tract or oral cavity of healthy adults
- . Formerly named as pleuropneumonia-like organism (PPLO).
- . The smallest living micro-organism capable of free living in nature self-replicating on laboratory media
- . Highly pleomorphic due to absence of rigid cell wall, instead bounded by a triple-layered "unit membrane"
  - . Completely resistant to penicillin and cephalosporin.
  - . Can reproduce in complex cell-free media.
  - . Have an affinity to mammalian cell membrane
  - . 14 species of mycoplasma is identified in humans and classification of species is based on biochemical reaction and serological tests

#### Antigenic structure

Glycolipids (CF antigens)

Proteins (ELISA antigens)

#### Mycoplasma species of medical importance

Mycoplasma pneumoniae

Mycoplasma hominis

Ureaplasma urealyticum

#### **Mycoplasma pneumoniae**

#### Clinical features:

Route of transmission: Infected respiratory secretion

Infection is initiated after adherence of bacterial polar tip adhesin protein to respiratory epithelial cells

IP=1-3 wks

It is a major cause of pneumonia in young age groups  
(5-20yrs.)

Extra pulmonary manifestations:

Hemolytic anemia

Skin rashes/lesions

Meningoencephalitis

Myelitis

Neuritis

Myopericarditis

Arthritis

Laboratory diagnosis:

Specimen: Sputum

Culture: Cultured in semisolid media-enriched with yeast extract and serum, incubated aerobically for 7-12 days

Identification: Observe for "fried-egg" colonies embedded into the surface of the medium or inhibition of growth around discs impregnated with specific antisera.

Serology: Complement fixation test

Indirect Immunofluorescent test

Haemagglutination inhibition test

NB: Cold hemagglutinins titer  $\geq 1:64$  suggests *M.pneumoniae* infection

Treatment: Tetracycline

Erythromycin

NB: The above antibiotics produce clinical improvement but do not eradicate the organism

Mycoplasmas are resistant to penicillin, cephalosporins and vancomycin

### **Mycoplasma hominis and Ureaplasma urealyticum**

- . Found as a normal flora in the lower genital tract.
- . Mycoplasma hominis causes genital infection and post-partum sepsis.
- . Ureaplasma urealyticum causes non-gonococcal urethritis.
- . Treatment is the same as M. pneumoniae.

### **2.9. GENUS: CHLAMYDIA**

Characteristics:

- . Obligate intracellular gram-negative bacteria.
- . Reproduce by binary fission.
- . Posses both DNA and RNA.
- . Have cell wall and ribosomes.
- . Sensitive to anti-microbial agents.
- . Have enzyme systems and make their own proteins, lipids, nucleic acids and vitamins.

Three species of medical importance

- C. tracomatis
- C. pneumoniae
- C. psittacii

NB: Chlamydia tachomatis is the main species of clinical importance in developing countries.

### Developmental cycle of chlamydia

The infectious environmental stable particle, named as elementary body is ingested by a host cell. The elementary body is reorganized into reticulate body in the host cell which is specifically adapted for intracellular growth. The reticulate body grows and divides many times to form inclusions in the host cell cytoplasm.

With in 24-48 hours of developmental cycle, the reticulate bodies rearrange them selves into infective elementary bodies and released after host cell rupture.

Antigenic structure: Group-specific antigen

Species-specific antigen

### Chlamydia trachomatis

. Stained with giemsa's and iodine stain.

. Appearance in giemsa's stain

Elementary body ----- Purple

Reticulate body----- Blue

Host cell cytoplasm----- Blue

. Appearance in iodine stain

Brown inclusions in host cell cytoplasm because of glycogen matrix surrounding the particle.

. There are 15 serotypes of *C. trachomatis*.

*C. trachomatis* serotype A, B, C causes trachoma.

*C. trachomatis* serotype D-K causes genital infection.

*C. trachomatis* serotype L<sub>1</sub>-L<sub>3</sub> causes lymphogranuloma venereum(LGV).

C. trachomatis serotype A, B, C causes trachoma.

Incubation period is 3-10 days

Route of transmission is through indirect contact like eye-to-eye by infected fingers or sharing towels.

It manifests as a chronic keratoconjunctivitis producing scarring and deformity of the eyelids, corneal vascularization and opacities which may lead to blindness.

It is associated with a low standard of living and poor personal hygiene.

Laboratory diagnosis:

Specimen: Conjunctival scraping from upper tarsal conjunctivae.

Smear: Giemsa's stain during early disease stage.

Culture: Mac coy cells or embryonated eggs

Serology: Immunofluorescent tests

Treatment:

Erythromycin

Tetracycline

Control measures:

- . Improving hygienic standard.
- . Treatment of cases with antibiotics.
- . surgical correction of eyelid deformities.

C.trachomatis serotype D-K causes

1. Genital infection.

. Male ----- non-gonococcal urethritis

Epididymitis

- . Females----- Urethritis
  - Cervicitis
  - Pelvic inflammatory diseases

If complicated in females, it causes infertility and ectopic pregnancy.

- 2. Inclusion conjunctivitis resembling trachoma.

Transmission is by self-inoculation of the eye with infected genital secretion.

- 3. Neonatal inclusion conjunctivitis and neonatal pneumonia

Transmission is during passage through the infected birth canal.

Laboratory diagnosis:

Specimen: Endocervical scraping

Culture: mac coy cells

Serology: Enzyme immunoassay for group-specific antigen.

Direct fluorescent antibody test for species- specific antigen.

*C. trachomatis* serotype L<sub>1</sub>-L<sub>3</sub> causes lymphogranuloma venereum (LGV).

It is a sexually transmitted disease (STD) which is characterized by suppurative inguinal adenitis.

Complication: . . Elephantiasis of penis, scrotum or vulva due to lymphatic obstruction.

- . Sinus formation at site of lesion.

Treatment

Tetracycline

Erythromycin

### **Chlamydia pneumoniae**

Humans are the only known host

Produces sulfonamide-resistant, round, dense, glycogen negative inclusions

Only one serovar has been demonstrated

Route of transmission: person-to-person transmission via Air borne

Clinical features:

Most infections are asymptomatic to mildly symptomatic

Symptomatic cases present with

Chlamydial pneumonia

Pharyngitis

Sinusitis

Otitis media

Laboratory diagnosis:

Culture: Grows better in HL and Hep-2 cells incubated at 35-37 °C for 3 days

Intracellular inclusions are detected by fluorescein staining with a genus and species specific antibodies or fluorescein conjugated C.pneumonia specific monoclonal antibodies

Serology: Micro immunofluorescence test

Most sensitive method for the diagnosis of C. pneumoniae infection

Single IgM titer of  $\geq 1:16$

Single IgG titer of  $\geq 1:512$

Four fold rise in either the IgM or IgG titers

Treatment: Tetracycline/doxycycline

Macrolids: Erythromycin/Azithromycin

Fluoroquinolones

## Review Questions

- Describe laboratory methods of differentiation of staphylococcus species
- List the kinds of laboratory tests used to differentiate the different streptococcus species
- Mention the criteria used to identify N. gonorrhoea and N. meningitidis
- Describe methods of collecting skin smears and staining of mycobacterium leprae
- Mention the diagnostic tests for syphilis at different stages of the disease

## CHAPTER FOUR

### Learning Objective



At the end of the session, the student should be able:

- To identify body tract defense mechanisms
- To identify the main pathogenic bacteria responsible to cause body tract infection
- To list the specimen of choice in body tract infection
- To choose the appropriate culture media, biochemical and Serological tests to identify the pathogenic bacteria in body tract infection

#### **4.1. HOST-PARASITE RELATIONSHIP**

Definition: It is a beneficial or harmful relationship between the host and the parasite.

On the basis of their life habits, microorganism is classified as saprophytes or parasites.

Saprophytes : Mode of life of free-living organisms which obtain their nourishment from soil and water. Saprophytes in general do not require a living host.

Parasitism: An Adaptation to life on or in the bodies of higher organisms. The association may take one of the three forms:

1. Symbiosis: The ability to live in the tissues of the host with mutual benefit.
2. Commensalism: The ability to live on the external or internal surface of the body with out causing disease.

*“Eating at the same table”*

3. Pathogenicity: The ability of an organism to cause disease.

The outcome of the host- parasite relationship depends on a balance between the virulence of the parasite and the resistance of the host.

**Aggressive mechanisms of the parasite**

1. Adherence factors
  - . Pili: Hair-like appendages extending from the bacterial cell surface.
2. Invasiveness of micro-organism
  - A high degree of bacterial invasiveness is usually associated with severe infection.
3. Bacterial toxins
  - These are of two types.
    - a. Exotoxins
    - b. Endotoxins

**Table 4.1 Characteristics of bacterial toxins**

<u>Character</u>	<u>Exotoxin</u>	<u>Endotoxin</u>
. Composition	Protein	ipopolysaccharide
. Action	Specific	on-specific
. Antigenicity	Strong	Weak
. Effect of heat	Labile	Stable
. Produced by	Gm+ve&Gm-ve Bacteria	m-ve bacteria only.
. Converted to toxoid	Yes	No
. Mode of release from bacteria	Excreted by	released on bacterial death

living cell	(Integral part of cell wall)
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#### 4. Enzymes

. Tissue degrading enzymes

. Collagenase: Degrade collagen, which is major protein of fibrous connective tissue.

. Hyaluronidase: (Early spreading factor) hydrolyzes hyaluronic acid, which is the ground substance of connective tissue.

. Lecithinase: Splits lecithin of cell membrane into phosphorylcholine and glycerides

. Staphylokinase/Streptokinase (fibrinolysin)

#### 5. Anti-phagocytic factors

. Protein A of *Staphylococcus aureus*

. M protein of *Streptococcus pyogenes*

#### 6. Intracellular pathogenicity

E.g. *Mycobacterium tuberculosis*

*Brucella* spp.

### Defense mechanisms of the host

#### 1. Non-specific defense mechanisms

##### a. Skin

. Many layered impermeable barrier to invasion of the tissues by microorganisms from the environment.

.Infection is frequent when this barrier is breached.

E.g. Wounds, Burns

- b. Mucus membrane: A single layer of epithelium and less protective than skin.
- c. Lysozyme: An enzyme which lyses the mucopeptide (peptidoglycan) of the Gram-positive bacteria.
- d. Normal flora: Prevents establishment of pathogenic bacteria.  
Flushing of tears, urine and respiratory secretion
- e. Tears: Keep the eye surface sterile due to lysozyme and flushing.
- f. Respiratory secretion: Traps bacteria and constantly moves them upward propelled by cilia on the cells of the epithelium.
- g. Urine: Voiding helps to flush out bacteria that have gained entry to the bladder.
- h.  $p^H$  of body tract  
Low  $p^H$  in stomach due to hydrochloric acid secretion kills ingested bacteria.  
Low  $p^H$  in vagina due to lactic acid conferred by lactobacilli spp. prevents entry of pathogenic bacteria.
- i. Phagocytosis: The process by which microorganisms are ingested and destrroyed by phagocytic cells.

There are two types of phagocytic cells.

1. Neutrophil polymorphonuclear leukocytes (The polymorphs)
  - . Produced and mature I bone marrow.
  - . Short lived cells; circulate in the blood stream for six hours.
  - . Act as an early defense against infection and are the “pus cells” seen in the exudate from acute infection.

- . Perform only one phagocytic event.

## 2. Macrophages

- . Produced in the bone marrow and found in blood stream as monocyte and in tissue as fixed macrophage.
- . Long-lived cells
- . Can perform many phagocytic events.

### **Major events in phagocytosis**

1. Chemotaxis: Attraction of the phagocytic cell to the site of the organism.
  2. Attachment: Adherence of the organism to the membrane of the phagocytic cell.
  3. Ingestion of the micro-organism by pseudopods of the phagocytic cells.
  4. Formation of phagosome and phagolysosome.  
Phagosome: The engulfed bacterium by a phagocyte.  
Phagolysosome: Fusion of phagosome and lysozyme (bag of hydrolytic and proteolytic enzymes found in phagocytic cells).
  5. Intracellular killing of microorganism.
  6. Exocytosis (removal) of degraded and killed bacteria.
- j. Complement system: Cascade of reactions mediated by complement components.

Complement components are a family of proteins present in serum.

### **Major functions of complement system**

1. Liberation of complement fragments that attract phagocytic cells.
2. Promotes and enhances phagocytosis.
3. Induces inflammatory reaction

k. Others

1. Nutrition: Malnutrition predisposes to infection.
2. Age: The very old and the very young are particularly liable to infection.
3. Sex: May be attributes to hormonal influence.
4. Impairment of the host immune response
  - . radiotherapy
  - . Immunosuppressive drugs including steroids
  - . Malignancy
  - . HIV
5. Race
6. Climate
7. Occupation

## 2. Specific defense mechanisms

There are two main mechanisms by which the host mounts a specific immune response against bacterial infection. These are:

1. The humoral(antibody) response
2. The cell mediated response

### The humoral response

Antibodies are proteins produced by B-lymphocytes in response to antigens (foreign substance which induces and binds with antibody).

### **Functions of antibodies**

1. Neutralization of toxin
2. Promotion of phagocytosis
3. Bacterial Lysis

### **The cell mediated response**

It is important in killing of intracellular pathogenic bacteria.

T-lymphocytes are population of lymphocytes conferring cell mediated immunity due to release of hormone-like mediators (lymphokines).

### **Functions of lymphokines**

1. Inhibition of macrophage migration: Localizes macrophage to the site of infection.
2. Chemotactic attraction of lymphocytes, macrophages and polymorphs to the site of infection.
3. Mitogenic activity: Stimulation of unsensitized lymphocytes to divide.

### **4.2 Normal microbial flora**

It denotes the population of micro-organisms that inhabit the skin and mucus membrane of healthy normal person.

There are two groups of normal flora. These are:

1. Resident normal flora
2. Transient normal flora

Resident normal floras are relatively fixed microorganisms regularly inhabiting the skin and mucus membrane of the normal host.

Transient normal floras are non-pathogenic or potentially pathogenic microorganisms that inhabit the skin and mucus membrane for a short period of time like hours, days and weeks.

#### **Roles of (Resident) normal flora**

1. Prevent colonization by pathogenic micro-organisms and possible disease through “bacterial interference”.
  - a. Competition for nutrition with pathogenic bacteria
  - b. Competition for binding sites with pathogenic bacteria.
  - c. Mutual inhibition by their toxic metabolites.
  - d. Mutual inhibition by bacteriocins.
2. Synthesis of vitamin K in the gastrointestinal tract
3. Aid in the absorption of nutrients in the small intestine.

NB: Normal flora can cause disease when the defense mechanisms of the body is breached or when the micro-organism is placed in the abnormal body site.

#### **Normal flora of the skin**

The skin is rich in resident bacterial flora, estimated at  $10^4$  microbes per square inch.

- . Staphylococcus epidermidis
- . Propionibacterium acne
- . Peptostreptococci and peptococci
- . Diphtheroids
- . Alpha-hemolytic streptococci and non-hemolytic streptococci



### **Normal flora of the mouth and nasopharynx and upper respiratory tract**

The upper respiratory tract is heavily colonized by normal flora but the lower respiratory tract is sterile.

#### **Mouth**

- . Viridans streptococci
- . Non-pathogenic *Neisseria* spp.
- . Diphtheroids
- . Anaerobes like *Prevotella* spp., *Fusobacterium* spp. and *Campylobacter* spp.
- . Commensal *Neisseria*
- . Spirochetes
- . Actinomyces

#### **Nasopharynx and upper respiratory tract**

- . *Staphylococcus epidermidis*
- . Diphtheroids
- . Alph-hemolytic streptococci
- . Commensal *Neisseria*
- . Pneumococci
- . *Haemophilus* spp.

#### **Normal flora of the gastrointestinal tract**

The normal flora of the stomach, duodenum, jejunum and upper ileum is scanty but the large intestine is very heavily colonized with bacteria.

. Coliforms except salmonella spp., shigella spp., vibrio spp., yersinia spp. and campylobacter spp.

. Enterococci

. Anaerobes like bacteroides, bifidobacteria, anaerobic lactobacilli, clostridia and peptostreptococci

Feces contain enormous number of bacteria, which constitute upto one third of the fecal weight.

### **Normal flora of the genitourinary tract**

For anatomical reasons the female genital tract is much more heavily colonized than that of the male.

a. Female

. Vulva

. Staphylococcus epidermidis

. Diphtheroids

. Coliforms

. yeasts

. Vagina

. lactobacilli

. Bacteroids

. Diphtheroids

. Group B beta-hemolytic streptococci

. Mycoplasma spp.

. Yeasts

b. Male and female distal urethra

. Staphylococcus epidermidis

. Diphtheroids

- . Alpha-hemolytic and non-hemolytic streptococci
- . Coliforms

#### **Normal flora of the eye**

- . Diphtheroids (*Corynebacterium xerosis*)
- . *Staphylococcus epidermidis*
- . Commensal *Neisseria*
- . Non-hemolytic streptococci

#### **Normal flora of the external auditory meatus**

It is an extension of skin normal flora and often profusely colonized.

- . *Staphylococcus epidermidis*
- . Diphtheroids
- . Alpha-hemolytic and non-hemolytic streptococci

### **4.3. INFECTION OF SKIN AND WOUND**

#### **A. Infection of skin**

Defense mechanisms of skin

- . The layers of skin
- . Enzymes in the skin
- . Fatty acids in the skin

#### **1. Superficial skin infection**

- a. Folliculitis
  - . Infection of one hair follicle by *S. aureus*
  - . Common in children.

- b. Furuncle (Boil)
  - . Infection of many hair follicle by S.aureus
  - . It can be single or multiple.
  - . It may extend to cause cellulitis.
- c. Carbuncle
  - . Infection of skin with hair follicle by S.aureus
  - . It may extend to cause cellulitis.

## 2. Deeper skin infection

- a. Impetigo
  - . Vesicular skin lesion with honey-colored crusts which is caused by S.aureus and S. pyogenes.
  - . It is common in young children and spread by contact.
- b. Erysipelas
  - . Rapidly spreading cellulitis on the face and limbs.
  - . It is caused by S. pyogenes.
- c. Cellulitis
  - . Acute inflammation of subcutaneous tissue.
  - . Furuncle and carbuncle may complicate to cause cellulitis.
  - . It is caused by S. aureus, S. pyogenes, Gram-negative rods, and anaerobes.
- d. Skin ulcers
  - . A break in the continuity of skin is named as skin ulcer.
  - . It is caused by S. aureus, S.pyogenes, B.anthraxis, M.ulcerans and C. diphtheriae.

## B. Infection of wound

- a. Soil contaminated wound
  - . It occurs after car accident and war.
  - . It is caused by gram-negative rods.
- b. Gas gangrene
  - . Extensive tissue destruction with necrosis of muscle, foul smelling discharge and gas under the skin.
  - . It is mainly caused by *C. perfringens*.
- c. Burns
  - . Infection of burn is by *P. aeruginosa*, *S. aureus*, *S. pyogenes* and gram-negative rods.
- d. Surgical wounds
  - . Types
    - . Clean wound
    - . Clean contaminated wound
    - . Contaminated wound
    - . Dirty wound

Laboratory diagnosis:

Specimen: Swab from lesion, ulcer and discharge.

Smear: Gram staining from primary specimen or culture.

Culture: Blood agar medium and Mac Conkey agar medium

Biochemical and sensitivity testing for microbe identification.

Treatment: Based on sensitivity testing.

#### 4.4. Infection of Respiratory Tract

Respiratory defense mechanisms

1. Mucociliary activity
2. Cough reflex
3. Secretory Ig A
4. Alveolar macrophages
5. Normal microbial flora

#### Infection of middle ear and sinuses

##### 1. Acute infection

- a. acute otitis media
- b. acute sinusitis

Acute infections of middle ear and sinuses are often due to secondary bacterial invasion following a viral infection of respiratory tract.

##### a. Acute otitis media

Causative agent: H.influenzae

S.pneumoniae

M. catarrhalis

Source: Endogenous; normal flora of the oropharynx

Clinical features: fever, headache, earache, ear discharge red tympanic membrane, pus discharging ear

Lab. diagnosis:

Specimen: Ear discharge (pus)

Procedures: Gram staining, culture, biochemical testing, serological testing, sensitivity testing

Treatment: Amoxicillin/ampicillin  
Co-trimoxazole

**b. Acute sinusitis**

Causative agent: H.influenzae

S.pneumoniae

S.pyogenes

Source: Endogenous: normal flora of the nasopharynx

Clinical features: Discomfort over the frontal or maxillary sinuses

Pain and tenderness of sinuses with  
purulent nasal discharge.

Lab. Diagnosis:

Specimen: Lavage/drainage of sinuses

Procedure: Gram staining, culture, biochemical testing,  
serological testing and sensitivity testing

Treatment: Amoxicillin/ampicillin

Co-trimoxazole

**2. Chronic infection**

**a. Chronic suppurative otitis media**

Long standing ear disease characterized by periods of exacerbation with profuse ear discharge and pain; and remission with relatively dry ear.

Risk factors: History of acute or chronic otitis media

Parental history of otitis media

Crowding

Causative agent: P. aeruginosa

S. epidermidis

Viridans streptococci

*S. pneumoniae*

Laboratory diagnosis:

Specimen: Swabs of pus from the ear

Procedure: Gram staining, culture, biochemical and serological test for microbe identification.

Treatment: Little role of oral antibiotic agents in the treatment of chronic suppurative otitis media.

Local and systemic antibiotics are given to treat the exacerbated symptoms.

Regular ear wicking is important for chronic suppurative otitis media.

**b. Chronic sinusitis**

Painful sinuses and head ache are prominent symptoms; often associated with mucoid or purulent nasal discharge and nasal obstruction.

Causal organisms are same as those implicated in acute sinusitis.

Laboratory diagnosis:

Specimen: Saline washings from the affected sinus

Procedure: Gram staining, culture, biochemical and serological test for microbe identification.

Treatment: Antibiotics often give disappointing results. If antibiotic is given, it should be guided by sensitivity pattern or "best-guess" basis.



## **Bronchitis**

### **1. Acute bronchitis**

It is an acute inflammation of the tracheobronchial tree generally self-limited and with eventual complete healing and return of function.

Etiology: Viruses: The commonest causative agents

Bacteria: M. pneumoniae

A. pneumoniae

B. pertussis

Predisposing factors: Chronic bronchopulmonary diseases

. Environmental irritants like indoor air pollution and tobacco smoking

Clinical features: Symptoms of upper respiratory infection proceed acute infectious bronchitis.

Initially dry cough followed by productive cough with mucoid or mucopurulent expectoration, low grade fever and substernal chest pain. Frank purulent sputum suggests super imposed bacterial infection..

Laboratory diagnosis:

Specimen: Sputum

Procedure: Gram staining, culture, biochemical and serological test for microbe identification.

Treatment: Antibiotics are indicated when:

.There is concomitant chronic obstructive pulmonary diseases.

- . Purulent sputum is present
- . High grade fever persists and the patient is more than mildly ill.

Drug of choice: Tetracycline

Cotrimoxazole

## 2. Chronic bronchitis

It is defined as chronic productive cough for at least three months in each of two successive years.

Causative factors: Cigarette smoking

Air pollution

Exposure to noxious stimuli

Clinical features:

Chronic productive cough with mucoid expectoration, low grade fever, weakness, and occasional chest pain.

It is characterized by remission and exacerbation of symptoms; the commonly exacerbating condition is superimposed bacterial infection.

Bacteria that exacerbate chronic bronchitis are:

Streptococcus pneumoniae

Haemophilus influenzae

Mycoplasma pneumoniae

Branhamella catarrhalis

Laboratory diagnosis:

Specimen: Sputum

Procedure: Gram staining, culture, biochemical and serological test for microbe identification.

Treatment:

- . To stop cigarette smoking
- . Avoid exposure to noxious stimuli
- . Treat the exacerbation with antibiotics like amoxicillin, cotrimoxazole.

### **Pneumonia**

It is infection of the lung parenchyma.

Causative agents: S. pneumoniae  
S. aureus  
H. influenzae  
M. pneumoniae  
Viruses

Route of entry of microbes to the lung

- . Aspiration of oral and gastric secretion
- . Haematogenous spread from distant foci
- . Direct inoculation and local spread from surrounding tissue
- . Inhalation

NB: Aspiration is the major route of infection.

Clinical features: Sudden onset of fever, chills, sweating, and productive cough of purulent or blood streaking sputum and pleuritic chest pain.

Complications:

- . Pleural effusion
- . Lung abscess
- . Septicemia

Laboratory diagnosis:

Specimen: Lower respiratory secretion

NB: Lower respiratory secretion is indicated by greater than 25 neutrophils and less than 10 squamous epithelial cells per high power field.

Procedure: Gram staining, culture, biochemical and serological test for microbe identification.

Treatment: Based on sensitivity pattern of offending micro-organism.

#### **4.5. Infection of gastrointestinal tract**

Host defense mechanisms to prevent GIT infection

- . Gastric acidity
- . Mucosal epithelium
- . Peristalsis
- . Normal flora
- . Luminal phagocytic cells
- . Copro antibodies

Microbial factors contributing to GIT infection

- . Invasiveness
- . Toxin production
- . Colonization factors

#### **DIARRHEA**

Definition: Diarrhea is a passage of three or more watery or loose stool in a day.

It is a common cause of death in under five children in developing countries.

Aetiology:

- a. Microbial causative agents
  - . Viral
    - . Rota virus

- . Norwalk virus
- . Adeno virus
- . HIV
- . Bacteria
  - . Salmonella spp.
  - . Shigella spp.
  - . E.coli
  - . Campylobacter spp.
- . Protozoa
  - . Giardia lamblia
  - . Entamoeba histolytica
  - . Cryptosporidium parvum
  - . Isospora belli
- . Fungus
  - . Candida albicans
- b. Non-microbial causative agents
  - . Congenital
    - . Congenital megacolon
  - . Enzyme deficiency
    - . Lactase deficiency
  - . Iatrogenic
    - . Antibiotic related enterocolitis
  - . Endocrine
    - . Diabetes mellitus, Hyperthyroidism
  - . Psychogenic
    - . Stress related gastroenteritis

### **Types of diarrhea**

1. Based on duration of diarrhea
  - . Acute diarrhea: Diarrhea occurring in less one week time.
  - . Persistent diarrhea: Diarrhea occurring in two weeks time.
  - . Chronic diarrhea: Diarrhea occurring in more than two weeks time.
2. Based on nature of diarrhea
  - . Secretary diarrhea: Watery diarrhea
  - . Invasive diarrhea: Bloody mucoid diarrhea
  - . Osmotic diarrhea Eg. Non-absorbed purgative intake (Magnesium sulfate)
  - . Motility diarrhea Eg. Hyperthyroidism

NB: Only secretary and invasive diarrhea are caused by microbes.

### **Examples of secretary diarrhea**

1. *V.cholerae* and *E.T.E.Coli* cause diarrhea by production of an enterotoxin which activates the adenylyclase of enterocytes to increase the cyclic AMP (CAMP) level, leading into increased secretion and decreased absorption of fluids and electrolytes in the intestinal wall.
2. *Giardia lamblia* causes diarrhea by physical coverage of the normal absorptive surface.
3. Viruses cause diarrhea by disrupting the normal absorptive surface.

### **Examples of invasive diarrhea**

*Shigella* spp.                      *Campylobacter* spp.  
*Salmonella* spp. *E.hystolytica*

E.I.E.coli

All of them cause extensive intestinal mucosal damage by invasion of the intestinal wall

Leading into bloody mucoid diarrhea.

### **Complication of diarrhea**

1. Local
  - . Intestinal wall perforation
  - . Intestinal vessel erosion leading to bleeding
2. Systemic (Generalized)
  - . Dehydration leading to hypovolemic shock
  - . Septicaemia leading to septic shock
- . Malnutrition due to loss of essential nutrients and electrolytes.

Laboratory diagnosis:

Specimen: Stool, rectal swab

Wet mount preparation: Pus cells, red blood cells, parasites and ova.

Culture: Mac Conkey agar, SS agar, TCBS agar, Campylo agar

Observe for colony appearance, pigmentation, hemolysis.

Biochemical reaction and serology: For species identification.

Treatment:

- . Fluid and electrolyte replacement
- . Provision of antibiotics
- . Continue feeding

**FOOD POISONING**

It is an out break related to common meal.

Characteristics:

- . Sharing same meal.
- . Multiple cases
- . Same clinical features
- . Developing same clinical features more or less at the same time.

There are two types of food poisoning

1. Food intoxication: Illness is caused by ingestion of food with preformed toxin.
2. Food infection: Illness is caused by ingestion of food with micro-organism.

Incubation period and severity of disease in food infection is determined by inoculum of micro-organism ingested.

**Table 4.2. Examples of food intoxication**

Organism	Incubation period	Clinical findings	Related food item
Bacillus cereus	1-6 hours	Vomiting, cramp	Rice, Pasta dishes
Staphylococcus aureus	2-4 hours	Vomiting	Meat, Salads
Clostridium botulinum	12-72 hours	Flaccid paralysis	Meat, Vegetables

**Table 4.3. Examples of food infection**

Organism	Incubation period	Clinical findings	Related food item
Bacillus cereus	6-24 hours	Watery diarrhea	Meat, Vegetables
Salmonella spp.	6-12 hours	Dysentery	Meat, Vegetables
Shigella spp.	12-48 hours	Dysentery	Variable



Laboratory diagnosis:

Specimen: Left over food, vomits, stool

Culture the specimen for microbial isolation.

Serological technique for toxin isolation.

Treatment: Depends on the causative agent.

#### **4.6. INFECTION OF URINARY TRACT**

Definition: The presence of significant numbers of micro-organisms any where in the urinary tract.

NB: Kidney and bladder are sterile at normal state.

Host defense mechanisms

- . Micturition (Urine flow)
- . Surface bladder mucosa
- . Normal microbial flora
- . Secretary IgA
- . Menstrual flow only in females.

Aetiology:

The commonest causative agents of UTI are gram-negative rods.

These are:

- Escherichia coli
- Pseudomonas aeruginosa
- Klebsiella pneumoniae
- Proteus spp.
- Enterobacter aerogens

Other important causative agents: Enterococci

Staphylococcus saprophyticus

### **Routes of infection**

1. Ascending route (passage of bacteria from urethra to bladder and kidney.)
2. Haematogenous route ( source of infection is blood)

NB: Ascending route is the commonest route infection of the urinary tract.

### **Contributing factors for urinary tract infection**

1. Age: Very young and very old individuals are more at risk for UTI.
2. sex: UTI is more common in females than males because females have short and wide urethra.
3. Instrumentation: Indwelling catheters and cystoscopic procedures
3. Neurogenic bladder dysfunction: Diabetes mellitus, Spinal injury
4. Obstruction: Congenital anomalies in youngs and prostatic adenoma, stricture and calculi in olds.
5. Underlying diseases: Diabetes mellitus, sickle cell disease
6. Vesico-ureteral reflex: Associated with recurrent acute pyelonephritis.

Clinical features:

1. Lower urinary tract infection: Infection of urethra and bladder which manifests with frequency of micturition, pain during micturition, blood-stained or cloudy urine, supra pubic tenderness.

Usually no fever.

2. Upper urinary tract infection: Infection of the kidney parenchyma and pylus which manifests with the lower UTI symptoms and signs, flank pain, fever and chills, nausea and vomiting, and flank tenderness.

Laboratory diagnosis

Specimen: Clean caught midstream urine

Catheterized urine

Suprapubic aspiration

Direct microscopic examination: WBCs, RBCs, Epithelial cells.

The presence of more than five WBCs and abundant epithelial cells per HPF supports infection of urinary tract.

Gram stain: The presence of one bacterium in Uncentrifuged gram stained urine confirms Urinary tract infection.

Culture: Blood agar medium, Mac Conkey agar medium

#### **Interpretation of culture results**

1.  $\geq 10^5$ cfu/ml of urine is significant to indicate UTI.
2.  $< 10^3$ cfu/ml of urine indicates contamination of specimen.
3.  $10^3$ - $10^5$ cfu/ml of urine is uncertain.

NB:  $10^3$ - $10^5$ cfu/ml of urine in symptomatic patient or suprapubic or catheterized specimen indicates UTI.

#### 4.7. INFECTION OF GENITAL TRACT

**Table 4.4. Genital tract infection manifests as either genital discharge or genital ulceration with or without inguinal lymphadenitis.**

<b>Causative agents</b>	<b>Diseases</b>
<b>1. Bacterial</b>	
Neisseria gonorrhoea	Gonorrhoea
Chlamydia trachomatis	Urethritis, cervicitis, LGV
Ureaplasma urealyticum	Urethritis
Gardnerella vaginalis	Vaginitis
Treponema pallidum	Syphilis
Haemophilus ducreyii	Chancroid
<b>2. Viral</b>	
Herpes simplex virus-2	Herpes genitalis
HIV	AIDS
Human papilloma virus	Genital warts, Cervical dysplasia
Molluscum contagiosum virus	Genital Molluscum contagiosum
<b>3. Protozoal</b>	
Trichomonas vaginalis	Vaginitis, Urethritis
<b>4. Fungal</b>	
Candida albicans	Vulvovaginitis, Balanitis
<b>5. Ectoparasites</b>	
Phthirus pubis	Pubic hair louse infestation
Sarcoptes scabiei	Scabies

## Urethral and vaginal discharge

### 1. Urethritis

It manifests with urethral discharge, pain during urination and frequency of urination.

#### Types

#### a. Gonococcal urethritis

Causative agent: *N. gonorrhoea*

Incubation period is 2-7 days.

It accounts for 1/3 of urethritis cases.

Clinical findings: Yellowish purulent discharge and dysuria.

#### b. Non-gonococcal urethritis

Causative agents: *C. trachomatis* ----50%

*U. urealyticum* ----30%

*M. hominis*

*T. vaginalis*

Incubation period is 2-3 weeks.

Clinical findings: White mucoid discharge

Laboratory diagnosis:

Specimen: Urethral discharge or swab (Before urination or antibiotics)

Wet mount: *T. vaginalis*

Gram stain: Gram-negative intracellular diplococci

Culture: Modified thayer-martin medium

Biochemical and serology: Species identification

### 2. Cervicitis / Vaginitis

It manifests with vaginal discharge.

Causative agents:

N. gonorrhoea --- Mucopurulent vaginal discharge.

T. vaginalis---Profuse foaming purulent, sometimes greenish vaginal discharge.

Non-specific vaginitis --- Yellowish homogenous vaginal discharge.

It is caused by anaerobes and G. vaginalis

C. albicans --- whitish curd-like vaginal discharge with itching and erythema of vulva.

Laboratory diagnosis:

Specimen: Vaginal discharge

Wet mount: . Clue cells i.e., distorted vaginal epithelial cells coated heavily with gram-negative coccobacilli which are diagnostic of infection with G. vaginalis.

. Yeast cells or pseudohyphae which indicates infection with C. albicans.

. Motile T. vaginalis

Gram stain, culture, biochemical and serology for species identification.

Treatment:

N. gonorrhoea ----- Ceftriaxone, Ciprofloxacin

B. trachomatis, U. urelyticum and M. hominis ---- Tetracycline

T. vaginalis, G. vaginalis and anaerobes ---- Metronidazole

C. albicans ----- Nystatin, Myconazole, Clotrimazole

**Table 4.5. Genital ulceration with or with out regional lymphadenopathy**

<b>Disease</b>	<b>Lesion</b>	<b>Inguinal lymphadenopathy</b>
Syphilis	Non tender, indurated clean based genital ulcer	Non tender, non-suppurating rubbery bilateral lymphadenitis
Chancroid	Tender, non-indurated shallow ragged ulcer	Suppurative, tender ymphadenitis
LGV	Occasionally small non tender genital papules is seen	Painless suppurative lymphadenitis with multiple draining sinuses
Genital herpes	Tender, multiple grouped vesicular lesions coalesce to form an ulcer	Non-suppurative, tender, bilateral lymphadenitis

**Laboratory diagnosis**

Specimen: Scrapings from base of lesion

Serous exudate squeezed from lesion

Pus from lymph node

Direct microscopic examination: Motile spirochetes under dark-field microscopy.

Giemsa's stain: Intranuclear inclusions for genital herpes.

Gram stain: Short gram-negative rods for H. ducreyii.

Culture: Chocolate agar medium for H. ducreyii

Serology: VDRL, FTA-ABs test

**Treatment:**

Syphilis ----- Penicillin

Chancroid ----- Erythromycin, Cotrimoxazole

Lymphogranuloma venereum ----- Tetracycline

Genital herpes ----- acyclovir

#### 4.8. INFECTION OF BLOOD

Bacteremia is defined as mere presence of bacteria in blood. It occurs in normal person after invasive medical procedures. The organism which causes bacteremia are less virulent and usually cleared from blood with in 30 min.

Complications of septicaemia

- . Infective endocarditis
- . Endotoxic shock
- . Acute pyogenic meningitis
- . Septic arthritis

##### **Infective endocarditis**

##### 1. Acute endocarditis

. It occurs in normal and abnormal heart valves and tissues with fulminant course resulting in death in six weeks time if not treated.

Etiologic agent: S. aureus

S. pyogenes

S. pneumoniae

N. gonorrhoea

##### 2. Sub acute endocarditis

. It occurs in acquired or congenitally damaged heart valves with less fulminant course resulting in death after six weeks if not treated.

.Etiologic agent: Viridans streptococci

Enterococci



S.epidermidis

S.aureus

S.epidermidis

Laboratory diagnosis:

Specimen: Blood

. Amount needed is 2ml for a child and 10 ml for an adult to give 1:10 dilution of the specimen.

Culture: Blood culture

. Blood culture bottle should have 18 ml and 90 ml of broth for a child and an adult respectively.

NB: Numbers of culture required are 3-6 with in 24 hrs.

Time of incubation of blood culture is 7 days and subculture is done in first, third and seventh day of incubation.

If the appearance of blood culture is changed to cloudy, it indicates bacterial growth.

#### **Interpretation of results**

- Positive bacterial growth in three of blood culture broth -----  
Definitive diagnosis
- Positive bacterial growth in two of blood culture broth-----  
Probable diagnosis
- Positive bacterial growth in one of blood culture broth-----  
Contamination

#### **Common contaminants of blood culture**

S. epidermidis

Corynebacterium spp.

Bacillus spp.

Treatment: Antibiotic should be started after collection of the specimen.

Medication should be given intravenously based on 'best guess basis'.

#### **4.9. INFECTION OF CENTRAL NERVOUS SYSTEM**

Defense mechanisms of CNS

- . Cranium (Bony covering of the brain)
- . Blood brain barrier

Route of infection

- . Haematogenous
- . Extension from middle ear, mastoids and sinuses
- . Congenital defects
  - . Meningomyelocele
  - . Spina bifida
- . Trauma
  - . Skull fracture
- . Intraneural pathway

#### **Common infection of central nervous system**

##### **1. Meningitis**

Definition: Meningitis is an inflammation of leptomeninges.

Types:

- . Bacterial (Pyogenic) Meningitis
- . Viral meningitis
- . Fungal meningitis

Bacterial meningitis

a. Acute form

Causative agents

- . In new born ----- Escherichia coli  
Streptococcus agalactiae
- . In children ----- Haemophilus influenzae  
Streptococcus pneumoniae  
Neisseria meningitidis
- . In adults ----- Streptococcus pneumoniae  
Neisseria meningitidis  
Haemophilus influenzae

Clinical features:

- . Sudden onset of head ache, fever, malaise, vomiting associated with neck and back stiffness, behavioral changes like irritability and drowsiness, convulsions and coma.

b. Chronic form

- . Mycobacterium tuberculosis
- . Partially treated acute pyogenic meningitis

It manifests with unexplained head ache of weeks to months duration associated with fever, weakness, neck and back stiffness, and behavioral changes.

#### Viral meningitis

The clinical features of viral meningitis are milder than bacterial meningitis.

Viruses causing viral meningitis are mumps virus, measles virus, coxsackie A and B virus, Enteroviruses and echo virus.

#### Fungal meningitis

Eg. Cryptococcal meningitis

The clinical features of fungal meningitis is similar to that of chronic bacterial meningitis.

#### **Complication of meningitis**

- . Cranial nerve damage
- . Convulsions
- . Brain abscess
- . Obstructive hydrocephalus due to blockage of CSF drainage system.
- . Subdural effusion of sterile or infected fluid.

Laboratory diagnosis:

Specimen: Cerebrospinal fluid by lumbar puncture

NB: Amount required for a child is 1-2 ml and for an adult is 2-4 ml.

The specimen should be processed within one hour of collection.

Smears: Gram staining and Ziehl-Neelson staining of centrifuged specimen

Culture: Blood agar medium

Mac Conkey agar medium

Lowenstein-jensen medium

Sabouraud's agar medium

Biochemical, serology and sensitivity testing for species differentiation.

Normal CSF findings:

- . Color ----- Crystal clear, colorless
- . Cells -----0-5 per ml, all lymphocytes
- . Microbiology----- Sterile
- . Protein----- 15-40 mg per 100 ml.
- . Glucose ----- 45-72 mg per 100 ml.

**Table 4.6. Cerebrospinal fluid findings in meningitis**

Meningitis type	Appearance	Cells per ml	Protein	Glucose
Acute pyogenic meningitis	Turbid	500-20,000	Markedly raised	Reduced or absent
Viral meningitis	Clear or Turbid	10-500 Predominantly Lymphocytes	Normal or slightly raised	Normal
Tuberculous Meningitis	Clear or Turbid	10-500 Predominantly Lymphocytes	Moderately raised	Usually reduced
Cryptococcal Meningitis	Clear or Turbid	10-500 Predominantly Lymphocytes	Raised	Reduced

Treatment: Antibiotic should be started based on 'best guess basis' after the CSF specimen was taken.

All medications for treating meningitis is given intravenously.

Encephalitis: It is an inflammation of brain substance.

It is commonly caused by viruses.

Brain abscess: It is an abscess with in the brain parenchyma.

It is a complication of meningitis, endocarditis and head injury.

#### **4.10. INFECTION OF BONE AND JOINTS**

The two main diseases are:

- . Osteomyelitis: infection of bone.
- . Septic arthritis: Infection of joints

Osteomyelitis

- . Acute osteomyelitis
- . Chronic osteomyelitis

##### **Acute osteomyelitis**

Infection of bone which occurs commonly under ten years old.

Distal femur, proximal tibia and proximal humerus are the classical sites.

Causative agents: *S. aureus* (80% of cases)

*H. influenzae* (especially in young children)

*S. pneumoniae*

*S. pyogenes*

Coliforms and Group B streptococci in new born.

Clinical features: Fever, bone pain, local tenderness and swelling, limitation of movement

Laboratory diagnosis:

Specimen: Blood culture, Pus from bone by needle aspiration

Gram reaction, culture, biochemical tests and serology for microbe identification.

Treatment: Antibiotics alone are usually effective if started early and continued for several weeks.

Surgery is needed if there is pus accumulation and bone destruction.

### **Chronic osteomyelitis**

It manifests with bone pain, bone destruction with formation of sequestra and discharging sinuses.

The most common causal organism is *S. aureus*; others include *M. tuberculosis*,

*S. typhi* and *Brucella* species.

Laboratory diagnosis: Same as acute osteomyelitis

Treatment:

- . Antibiotics for several weeks.
- . Surgery is usually necessary for pus drainage and sequestra removal.

### **Septic arthritis**

It is usually seen as a complication of septicemia or an extension of osteomyelitis.

Causative agents:     *S. aureus*  
                              *S. pneumoniae*  
                              *H. influenzae*

Neisseria gonorrhoea

Neisseria meningitidis

M. tuberculosis

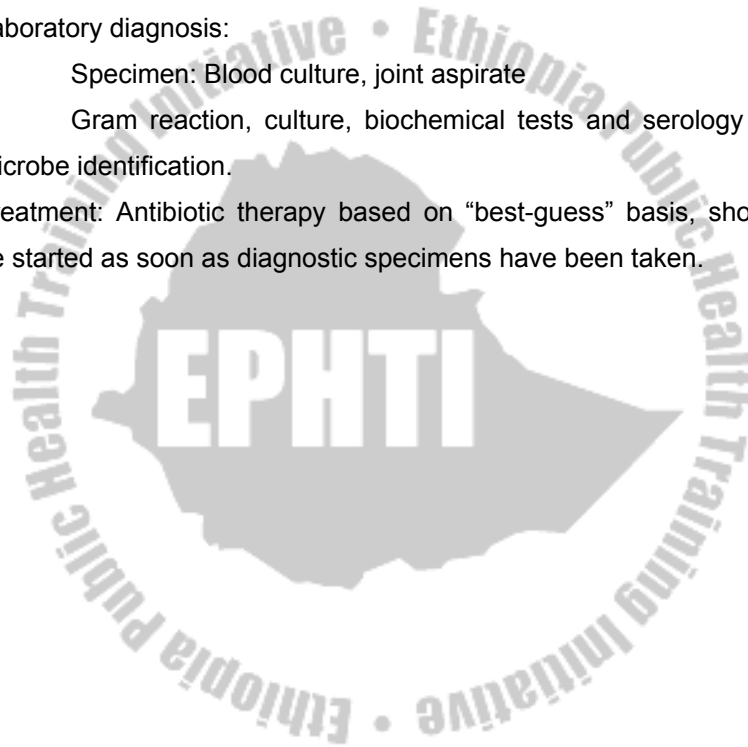
Clinical features: The onset is sudden with fever, swelling and redness over the joint and severe pain which limits movement of the affected joint.

Laboratory diagnosis:

Specimen: Blood culture, joint aspirate

Gram reaction, culture, biochemical tests and serology for microbe identification.

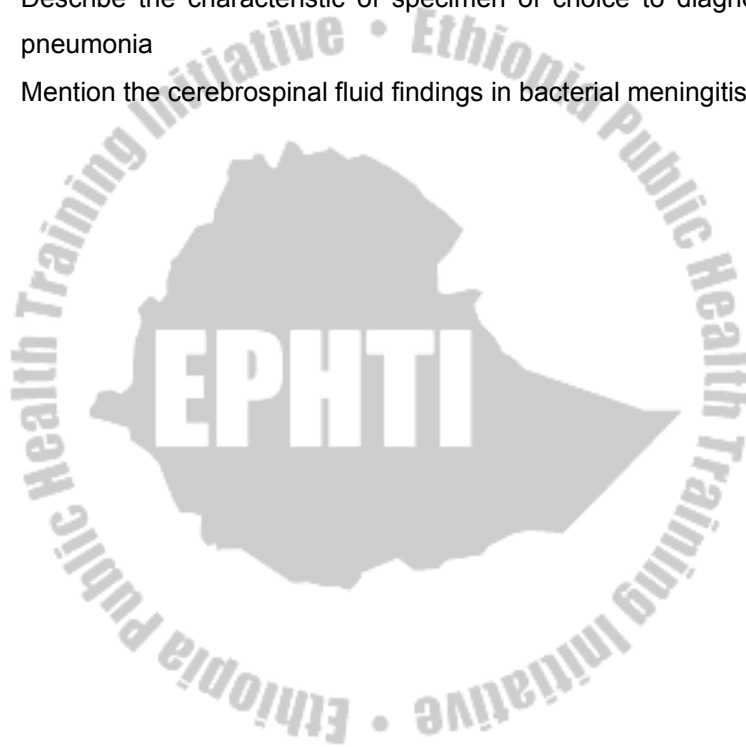
Treatment: Antibiotic therapy based on "best-guess" basis, should be started as soon as diagnostic specimens have been taken.





## Review Questions

- Discuss defense mechanisms of different body tracts
- Describe the interpretation of urine culture result
- Discuss the characteristics and types of food poisoning
- Describe the characteristic of specimen of choice to diagnose pneumonia
- Mention the cerebrospinal fluid findings in bacterial meningitis



## CHAPTER FIVE

### Learning objective:

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

- . Perform bacteriological analysis of water sample

### Bacteriology of water

Good quality of water is odorless, colorless, tasteless and free from fecal pollution and harmful chemicals.

Human illness is caused by water supplies becoming contaminated from feces being passed or washed into rivers, streams, or being allowed to seep into wells.

Feces contain microorganisms like *Escherichia coli*, *Streptococcus faecalis* and *Clostridium perfringens*, which contaminate safe water.

Determining whether a water supply is fecally polluted is to test for the presence of normal fecal organism.

Testing for normal fecal organisms as indicators of fecal pollution is a reliable way of determining whether water is bacteriologically safe to drink.

A single laboratory examination of any water does not justify the conclusion that the supply is safe for drinking so bacteriologic analysis of water should be performed at regular intervals.

Organisms used as indicators of fecal pollution are the coliform group particularly *E. coli*.

*E. coli* is a coliform organism, which is capable of fermenting lactose with the production of acid and gas at 37 °C and 44 °C in less than

48 hours, produces indole in tryptophan-containing peptone water and gives positive result in methyl red test.

When organisms of coliform group other than E.coli are found in a water sample,

Presence of S.faecalis confirms fecal pollution of water.

The presence of C. perfringenes in the absence of coliform group in water suggests fecal pollution of water at some remote date since clostridial spores are capable of surviving in water for a long duration.

### **Collection of a water sample**

Technique:

- . Sample of water must be collected in sterile bottles.
- . Hold the base of sterile bottle in one hand, remove the stopper and cover together with the other hand.
- . Fill the bottle with sample water, replace the stopper and cover together.

### **Collecting a sample from tap:**

- . Remove any external fittings from the tap.
- . Clean carefully the outside nozzle of the tap.
- . Turn on the tap and allow the water to run for 1 min.
- . Sterilize the tap using the flame by igniting a piece of cotton wool soaked in alcohol holding with a pair of tongs.
- . Allow the tap to cool by running the water for few seconds.
- . Fill the sample bottle from a gentle flow of water, and replace the bottle cap.

**Collecting a sample from an open well:**

- . Tie a sterile sample bottle on to a weighted length of rope; attach ½ Kg weighing stone as a weight below the bottle.
- . Remove the cap from the bottle septically and lower the bottle into the well to a depth of one meter.
- . Raise the bottle out of the well when no more bubbles raise to the surface.
- . Replace the bottle cap and label the bottle.

**Transport of water sample**

Water sample should be placed in an insulated cold box immediately after collection, and should be processed within six hours of collection.

**Frequency of sampling**

<b>Population served</b>	<b>Sampling interval</b>
< 20,000	Four weeks
20,000-50,000	Two weeks
50,000-100,000	Four days

**Multiple tube technique for counting fecal coliforms**

A 100 ml water sample is distributed (five 10 ml amounts and one 50 ml amount) in bottles of sterile selective culture broth containing lactose and an indicator.

After incubation, count the number of bottles in which lactose fermentation with acid and gas production has occurred.

Estimate the most probable number of coliforms in the 100 ml water by referring to probability tables.

Required:

- . Bottles of sterile Mac Conkey broth (Purple)
- . Water samples

	No. of bottles	ml of broth	strength of broth
Treated water sample	1	50	double
	5	10	double
Untreated water sample	1	50	double
	5	10	double
	5	5	single

NB: Double strength broth refers to broth made up using twice the normal amount of broth powder. Single strength broth contains the normal amount of broth powder.

Bottle broth contains an inverted durham tube for gas collection.

Method:

- Label the bottles.
- Mix thoroughly the sample of water by inverting the bottle several times.
- Remove the bottle cap and cover, flame the mouth of the bottle, and inoculate the bottles of sterile broth as follows:
- Add 50 ml of water to the bottle containing 50 ml of broth for treated and untreated water samples.
- Add 10 ml of water to each of five bottles containing 10 ml of broth for treated and untreated water samples.
- Add 1 ml of water into each of five bottles containing 5ml of broth in untreated water sample.

- Mix the contents of each bottle.
- Incubate the inoculated broth in a water bath at 44°C for 24 hours with the bottles loosely capped.
- After incubation, examine and count each bottle which has produced acid and gas.
- Determine the most probable number (MPN) of fecal coliform bacteria in the 100 ml of treated and 105 ml of untreated water sample with referring to probability tables.

NB: Acid production is shown by a change in color of the MacConkey broth from purple to yellow and gas production by the collection of a bubble in the Durham tube.

### Interpretation of results

1. For treated water sample  
The E. coli count should never exceed 5 per 100ml.

<b>E. coli count</b>	<b>Comment</b>
0	Excellent
≤ 5	Acceptable
> 5	Unacceptable

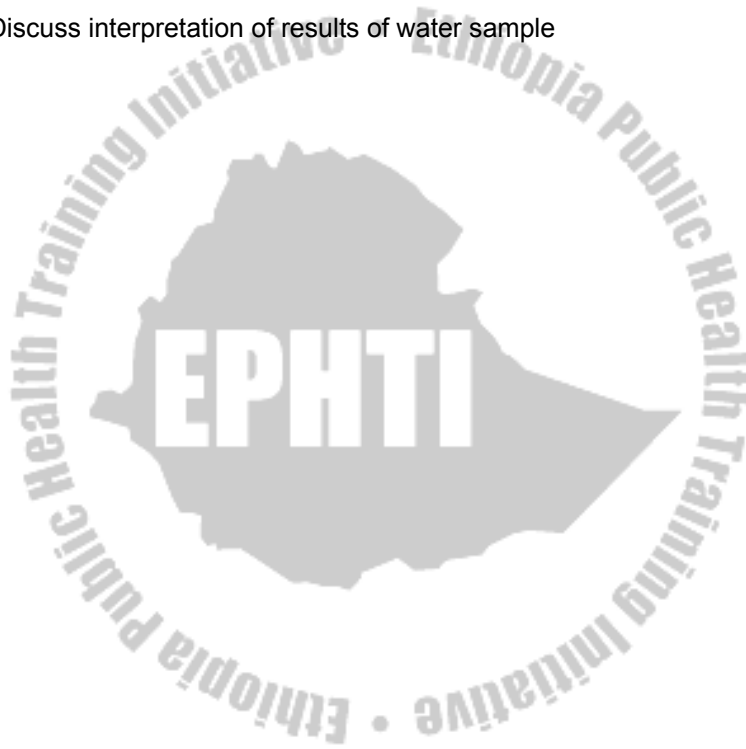
2. For untreated water sample

<b>E. coli count</b>	<b>Category</b>	<b>Comment</b>
0	A	Excellent
1-10	B	Acceptable

10-50	C	Unacceptable
>50	D	Grossly polluted

**Review question**

- . Describe method of bacteriological analysis of water sample
- . Discuss interpretation of results of water sample



## CHAPTER SIX

### Learning objective:

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

- Know the simple, common and applicable methods
- Analysis difference food samples
- Know reasons for microbials food analysis
  - to meet certain set standards
  - to estimate the shelf – life of the product
  - to determine the quality of the food
  - to determine the safety of the food for public health

### Food bacteriology

- Food are essential substance for life.
- They are also important vehicles for micro-organisms that cause food borne infections and intoxications.
- Food is essentially complex, and predicting whether, or how fast micro organisms will grow in any food is difficult. most food contains sufficient nutrients to support microbial growth.
- To provide consumers with claimable qualities of food, these substance must be controlled, regulated and inspected from the microbiological view – point. Such activities are usually carried out by FAO, WHO, UNICEF, Federal state and other agencies.



- In Ethiopia the quality and safety of food have been controlled, regulated and inspected by the National Research Institute of Health and regional laboratories.

### **Sources of food contamination**

Food may acquire their micro-organism from various sources and the following are the important sources.

#### 1. Animals

- Animals could be a source of contamination of food.
- The surface of animals, the respiratory tract, the gastrointestinal tract, hides, Loofs and waste products of animals are important sources of contamination.

#### 2. Plants

- Food may get their microbial contamination from green plants.
- The natural flora of growing plants includes pseudomonas, alcaligenes, bacissus, Micrococcus, coliform etc.

#### 3. Sewage

- Gastrointestinal pathogens, coliforms, Enterococci of untreated domestic sewage could be source of contamination of raw plant foods.
- Sewage can also contaminate natural waters and contribute micro-organisms to shellfish, fish and other sea foods.

4. Soil

- Soil is a very rich environment in microbes and is a major source of contamination of food.
- Bacillus, clostridium, enterobacter, Escherichia, Micrococcus, Alkaligens, Flavobacterium, Pseudomonas, proteus, Aerobacter, molds and yeast are kinds of organisms that contaminate food from soil.

5. Air and water:- are also important source of food contamination.-

**Factors influencing microbial activity in food**

1. Nutrient found in foods

- Organisms obtain their energy for carrying their metabolic activity mainly from the food.

2. Hydrogen ion concentration (pH)

- The optimum pH for many microorganisms is near the neutral point of pH 7. However molds and yeasts as a rule are acid tolerant. This is one of the reasons why fungi are usually associated with acid foods especially fruits.
- Many bacteria are not acid tolerant, accordingly, several acid like acetic, benzoic, propionic acids are used to preserve foods.

- It has been found that pH of 4.5 or below is lethal to salmonellae and staphylococci.
3. Oxidation reduction potential (O – R)
- Organisms can be classified into aerobic and anaerobic based on their oxygen requirements. There fore, the reducing and oxidizing power of the food influences the type of organism that growth on it.
  - Foods with high oxidation potential favours the growth of aerobes and facultative anaerobic organisms.
  - Foods with low oxidation potential favours the growth of anaerobic and facultative anaerobic organism.
4. Growth inhibitors: - These are chemicals such as sodium chloride (NaCl), Nitrate, Nitrite, Sulphur dioxide and hypochlorites that are added to foods to the growth of certain organisms.
5. Temperature
- It was found that E.coli, for example, requires the following periods for a cell to divide at different temperatures.
- |                                   |                                   |
|-----------------------------------|-----------------------------------|
| - 60minutes at 20 <sup>0</sup> C  | - 17 Minutes at 37 <sup>0</sup> C |
| - 40 minutes at 25 <sup>0</sup> C | - 19 minutes at 40 <sup>0</sup> C |
| - 29 minutes at 29 <sup>0</sup> C | - 32 minutes at 45 <sup>0</sup> C |
|                                   | - No growth at 50 <sup>0</sup> C  |

It is clear that rate of growth is optimal at 37°C, but decreases when the temperature is lowered below, or raised above 37°C.

6. Water activity ( $a_w$ )

- No microbial activity can occur unless water is available.
- Water activity is the ratio between the vapour pressure of the food and that of pure water.
- Pure water has  $a_w = 1.0$  and a relative humidity of 100%. This means that  $a_w \times 100$  indicates the equilibrium relative humidity, which the particular food would produce if enclosed in a sealed container at a constant temperature.
- Organisms have their own characteristic optimal  $a_w$  and range of  $a_w$  for their growth.

The lowest  $a_w$  values permitting growth of spoilage organisms are

- Normal bacteria	0.91	- xalophilic	0.77
- Normal yeast	0.88	- xalophilic fungi	0.65
-Normal moulds	0.80	- osmophilic yeasts	0.60

- As can be seen from the above values dry foods are most likely spoiled by moulds.
- Syrups and honey favours the growth of osmophilic yeasts.
- Moist neutral foods like milk and meats are spoiled by bacteria.

### **Microorganism of importance in food bacteriology**

Micro-organisms of interest in food bacteriology are:

- (i) Indicator organism(s):-
- An indicator organism or group of organisms is/are one whose numbers in a product reflects the success or failure of good manufacturing practices.
  - Indicator organisms are bacterial groups (or species) whose presence in foods, above a certain numerical limits, is considered to indicate exposure of food to conditions that might introduce hazardous organisms and/or allow proliferation of pathogenic or toxinogenic species.
  - They have value in assessing both the microbiological safety and quality of foods.
  - Indicator organisms include coliforms and faecal streptococci.
  - The best example of indicator organisms are the coliforms.
  - Coliform group is defined to include all aerobic and facultative anaerobic, gram negative, non-spor forming, rod shaped species which ferment lactose with the production of acid and gas within 48 hours at 35<sup>o</sup>C
  - Coliform are either part of the normal flora of intestinal tract of man and animals or found in environments such as soil and plants.
  - Coliform that are commonly found in the intestinal tracts of man and animals (faeces) are called faecal

coliform and those coliforms that are normal inhabitants of soil and plants are called non faecal coliform.

- The recovery of coliform from food or water above a certain numerical limits implies that diseases causing organism may be present and the food is potentially dangerous for human consumption.

**Differentiation of faecal from non faecal coliform:-**

- In many laboratories differentiation of faecal coliforms from non faecal coliform is considered of limited value in determining the suitability of water or food for human consumption, as contamination with either type renders water or food potentially dangerous and unsafe from a sanitary stand point. However, differentiation may be advantageous under some conditions where the identity of specific members of the group present may indicate the source of pollution.
- Coliform such as *Aerogenes* and *E. freundii* only occasionally found in the intestinal contents of man and animals (6%). They are normally found in soil. Therefore, their presence in food or water is of doubtful sanitary significance.
- On the other hand *E. coli*, rarely found in the soil but constitute the predominant organisms in the intestinal contents of man and animals. Therefore, isolation of *E. coli* in food may strongly suggest the

food has been polluted with faeces than isolation of *A.aerogenes* or *E.freundii*.

Methods used for differentiation of faecal from non faecal coliforms indole, methyl red, voges proskauer, citrate test (IMVic). IMvic test is one of the test used to differentiate faecal coliforms (*E.coli*) from non faecal coliforms (*A.aerogenes* and *E.freundii*) as shown in the table below.

Organisms	Indole	Methyl red	V – P	Sodium citrate
<i>E.coli</i> type I	+	+	-	-
<i>E.coli</i> type II	-	+	-	-
<i>E. freundii</i> I	-	+	-	+
<i>E. freundii</i> II	+	+	-	+
<i>A. aerogenes</i> I	-	-	+	+
<i>A. aerogenes</i> II	+	-	+	+

#### Elevated temperature test

- In elevated temperature tests faecal coliforms are defined to be bacterial species that grow and ferment lactose with the production of acid and gas at temperatures higher than the normal (44-44.5°C).
- *E.coli* type I and II grow and ferment lactose at elevated temperature while *A. aerogenes* and

*E. freundii* fail to grow and ferment lactose at elevated temperature.

ii) **Index organisms:** is one whose presence implies the possible occurrence of a similar but pathogenic organism. *E. coli* is used as an index organism and its presence indicates possible presence of pathogenic enterobacteriaceae e.g. salmonellae species.

iii) **Food poisoning organisms**

- those which cause the disease by infection
- those which produce toxin in food
  - ✓ Those which cause infection must grow in food in large numbers and cause infection when consumed together with food. The most most common microorganisms includes salmonella typhimurium, enteropathogenic *E. coli*, *Vibrio parahaemolyticus* etc.
  - ✓ Those which cause intoxication must grow in food large numbers and produce enough toxin and when consumed together with food cause intoxication. The most common microorganism in this group are clostridium botulinum, staphylococcus and toxigenic fungi e.g. *Aspergillus flavus*.



**iv) Infectious microorganisms**

Organisms whose presence in small numbers in food and when consumed can cause infection. In this case the food acts as a vector but not necessarily as a growth medium. Organisms in this group are, *Vibrio cholerae* O1, *Salmonella typhi*, *Shigella sonnei*, *Bacillus anthracis*, Hepatitis B virus etc.

**The spoilage micro-organisms**

. The spoilage microorganisms include bacteria, yeasts and molds that cause undesirable changes of the appearance, odour, texture or taste of the food. They are commonly grouped according to their type of activity or according to their growth requirements.

**Psychrophilic microorganisms**

. Are those organisms capable of growing relatively rapidly at commercial refrigeration temperatures without reference to optimum temperature for growth.

. Species of *Pseudomonas*, *Achromobacter*, *Flavobacterium* and *Alcaligenes* are examples of psychrophilic bacteria.

. Many psychrophilic bacteria when present in large numbers can cause a variety of off flavors as well as defects in foods

. The presence of large number of psychrophilic bacteria in refrigerated foods such as dairy products, meat, poultry and sea food may reflect growth of initial population during storage and /or massive contamination at some point prior to or during refrigerated storage.

### **Thermoduric microorganisms**

. Thermoduric organisms are those organisms which will survive so significant measure of heat treatment

. The thermophilic organisms not only survive the heat treatment but also grow at the elevated temperature

. Thermoduric bacteria are important with regard to milk and milk products as they may survive pasteurisation temperature

The genera Micrococcus, Streptococcus primary the enterococci, Lactobacillus, Bacillus and Clostridium are recognized as containing some species which will qualify as thermoduric.

The thermoduric count may be useful as a test of the care employed in utensil sanitation and as means of detecting sources of organisms responsible for high bacterial count in pasteurized.

### **Lipolytic Microorganisms**

Are those organisms capable of hydrolytic and oxidative deterioration of fats, mostly cream, butter, margarine, etc

The genera Pseudomonas, Achromobacter, and staphylococcus among other bacteria, Rhizopus, Geotrichum, Aspergillus and penicillium among the moulds and the yeast genera Candida, Rhodotorula, and Hansenula contain many lipolytic species.

### **Proteolytic microorganisms**

Proteolytic microorganisms are those microorganisms capable of hydrolyzing proteins producing a variety of odour and flavour defects

Proteolytic species are common among the genera Bacillus, Clostridium, Pseudomonas, and proteus.

Acid proteolytic organisms are those organisms which carry out protein hydrolysis and acid fermentation as streptococcus faecalis var. liquefaciens

### **Halophilic microorganisms**

Halophilic microorganisms are those organisms which require certain minimal concentrations of salt (NaCl) for growth.

Slight halophiles grow optimally in media containing 2.5%. Most of the slight halophilic bacteria originate from marine environments

Marine psychrophilic bacteria of the genera pseudomonas, Moraxella, Acinetobacter, and Flavobacterium contribute to the spoilage of marine fish and shellfish

Moderate halophiles grow optimally in media containing 5-20% salt and most of the moderately halophilic bacteria involved in the spoilage of salted foods are gram positive species of the Bacillaceae and Micrococcaceae

The extreme halophiles grow optimally in media containing 20-30% salt

The extreme halophiles are principally species of the genera Halobacterium and Halococcus which produce bright red or pink pigments, grow very slowly.

They have been incriminated in spoilage of fish, and hides preserved in sea salts.

### **Halotolerant organisms**

Are those organisms capable of growth in salt concentrations exceeding 5%.

Some halotolerant microorganisms are involved in food spoilage while some others such as staphylococcus aureus and cl.perfringens are pathogenic.

### **Osmophilic microorganisms**

Are those organisms that grow in concentrated food products

Osmophilic microorganisms most commonly encountered in food industry are yeasts

They can grow in highly concentrated sugar solutions

They are frequently the cause of honey, chocolate, candy, jams etc.

Almost all of the known osmophilic yeasts are species of saccharomyces species.

### **Pectinolytic microorganism**

Are those microorganisms capable of degrading pectins found in fruits and vegetables. The destruction of the pectin can cause subsequent loss of jelly power of fruits and softening of the stored fruits and vegetables.

The pectinolytic organisms include species of Achrobacterium, Aeromonas, Arthrobacter, Bacillus, Enterobacter etc.

It also includes many yeasts and moulds

### **Acid producing microorganisms**

An important group of acid producing bacteria in the food industry is the lactic acid bacteria

This group is subdivided into the genera streptococcus, Leuconostoc, Pediococcus and Lactobacillus.

The homofermentative species produce lactic acid from the available sugar, while the heterofermentative types produce in addition to lactic acid, mainly acetic acid, ethanol and CO<sub>2</sub>.

Many sporeforming species belonging to the genera *Bacillus* and *Clostridium* are also important acid producers

Some moulds and yeasts produce citric acid, oxalic acid, etc.

#### **Yeasts and moulds**

Yeasts and moulds can be responsible for spoilage of many types of foods

They often manifest themselves in foods of low pH, low moisture, high salt or sugar content, etc.

They are resistant to heat, freezing, antibiotics

#### **Mesophilic spore forming aerobes**

The mesophilic, aerobic spore forming bacteria are all strains of *Bacillus* species that grow at 35°C but not at 55°C.

They cause spoilage in canned low acid (pH > 4.6) foods is usually of the sour type.

Inadequate heat processing is commonly responsible since spores of mesophilic bacteria are moderately resistant to moist heat.

#### **Thermophilic anaerobes**

These organisms are obligatory anaerobes and are strongly saccharolytic, producing abundant gas from different sugars they are non hydrogen sulphide producers

They are responsible for spoilage of canned food products.

## **Microbiological Examination of Food**

### **Sampling**

It is important to note that samples of foods collected for microbiological analysis should reflect the microbiological condition at the time of collection. This implies that

- Sampling should be carried out aseptically
- Samples should be protected against extraneous contamination
- Moreover, samples must be held under conditions that permit neither die off nor multiplication of the original microflora present in the food.

Definition of terms

#### **A lot**

- Is a quantity of food produced and handled under uniform condition. This means that food produced within a batch or in a continuous process a food produced within a limited period of time
- The number of field samples collected are usually five. But for the investigation of food for salmonella the number of field sample is ten

#### **Filed sample:**

- The amount of material actually used in the analysis of food for microorganisms. The sample unit is recommended to be 25 g for all types of food

### **Microbiological criteria**

A microbiological criteria is a microbiological value (eg. Number of microorganism per g of food) or a range established by use of defined procedures and includes the following information.

- A statement of the microorganism of concern and/or the toxin
- The analytical methods of their detection and quantification
- A plan refining the number of field sample to be withdrawn and the size of the sample unit
- The microbiological limits considered appropriate to the food
- The proportion of the sample units that should conform to these limits.

### **MICROBIOLOGICAL EXAMINATION OF DIFFERENT FOOD**

For example Egg and egg products ‘

#### **Types of contaminating microorganisms**

- The shell of eggs are either sterile or harbour very low numbers of microorganisms at the time of ovipositor.
- It gets its bacterial contamination after oviposition from nesting material, dirt, and faecal matter.
- The flora of the egg shell is dominated by Gram-positive cocci. Whereas the Gram-negative rods are present in low numbers
- They penetrate more easily through the egg shell membrane and multiply more readily than do the Gram-positive cocci.
- The number of bacteria in liquid egg product depend upon the bacteriological condition of shell eggs used for breaking, plant sanitation and conditions under which the liquid may be stored.
- The most common bacterial genera found in liquid eggs are members of pseudomonas, Alcaligenes, Proteus and

Escherichia. These organisms are the main cause of spoilage resulting in characteristic off odours and off colours.

- The major pathogen associated with eggs and egg products is Salmonella.

### **Microbiological examination**

Eggs can be given as liquid egg frozen egg, dried egg

### **Methods of analysis**

- a) Enumeration of mesophilic aerobic bacteria
- b) Enumeration of coliforms
- c) Detection of salmonella

### **Sampling plan and microbiological limit**

Mesophilic aerobic bacteria should not be recovered from any of the five sample units examined, when the test is carried out according to the method described, in a number exceeding  $10^6$  per g, nor in a number exceeding  $5 \times 10^4$  per g from three or more of the five sample units examined ( $n=5, c=2, m=5 \times 10^4, M=10^6$ )

$n$  = the number of sample units comprising the sample

$m$  = the threshold value for the number of bacteria; the result is considered to be satisfactory if the number of bacteria in all sample units does not exceed this value

$M$  = is the maximum value for the number of bacteria, the result is considered to be unsatisfactory if the number of bacteria in one or more sample units is equal to or greater than this value.

$C$  = is the number of sample units where the bacterial count may be between  $m$  and  $M$ . the sample is considered to be acceptable if the



bacterial counts of the other sample unit are equal to or less than the value of  $m$  Coliform bacteria should not be recovered from any of five sample units examined, when the test is carried out according to the method described, in a number exceeding  $10^3$  per g, nor in a number exceeding 10 per g from three or more of the five sample units examined ( $n=5$ ,  $c=2$ ,  $m=10$ ,  $M=10^3$ ).

Salmonella organisms should not be recovered from any of ten sample units examined when the test is carried out according to the method described; ( $n=10$ ,  $c=0$   $m$   $M=0$ ).

## **METHODS OF MICROBIOLOGICAL ANALYSIS OF FOOD AND WATER**

### **1. ENUMERATION OF MESOPHILIC AEROBIC BACTERIA (Aerobic plate count)**

#### **Principle**

- This method is based on the assumption that the microbial cells present in a sample mixed with an agar medium each form visible, separated colonies.
- This is obtained by mixing decimal dilutions of the food sample homogenate or water with the medium
- After incubation of the plates at  $35^{\circ}\text{C}$  72 hours the number of mesophilic aerobic bacteria per g of food sample is calculated from the number of colonies obtained in selected petridishes at levels of dilutions giving a significant result.

However, it should be borne in mind that this method, as all other methods, has some limitations

- Microbial cells often occur as clumps, clusters, chains, or pairs in foods, and may not be well distributed irrespective of the mixing and dilution of the sample.
- Consequently, each colony that appears on the agar plate may arise from a single cell or from groups of cells. Moreover, some microorganisms may fail to grow and form visible colonies on the agar medium as a result of unfavourable conditions of temperature, oxygen or nutrition, or because the cells are weak.
- Hence the colony count may not reflect the actual number of viable bacteria in the food.

#### **Apparatus and Glassware**

- a) Petri dishes 90-100mm, glass or plastic
- b) Pipettes 1,5 and 10ml, graduated (total-flow)
- c) Water bath,  $45 \pm 1^{\circ}\text{C}$
- d) Colony counter
- e) Incubator,  $35 \pm 1^{\circ}\text{C}$

#### **Culture media and diluent**

- a) Buffered peptone water (BPW)
- b) Plate count agar (PCA)

#### **Procedure**

##### **Preparation of food homogenate**

- Weigh 25 g of the mixed sample aseptically into a sterile blender jar or into a stomacher bag and add 225ml of BPW.

- Blend the food at a speed of 15000-20000rpm for not more than 2.5 min or mix in the stomacher for 20 sec.

#### **Dilution**

- a) Mix the food homogenate by shaking and pipette 1.0ml into a tube containing 9ml of the of the BPW, mix with a fresh pipette, and
- b) From the first dilution, transfer with the same pipette 1.0ml to 2<sup>nd</sup> dilution tube containing 9ml of the BPW, mix with a fresh pipette, and
- c) Repeat using a 3<sup>rd</sup>, 4<sup>th</sup> tube or more until the required numbers of dilutions are made.
- d) Shake all dilution carefully.

#### **Pour plating**

- a) Pipette 1.0ml of the food homogenate and of each dilution of the homogenate into each of the appropriately marked duplicate dishes

- b) Pour into each petri dish 15ml of the PCA (kept at  $45 \pm 1^{\circ}\text{C}$  in water bath ) within 15 minute of the time of original dilution.
- c) Mix the sample dilution and agar medium thoroughly and uniformly; allow to solidify.

### **Incubation**

Incubate the prepared dishes, inverted, at  $35 \pm 1^{\circ}\text{C}$  for  $72 \pm 3$  hours.

### **Counting the colonies**

Following incubation, count all colonies on dishes containing 30 -300 colonies and record the results per dilution counted.

### **Calculation**

- a) When the dishes examined contain no colonies, the result is expressed as; less than  $1 \times 10^1$  bacteria per g or ml.
- b) When the dishes (dilution 1 in 10) contain less than 30 colonies, the result is expressed as: less than  $3 \times 10^2$  ( $30 \times 10 = 3 \times 10^2$ ).
- c) When the colonies are more than 30, count the colonies in both plates of a dilution and calculate the average, retaining only two significant digits and multiply by the inverse of the

corresponding dilution to obtain the number of bacteria per g or ml.

Example: dilution 1/100 dish 1: 175 colonies

Dish 2: 208 colonies

**Calculation:**  $175+208=383/2=191 \rightarrow 190 \times 100$

Result:  $1.9 \times 10^4$  bacteria per g of food.

### ENUMERATION OF COLIFORM BACTERIA

Standard multiple tube fermentation technique (determination of the most probable number, **MPN**)

#### Principle

The standard tests are

- Presumptive test
- Confirmed test and
- Completed test

#### Presumptive

- Graduated amount of food are transferred to series of fermentation tubes containing lactose broth or lauryl sulphate tryptose broth of proper strength. It is usual practice to inoculate to five fermentation tubes
- The tubes are incubated at  $35 \pm 0.5^\circ\text{C}$  and examined at the end of  $24 \pm 2$  hours. Tubes showed no gas productions at the end of  $24 \pm 2$  hours are reincubated and examined at the end of  $48 \pm 3$  hrs.

- The formation of gas in any of the tubes within  $48 \pm 3$  hours, regardless of the amount, constitutes a positive presumptive test.
- The absence of gas formation within that period constitutes a negative test, and no further tests need be performed.

#### **Confirmed test**

- All fermentation tubes showing gas production in presumptive tests within 48 hours at  $35^{\circ}\text{C}$  shall be utilized in the confirmed test
- Eosin methylene blue (E.M.B) agar, Endo agar or brilliant green lactose bile broth fermentation tubes may be used in the test
- A loop-full of culture from each positive fermentation tubes is streaked over the surface of E.M.B agar or Endo agar. Development of typical colonies (nucleated, with or without metallic sheen) or atypical colonies (opaque, nonnucleated mucoid, pink) the confirmed test may be considered positive
- If no colonies develop within the incubation period the confirmed test may be considered negative.

#### **Brilliant green lactose bile broth (BGLBB)**

- A loop-full of culture from each positive presumptive tube is transferred to brilliant green lactose bile broth fermentation tubes and incubated at appropriate temperature and time
- Presence of gas in any amount in the inverted vial within the incubation period may be positive confirmed test

### **Completed test**

- Transfer typical or atypical colonies from E.M.B or Endo agar to lactose fermentations tubes and nutrient agar slants and incubate at appropriate temperature for a period not to exceed 48hours
- If Brilliant green lactose bile broth is used in the confirmed test, an E.M.B or Endo agar plate is streaked from each fermentation tube showing gas and all plates should be incubated at appropriate temperature and period

### **The purpose of the completed test is to determine**

- The colonies developing on E,M.B or Endo agar are again capable fermenting lactose with the formation of acid and gas .
- Organisms transferred to agar slants show the morphological and tinctorial picture of members are the coliform group
- The formation of gas in any amount in the fermentation tube and the demonstration gram negative, non spore forming rods on the agar slants shall constitute a positive completed for members of the coliform group
- The absence of or failure to show the presence of rods answering to the above description in gas-forming culture shall constitute a negative completed group.

### **Apparatus and Glassware**

- a) Test tubes (18mmx180mm)
- b) Durham tubes (10mmx75mm)
- c) Pipettes 1(total-flow)
- d) Incubators,  $35\pm 1^{\circ}\text{C}$ ,  $37\pm 1^{\circ}\text{C}$
- e) Water bath,  $45.5\pm 0.05^{\circ}\text{C}$

### Culture media and reagent

- a) Brilliant-Green lactose bile broth 2% (BGLB)
- b) Buffered peptone water
- c) Indole medium and reagent
- d) Koser's citrate
- e) Lauryl sulphate tryptose broth (LST)
- f) Levine's eosin methylene blue agar(L-EMB)
- g) Voges-proskauer (VP) medium

### Procedure

#### Preparation of food homogenate

- Weigh 25 gm of the mixed sample aseptically into a sterile blender jar or into a stomacher bag and add 225ml of buffered peptone water(BPW)
- Blend the food at a speed of 15000 – 20000 rpm for not more than 2.5 minute or mix the stomacher for 20 seconds

#### Dilution

- a) Mix the food homogenate by shaking and pipette 1.0ml into a tube containing 9ml of the of the BPW, mix with a fresh pipette, and
- b) From the first dilution, transfer with the same pipette 1.0ml to 2<sup>nd</sup> dilution tube containing 9ml of the BPW, mix with a fresh pipette, and
- c) Repeat using a 3<sup>rd</sup>, 4<sup>th</sup> tube or more until the required numbers of dilutions are made.
- d) Shake all dilution carefully.



### **Inoculation**

- a) Inoculate each of 3 tubes of LST broth (containing inverted Durham tubes) with 1.0ml of the food homogenate (1 in 10).
- b) Carry out the same operation from the first (1 in 100) and the second (1 in 1000) dilution tubes, using a new sterile pipette for each dilution.

### **Incubation**

Incubate the LST tubes at  $37 \pm 1^\circ\text{C}$  for 24 and 48 hours

### **Reading of enrichment tubes (presumptive test)**

Record tubes showing gas production after 24 hours, and re-incubate negative tubes for further 24 hours, and then record tubes showing gas production.

### **Confirmed test for coliforms**

- a) Transfer a loopful from each gas-positive tube of LST to a separate tube of BGLB broth.
- b) Incubate the BGLB tubes at  $35 \pm 1^\circ\text{C}$  for 48 hours.
- c) The formation of gas confirms the presence of coliform bacteria. Record the number of positive

tubes that were confirmed positive for coliforms.

**Calculation (MPN)**

Note the MPN appropriate to the number of positive tubes from the following table for example:

3 in 1:10, 1 in 1:100 and 0 in 1:1000 the table shows that MPN=43 coliforms/g or ml

**MPN index and 95% confidence limits when 3 tubes are used**

Number of positive tubes			MPN per g or ml	95% confidence limits	
1:10	1:100	1:1000		Lower	Upper
0	0	0	<3		
0	0	1	3	<0.5	9
0	1	0	3	<0.5	13
1	0	0	4	<0.5	20
1	0	1	7	1	21
1	1	0	7	1	23
1	1	1	11	3	36
1	2	0	11	3	36
2	0	0	9	1	36
2	0	1	14	3	37
2	1	0	15	3	44
2	1	1	20	7	89
2	2	0	21	4	47
2	2	1	28	10	150
3	0	0	23	4	120

3	0	1	39	7	130
3	0	2	64	15	380
3	1	0	43	7	210
3	1	1	75	14	230
3	1	2	120	30	230
3	2	0	93	15	380
3	2	1	150	30	440
3	2	2	210	35	470
3	3	0	240	36	1300
3	3	1	460	71	2400
3	3	2	1100	150	4800
3	3	3	>2400		

#### Differentiation of non-faecal coliforms faecal coliforms

- a) Elevated temperature test
  - Simultaneously with the confirmatory procedure using brilliant green lactose broth, transfer should be made from all positive presumptive tubes to EC broth medium.
  - The inoculated EC tubes are incubated at 45.5 °C for 24hours, and gas formation is recorded and the bacterial density is estimated from the tables of MPN.
- b) Run IMViC Test
  - Streak E.M.B agar or Endo agar from each positive tubes in a way to obtain discrete colonies and incubate for 18-24 hours at 35 °C.
  - Perform indole, methyl red, v-p and citrate tests (IMViC).

**Classification of Coliforms by IMViC test**

Indole	MR	VP	Citrate	Type
+	+	-	-	Typical E.coli
-	+	-	-	Atypical E.coli
+	+	-	+	Typical intermediate
-	+	-	+	Atypical intermediate
-	-	+	+	Typical E.aerogenes
+	-	+	+	Atypical E.aerogenes

**Procedure**

**Preparation of food homogenate**

Prepare as described above

**Dilution**

Prepare as described above

**Inoculation**

Inoculate three 10 ml portions of 1:10 dilutions into 10 ml each of double strength GSTB, and then inoculate three 1ml portions of 1:10, 1:100, 1:1000 and 1:10000 dilutions into single strength GSTB.

**Incubation**

Incubate broth tubes overnight at 35<sup>0</sup>C.

**Confirmation**

- a) After incubation, streak a loopful of the culture from the three highest dilutions of GSTB showing growth onto TCBS agar plates.
  - b) Incubate the plates for 18 hours at 35<sup>0</sup>C.
  - c) The colonies of V.parahaemolyticus on TCBS appear round, 2-3mm in diameter with green or blue centres. V.alginolyticus

colonies are larger and yellow. Coliforms, proteus and enterococci colonies are small and translucent.

### Biochemical identification

- a) TSI: Streak the slant and stab the butt and incubate overnight at 35<sup>o</sup>C. *V. parahaemolyticus* produces alkaline slant and acid butt, no gas and no H<sub>2</sub>S (typical shigella-like reaction).
- b) Motility medium: inoculate 4 tubes by stabbing. Diffuse circular growth occurs after 24hours incubation at 35<sup>o</sup>C.
- c) Make a Gram stain from growth on TSA slant.
- d) Halophilic nature: inoculate 4 tubes of STB containing 0, 6, 8 and 10% NaCl, incubate; *V. parahaemolyticus* will grow well in 6 and 8% NaCl but not in 0 and 10% concentrations.
- e) MR-VP test
- f) Indole test
- g) Carbohydrate fermentation: Inoculate one tube each of glucose, lactose, sucrose, maltose, mannitol, etc. from TSA slant. After incubation check for acid production.
- h) Glucose fermentation: stab 2 tubes of HLGB medium, overlay one tube with

sterile paraffin oil and incubate for 2 day at 35 °c. yellow coloration of both tubes indicates fermentation, in the tube without oil only indicates oxidation. V.paraphaemolyticus is a glucose fermenter producing no gas.

- i) Cytochrome oxidase test: Allow 2-3 drops of alphanaphthol solution to flow over a fresh slant of V.parahaemolyticus or over a colony on a plate, then follow this by an equal amount of phenylenediamine solution. The development of dark blue colour within 2 minute is positive
- j) LDC
- k) Growth at 42<sup>0</sup>C: incubate an inoculated TSB at 42<sup>0</sup>C in a water bath for 24 hours.

The characteristics features of V.parahaemolyticus are:

- a) Gram-negative curved rods
- b) Cytochrome oxidase positive
- c) Glucose oxidation/fermentation (O/F) positive, no gas
- d) Colony on TCBS typical blue-green in colour
- e) TSI, alkaline slant, acid butt, no gas, no H<sub>2</sub>S
- f) Positive growth at 42<sup>0</sup>C

- g) Positive growth in 8% but not in 10%NaCl
- h) Positive LDC
- i) Negative VP
- j) Negative sucrose

### **Calculation**

When the blue green colonies on TCBS are finally identified biochemically as vibrio parahaemolyticus, refer to the original positive dilutions of GSTB and apply the 3 tube MPN table for final enumeration of the organism.

### **ENUMERATION OF BACILLUS CEREUS**

#### **Principle**

This method is based on surface plating technique using a medium containing egg yolk on which the colonies of *B. cereus* are recognized by being surrounded by zones of turbidity.

#### **Apparatus and Glassware**

- a. Petridishes
- b. Pipettes, 1 ml
- c. Incubators, 20<sup>o</sup>C, 30<sup>o</sup>C, 35<sup>o</sup>C

#### **Culture media and reagents**

- a. Buffered peptone water
- b. KG agar
- c. Nitrate broth
- d. Nutrient agar
- e. Nutrient gelatin
- f. VP medium

g. Gram stains

**Procedure**

**Preparation of food homogenate**

Prepare as described as above

**Dilution**

Prepare as described above.

**Inoculation**

Pipette 0.25ml of homogenate and dilutions of the homogenate on the surface of previously dried KG agar plates and spread with a sterile bent glass rod.

**Incubation**

Incubate the plates at 30<sup>0</sup>C for 20-24hours

**Counting of the colonies (presumptive *B. cereus*)**

Count the colonies surrounded by a halo of dense precipitate (lecithinase activity) and calculate the total number per gram of specimen by multiplying by 4 and by the dilution factor.

**Confirmation**

- a) From typical colonies make smear and stain with Gram and examine microscopically.
- b) At the same time transfer some of the typical colonies to nutrient agar slants, incubate at 30<sup>0</sup>C for 24hours and from the growth inoculate the following:
  - i. Gelatine tube: examine for liquefaction after 24 hours incubation
  - ii. Nitrate broth tube: after 24 hours incubation at 35<sup>0</sup>C test for the reduction nitrate to nitrite



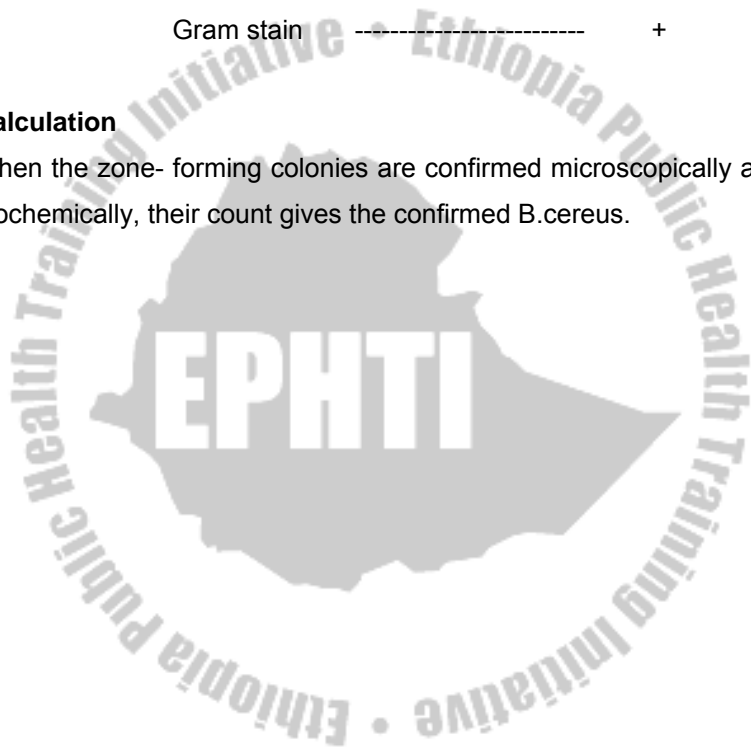
iii. VP medium

c) Characteristics of B.cereus

Glatine liquefication-----	+
Nitrate reduction -----	+
Egg yolk reaction -----	+
VP – reaction -----	-
Gram stain -----	+

**Calculation**

When the zone- forming colonies are confirmed microscopically and biochemically, their count gives the confirmed B.cereus.



## ANNEX

### Preparation of media

Presently, a wide range of culture media are available commercially in the form of dehydrated media. These media are simply reconstituted by weighing the required quantities and by adding distilled water, as per the manufacturer's instructions.

The pH determination can be conveniently done with the use of Lovibond comparator with phenol red indicator disc.

- Take two clean test tubes and add 5 ml of the medium to each of the tubes. One serves as a blank while phenol red indicator is added to the other tube.
- Compare the colour of the medium with the phenol red indicator at the appropriate pH marking.
- Add N/10 NaOH or N/10 HCl, drop by drop till the colour of the medium matches the colour of the disc at the required pH reading.
- Calculate the volume of the NaOH or HCL of 1/10 strength for 5 ml of the medium to get the required pH.
- Based on the calculation, the volume of 1N NaOH or 1N HCl required for the total volume of medium can be calculated and added.
- Check the pH of the medium once again before use.

The quantity of agar given in the formulae of media may have to be changed depending upon the quality of agar used. The concentration varies from batch to batch and should be such that will produce a sufficiently firm surface on solidification. This can be tested by streaking with inoculating wire.

In some laboratories media are prepared by individual measurement of ingredients and then mixing the same. Hence the method of preparation is given likewise:

#### **Nutrient broth**

Meat extract	10.0 gm
Peptone	10.0 gm
Sodium chloride	5.0 gm
Distilled water	1000 ml

Mix the ingredients and dissolve them by heating in a steamer. When cool, adjust the pH to 7.5-7.6.

#### **Nutrient agar**

To the ingredients as in nutrient broth, add 15 gm agar per litre. Dissolve the agar in nutrient broth and sterilize by autoclaving at 121°C for 15 minutes. Prepare plates and slopes as required.

### **Glucose broth**

Nutrientbroth 900ml

Glucose (10% solution) 100 ml

- Dissolve 9 gm glucose in distilled water and sterilize by tyndallisation.
- Add 100 ml of the glucose solution to 900 ml of sterile nutrient broth.
- Dispense 60 ml each in 100 ml pre-sterilized culture bottles.
- Sterilize by open steaming at 100°C for one hour.

### **Blood agar**

Nutrient agar 100 ml

Sheep blood (defibrinated) 10 ml

- Melt the sterile nutrient agar by steaming, cool to 45°C.
- Add required amount of sheep blood aseptically with constant shaking.
- Mix the blood with molten nutrient agar thoroughly but gently, avoiding froth formation.
- Immediately pour into petri dishes or test tubes and allow to set.

### Chocolate agar

The ingredients are essentially the same as in blood agar.

- Melt the sterile nutrient agar by steaming and cool to about 75°C.
- Add blood to the molten nutrient agar and allow to remain at 75°C after gently mixing till it is chocolate brown in colour.
- Pour in petri dishes or test tubes for slopes as desired.

### XLD agar

Xylose	3.5gm
l-lysine	5.0gm
Lactose	7.5gm
Sucrose	7.5gm
Sodiumchloride	5.0gm
Yeastextract	3.0gm
Sodiumdesoxycholate	2.5gm
Sodiumthiosulphate	6.8gm
Ferricammoniumcitrate	0.8gm
Phenolred	0.08gm
Agaragar	5.0gm
Water	1000 ml

Weigh the ingredients into a flask and add distilled water. Mix the contents well and steam it for 15 minutes (do not autoclave). Cool to 56°C and pour in plates.

**Buffered glycerol saline**

Glycerol	300	ml
Sodiumchloride	4.2	gm
Disodiumhydrogenphosphate	10.0	gm
Na <sub>2</sub> HPO <sub>4</sub> Anhydrous	15.0	gm
Phenolredaqueoussolution0.02percent	15.0	ml
Water	700	ml

- Dissolve NaCl in water and add glycerol.
- Add disodium hydrogen phosphate to dissolve.
- Add phenol red and adjust pH to 8.4.
- Distribute 6 ml in universal containers (screw -capped bottles of 30 ml capacity). Autoclave at 115°C for 15 minutes.

**Loeffler serum medium**

Nutrientbroth	100ml
Serum(sheeporhorseorox)	300ml
Glucose	1.0 gm

- Dissolve glucose in nutrient broth and sterilize at 121°C for 15 minutes.
- Add serum aseptically.
- Mix thoroughly but gently, avoiding froth formation.
- Distribute in sterile test tubes or quarter ounce screw-cap bottles.

- Inspissate the medium in a slanting position in a water inspissator at 82°C for two hours.
- In the absence of an inspissator, the medium may be coagulated by standing over the top of a steam sterilizer for 6-7 minutes.

### **Blood tellurite agar**

#### **Agar base**

Meatextract	5.0 gm
Peptone	10.0 gm
Sodium chloride	5.0 gm
Agar	25.0 gm
Water	1000 ml

Dissolve the ingredients and adjust the pH to 7.6. Distribute in 100 ml quantities in a bottle and autoclave at 121°C for 15 minutes.

#### **Glycerolated blood tellurite mixture**

Sterile defibrinated sheep blood 14 ml  
Sterile glycerol 6 ml  
Sterile potassium tellurite solution  
(1% in water) 4 ml

- Sterilize the glycerol in hot air oven at 160°C for 60 minutes and the tellurite solution by autoclaving at

- 115°C for 20 minutes. Mix the ingredients in a sterile flask, incubate for 1-2 hrs. at 37°C, then refrigerate.
- Haemolysis is complete after 24 hrs. The mixture keeps well in a refrigerator. One per cent solution of
- good quality tellurite is sufficient but 2% of some batches may be required.

#### **Preparation of complete medium**

Glycerolated blood tellurite mixture 24 ml

Agar base 100 ml

Melt the agar, cool to 45°C, add blood and tellurite and pour in sterile petri dishes.

#### **M1. A-1 Medium**

Tryptone 20 g

Lactose 5 g

NaCl 5 g

\*Triton X-100 (Rohm & Haas) 1 ml

Salicin 0.5 g

Distilled water 1 liter

Dissolve ingredients in 1 liter distilled water. Adjust pH to  $6.9 \pm 0.1$ . Dispense 10 ml portions of single strength broth into 18 x 150 mm tubes containing inverted fermentation vials. For double strength broth, use 22 x 175 mm tubes containing inverted fermentation vials. Medium may be cloudy before sterilization. Autoclave 10 min at



21°C. Store in dark up to 7 days. (Commercially available A-1 medium is unacceptable.)

### **M2. Acetate Agar**

Sodium acetate 2 g

NaCl 5 g

Mg SO<sub>4</sub> (anhydrous) 0.2 g

Ammonium phosphate 1 g

K<sub>2</sub>HPO<sub>4</sub> 1 g

Bromthymol blue 0.08 g

Agar 20 g

Distilled water 1 liter

Add all ingredients except Mg SO<sub>4</sub> to 1 liter distilled water. Heat to boiling with stirring. Add Mg SO<sub>4</sub> and adjust pH. Dispense 8 ml portions into 16 x 150 mm tubes. Autoclave 15 min at 121°C. Incline tubes to obtain 5 cm slant. Final pH, 6.7

### **M3. Acid Broth**

Proteose peptone 5 g

Yeast extract 5 g

Dextrose 5 g

K<sub>2</sub>HPO<sub>4</sub> 4 g

Distilled water 1 liter

Dissolve ingredients and dispense 12-15 ml portions into 20 x 150 mm tubes. Autoclave 15 min at 121°C. Final pH, 5.0.

**M4. AE Sporulation Medium, Modified (for *C. perfringens*)**

**(AE base is available commercially)**

Polypeptone 10.0 g

Yeast extract 10.0 g

Na<sub>2</sub>HPO<sub>4</sub> 4.36 g

KH<sub>2</sub>PO<sub>4</sub> 0.25 g

Ammonium acetate 1.5 g

Mg SO<sub>4</sub>·7H<sub>2</sub>O 0.2 g

Distilled water 1 liter

Dissolve ingredients and adjust to pH 7.5 ± 0.1, using 2 M sodium carbonate. Dispense 15 ml into 20 x 150-mm screw-cap tubes and sterilize by autoclaving for 15 min at 121°C. After sterilization, add 0.6 ml of sterilized 10% raffinose and 0.2 ml each of filter-sterilized 0.66 M sodium carbonate and 0.32% cobalt chloride (CoCl<sub>2</sub>·6H<sub>2</sub>O) dropwise to each tube. Check pH of one or two tubes; it should be 7.8 ± 0.1. Just before use, steam medium for 10 min; after cooling, add 0.2 ml of filter-sterilized 1.5% sodium ascorbate (prepared daily) to each tube.

**M5. Alkaline Peptone Agar**

Peptone 10 g

NaCl 20 g

Agar 15 g

Distilled water 1 liter

Boil to dissolve ingredients. Adjust pH so that value after sterilization is  $8.5 \pm 0.2$ . Autoclave 15 min at  $121^{\circ}\text{C}$ . Solidify agar in tubes as slants.

**M6. Alkaline Peptone Salt Broth (APS)**

Peptone 10 g

NaCl 30 g

Distilled water 1 liter

Dissolve ingredients. Adjust pH so that value after sterilization is  $8.5 \pm 0.2$ . Dispense 10 ml into tubes. Autoclave 10 min at  $121^{\circ}\text{C}$ .

**M7. Alkaline Peptone Water**

Peptone 10 g

NaCl 10 g

Distilled water 1 liter

Adjust pH so that value after sterilization is  $8.5 \pm 0.2$ . Dispense into screw-cap tubes. Autoclave 10 min at  $121^{\circ}\text{C}$ .

**M8. Anaerobe Agar**

**Base**

Trypticase (tryptic) soy agar 40 g

Agar 5 g

Yeast extract 5 g

L-Cysteine (dissolved in 5 ml 1 N NaOH) 0.4 g

Distilled water 1 liter

Heat with agitation to dissolve agar. Adjust pH to  $7.5 \pm 0.2$ . Autoclave 15 min at  $121^{\circ}\text{C}$ . Cool to  $50^{\circ}\text{C}$ .

**Hemin solution.** Suspend 1 g hemin in 100 ml distilled water. Autoclave 15 min at  $121^{\circ}\text{C}$ . Refrigerate at  $4^{\circ}\text{C}$ .

**Vitamin K<sub>1</sub> solution.** Dissolve 1 g vitamin K<sub>1</sub> (Sigma Chemical Co., St. Louis, MO) in 100 ml 95% ethanol. Solution may require 2-3 days with intermittent shaking to dissolve. Refrigerate at  $4^{\circ}\text{C}$ .

**Final medium.** To 1 liter base add 0.5 ml hemin solution and 1 ml Vitamin K<sub>1</sub> solution. Mix and pour 20 ml portions into 15 x 100 mm petri dishes. Medium must be reduced before inoculation by 24 h anaerobic incubation in anaerobic glove box or GasPak jar.

### **M9. Anaerobic Egg Yolk Agar**

#### **Agar base**

Yeast extract 5 g  
Tryptone 5 g  
Proteose peptone 20 g  
NaCl 5 g  
Agar 20 g  
Distilled water 1 liter

Autoclave 15 min at  $121^{\circ}\text{C}$ . Adjust pH to  $7.0 \pm 0.2$ .

## 2 Fresh Eggs

**Treatment of eggs.** Wash 2 fresh eggs with stiff brush and drain. Soak eggs in 70% ethanol for 1 h. Crack eggs aseptically. Retain yolks. Drain contents of yolk sacs into sterile stoppered graduate and discard sacs. Add yolk to equal volume of sterile 0.85% saline. Invert graduate several times to mix. Egg yolk emulsion (50%) is available commercially.

**Preparation of medium.** To 1 liter melted medium (48-50°C) add 80 ml yolk-saline mixture (available from Difco as Bacto Egg Yolk Enrichment 50%), and mix. Pour plates immediately. After solidification dry 2-3 days at ambient temperature or at 35°C for 24 h. Check plates for contamination before use. After drying, plates may be stored for a short period in refrigerator.

### **M10. Antibiotic Medium No. 1 (Agar Medium A)**

Gelatone or gelysate 6 g

Tryptone or trypticase 4 g

Yeast extract 3 g

Beef extract 1.5 g

Dextrose 1 g

Agar 15 g

Distilled water 1 liter

Autoclave 15 min at 121°C. Final pH, 6.5-6.6. Commercially available in dehydrated form as Difco Penassay Seed Agar or BBL Seed Agar.

**M11. Antibiotic Medium No. 4 (Agar Medium B)**

Tryptone or trypticase 6 g

Yeast extract 3 g

Beef extract 1.5 g

Dextrose 1 g

Agar 15 g

Distilled water 1 liter

Autoclave 15 min at 121°C. Final pH, 6.5-6.6. Commercially available in dehydrated form as Difco Yeast Beef Agar or BBL Yeast Beef Agar.

**M12. Arginine-Glucose Slant (AGS)**

Peptone 5 g

Yeast extract 3 g

Tryptone 10 g

NaCl 20 g

Glucose 1 g

L-Arginine (hydrochloride) 5 g

Ferric ammonium citrate 0.5 g

Sodium thiosulfate 0.3 g

Bromocresol purple 0.02 g

Agar 13.5 g

Distilled water 1 liter

Suspend ingredients in distilled water and boil to dissolve. Dispense into tubes (for 13 x 100 mm tubes use 5 ml). Autoclave 10-12 min at 121°C. After sterilization, solidify as slants. Final pH, 6.8-7.0.

**M13. Bile Esculin Agar**

Beef extract 3 g

Peptone 5 g

Esculin 1 g

Oxgall 40 g

Ferric citrate 0.5 g

Agar 15 g

Distilled water 1 liter

Heat with agitation to dissolve. Dispense into tubes, autoclave 15 min at 121°C, and slant until solidified. Final pH, 6.6 ± 0.2.

**M13Bismuth Sulfite Agar (Wilson and Blair)**

Polypeptone (or peptone) 10 g

Beef extract 5 g

Dextrose 5 g

Na<sub>2</sub>HPO<sub>4</sub> (anhydrous) 4 g

FeSO<sub>4</sub> (anhydrous) 0.3 g

Bismuth sulfite (indicator) 8 g

Brilliant green 0.025 g

Agar 20 g

Distilled water 1 liter

Mix thoroughly and heat with agitation. Boil about 1 min to obtain uniform suspension. (Precipitate will not dissolve.) Cool to 45-50°C. Suspend precipitate by gentle agitation, and pour 20 ml portions into sterile 15 x 100 mm petri dishes. Let plates dry about 2 h with lids partially removed; then close plates. Final pH, 7.7 ± 0.2 **DO NOT**

**AUTOCLAVE.** Prepare plates on day before streaking and store in dark. Selectivity decreases in 48 h.

**M14. Blood Agar**

Tryptone 15 g  
Phytone or soytone 5 g  
NaCl 5 g  
Agar 15 g  
Distilled water 1 liter

Heat with agitation to dissolve agar. Autoclave 15 min at 121°C. Cool to 50°C. Add 5 ml defibrinated sheep red blood cells to 100 ml melted agar. Mix and pour 20 ml portions into sterile 15 x 100 mm petri dishes. Final pH of base,  $7.3 \pm 0.2$ . Tryptic soy agar, tryptic soy agar blood base, or trypticase soy agar [soybean-casein digest agar (M152)] may be used as the basal medium. Commercially available sheep blood agar plates are satisfactory. For *Vibrio hollisae*, add NaCl to a final concentration of 1%.

**M15. Blood Agar Base (Infusion Agar)**

Heart muscle, infusion from 375 g  
Thiotone 10 g  
NaCl 5 g  
Agar 15 g  
Distilled water 1 liter



Heat gently to dissolve. Autoclave 20 min at 121°C. Final pH, 7.3 ± 0.2. Commercially available dehydrated heart infusion agars may be used.

**M16. Blood Agar Base #2 (Difco)**

Proteose peptone 15 g  
Liver digest 2.5 g  
Yeast extract 5 g  
NaCl 5 g  
Agar 12 g  
Distilled Water 1 liter

Autoclave at 121°C for 15 min. For blood agar, reduce water to 950 ml. Add 50 ml defibrinated (whole or lysed) horse blood and FBP (4 ml to agar + blood) after autoclaving and cooling to 48°C. Final pH, 7.4 ± 0.2.

**M17. Brain Heart Infusion (BHI) Agar (0.7%)  
(for staphylococcal enterotoxin)**

Prepare a suitable quantity of brain heart infusion broth. Adjust pH to 5.3 with 1 N HCl. Add agar to give 0.7% concentration. Dissolve by minimal boiling. Dispense 25 ml portions into 25 x 200 mm test tubes. Autoclave 10 min at 121°C.

**M18. Brain Heart Infusion (BHI) Broth and Agar**

Formulations used by selected manufacturers are represented below; these media are normally available as pre-mixed dry powder.

**Medium 1**

Calf brain, infusion from 200 g  
Beef heart, infusion from 250 g  
Proteose peptone (Difco) or polypeptone (Bioquest) 10 g  
NaCl 5 g  
Na<sub>2</sub>HPO<sub>4</sub> \* 2.5 g  
Dextrose 2.0 g  
Distilled water 1 liter

**Medium 2**

Brain heart-infusion 6.0 g  
Peptic digest of animal tissue 6.0 g  
NaCl 5.0 g  
Dextrose 3.0 g  
Pancreatic digest of gelatin 14.5 g  
Na<sub>2</sub>HPO<sub>4</sub> \*2.5 g  
Distilled water 1 liter

Dissolve ingredients of Medium 1 in distilled water with gentle heat. Suspend ingredients of Medium 2 in distilled water and boil for 1 min to completely dissolve. For both Medium 1 and Medium 2, dispense broth into bottles or tubes for storage. Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2. Commercially available BHI is acceptable.

To prepare brain heart infusion *agar*, add 15 g agar to 1 liter BHI broth. Heat to dissolve agar before dispensing into bottles or flasks. Autoclave 15 min at 121°C.

For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

#### **M19. Brilliant Green Lactose Bile Broth**

Peptone 10 g  
Lactose 10 g  
Oxgall 20 g  
Brilliant green 0.0133 g  
Distilled water 1 liter

Dissolve peptone and lactose in 500 ml distilled water. Add 20 g dehydrated oxgall dissolved in 200 ml distilled water. The pH of this solution should be 7.0-7.5. Mix and add water to make 975 ml. Adjust pH to 7.4. Add 13.3 ml 0.1% aqueous brilliant green in distilled water. Add distilled water to make 1 liter. Dispense into fermentation tubes, making certain that fluid level covers inverted vials. Autoclave 15 min at 121°C. Final pH,  $7.2 \pm 0.1$ .

#### **M20. Bromcresol Purple Broth**

##### **Base**

Peptone 10 g  
Beef extract 3 g  
NaCl 5 g  
Bromcresol purple 0.04 g  
Distilled water 1 liter

Dispense 2.5 ml portions of base solution into 13 x 100 mm test tubes containing inverted 6 x 50 mm fermentation tubes. Autoclave

10 min at 121°C. Final pH,  $7.0 \pm 0.2$ . Sterilize stock solutions of carbohydrates (50% w/v) separately by autoclaving or, preferably, by filtration (0.2  $\mu\text{m}$  pore size). Add  $0.278 \pm 0.002$  ml stock carbohydrate solution to 2.5 ml basal medium to give 5% w/v final carbohydrate concentration.

For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

**M21. Bromcresol Purple Dextrose Broth (BCP)**

Dextrose 10 g

Beef extract 3 g

Peptone 5 g

Bromcresol purple (1.6% in ethanol) 2 ml

Distilled water 1 liter

Dissolve ingredients in distilled water. Dispense 12-15 ml into tubes. Autoclave 15 min at 121°C. Final pH,  $7.0 \pm 0.2$ .

**M22. Egg Yolk Emulsion, 50%**

Wash fresh eggs with a stiff brush and drain. Soak eggs 1 h in 70% ethanol. Drain ethanol. Crack eggs aseptically and discard whites. Remove egg yolks with sterile syringe or wide-mouth pipet. Place yolks in sterile container and mix aseptically with equal volume of sterile 0.85% saline. Store at 4°C until use. Egg yolk emulsion (50%) is available commercially.

**M23. Heart Infusion (HI) Broth and Agar (HIA) (for *Vibrio*)**

Beef heart, infusion from 500 g 1 liter

Tryptose 10 g

NaCl 5 g

Dissolve ingredients and dispense into tubes. For halophilic *Vibrio* spp. add an extra 15 g NaCl/liter. Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2. For heart infusion agar, add 15 g agar/L and boil to dissolve before dispensing and sterilizing. Commercially available from Difco.

**M24. Kligler Iron Agar**

Polypeptone peptone 20 g

Lactose 20 g

Dextrose 1 g

NaCl 5 g

Ferric ammonium citrate 0.5 g

Sodium thiosulfate 0.5 g

Agar 15 g

Phenol red 0.025 g

Distilled water 1 liter

Heat with agitation to dissolve. Dispense into 13 x 100 mm screw-cap tubes and autoclave 15 min at 121°C. Cool and slant to form deep butts. Final pH, 7.4 ± 0.2. Commercially available from Difco, BBL, and Oxoid. For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

M25. Lysine Decarboxylase Broth (Falkow)

(for *Salmonella*)

Gelysate or peptone 5 g

Yeast extract 3 g  
Glucose 1 g  
L-Lysine 5 g  
Bromcresol purple 0.02 g  
Distilled water 1 liter

Heat until dissolved. Dispense 5 ml portions into 16 x 125 mm screw-cap tubes. Autoclave loosely capped tubes 15 min at 121°C. Screw the caps on tightly for storage and after inoculation. Final pH,  $6.8 \pm 0.2$ . For halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

M26. Lysine Decarboxylase (LDC) Medium  
(for Gram-negative nonfermentative bacteria)

L-Lysine HCl 0.5 g  
Dextrose 0.5 g  
KH<sub>2</sub>PO<sub>4</sub> 0.5 g  
Distilled water 100 ml

Dissolve ingredients. Adjust pH to  $4.6 \pm 0.2$ . Autoclave 15 min at 121°C. Aseptically dispense 1 ml portions to sterile 13 x 100 mm tubes.

M27. Lysine Iron Agar (Edwards and Fife)

Gelysate or peptone 5 g  
Yeast extract 3 g  
Glucose 1 g  
L-Lysine hydrochloride 10 g

Ferric ammonium citrate 0.5 g  
Sodium thiosulfate (anhydrous) 0.04 g  
Bromcresol purple 0.02 g  
Agar 15 g  
Distilled water 1 liter

Heat to dissolve ingredients. Dispense 4 ml portions into 13 x 100 mm screw-cap tubes. Autoclave 12 min at 121°C. Let solidify in slanted position to form 4 cm butts and 2.5 cm slants. Final pH, 6.7 ± 0.2.

M28. MacConkey Agar

Proteose peptone or polypeptone 3 g  
Peptone or gelysate 17 g  
Lactose 10 g  
Bile salts No. 3 or bile salts mixture 1.5 g  
NaCl 5 g  
Neutral red 0.03 g  
Crystal violet 0.001 g  
Agar 13.5 g  
Distilled water 1 liter

Suspend ingredients and heat with agitation to dissolve. Boil 1-2 min. Autoclave 15 min at 121°C, cool to 45-50°C, and pour 20 ml portions into sterile 15 x 100 mm petri dishes. Dry at room temperature with lids closed. DO NOT USE WET PLATES. Final pH, 7.1 ± 0.2.

M29. Motility-Indole-Ornithine (MIO) Medium

Yeast extract 3 g

Peptone 10 g

Tryptone 10 g

L-Ornithine HCl 5 g

Dextrose 1 g

Agar 2 g

Bromcresol purple 0.02 g

Distilled water 1 liter

Dispense 4 ml portions into 13 x 100 mm tubes. Autoclave 15 min at 121°C. Final pH, 6.5 ± 0.2.

M30. Motility Medium (for *B. cereus*)

Trypticase 10 g

Yeast extract 2.5 g

Dextrose 5 g

Na<sub>2</sub>HPO<sub>4</sub> 2.5 g

Agar 3 g

Distilled water 1 liter

Heat with agitation to dissolve agar. Dispense 100 ml portions to 170 ml bottles. Autoclave 15 min at 121°C. Final pH, 7.4 ± 0.2. Cool to 50°C. Aseptically dispense 2 ml portions to sterile 13 x 100 mm tubes. Store at room temperature 2 days before use.



M31. Motility-Nitrate Medium, Buffered (for *C. perfringens*)

Beef extract 3 g

Peptone (Difco) 5 g

KNO<sub>3</sub> 1 g

Na<sub>2</sub>HPO<sub>3</sub> 2.5 g

Agar 3 g

Galactose 5 g

Glycerin (reagent grade) 5 ml

Distilled water 1 liter

Dissolve all ingredients except agar. Adjust pH to  $7.3 \pm 0.1$ . Add agar and heat to dissolve. Dispense 11 ml portions into 16 x 150 mm tubes. Autoclave 15 min at 121°C. If not used within 4 h, heat 10 min in boiling water or flowing steam. Chill in cold water.

M32. Motility Test Medium (Semisolid)

Beef extract 3 g

Peptone or gelysate 10 g

NaCl 5 g

Agar 4 g

Distilled water 1 liter

Heat with agitation and boil 1-2 min to dissolve agar. Dispense 8 ml portions into 16 x 150 screw-cap tubes. Autoclave 15 min at 121°C. Final pH,  $7.4 \pm 0.2$ .

For *Salmonella*: Dispense 20 ml portions into 20 x 150 mm screw-cap tubes, replacing caps loosely. Autoclave 15min at 121°C. Cool

to 45°C after autoclaving. Tighten caps, and refrigerate at 5-8°C. To use, remelt in boiling water or flowing steam, and cool to 45°C. Aseptically dispense 20 ml portions into sterile 15 x 100 mm petri plates. Cover plates and let solidify. Use same day as prepared. Final pH, 7.4 ± 0.2. For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

M33. Nitrate Broth

Beef extract 3 g

Peptone 5 g KNO<sub>3</sub> (nitrite-free) 1 g

Distilled water 1 liter

Dissolve ingredients. Dispense 5 ml portions into 16 x 125 mm tubes. Autoclave 15 min at 121°C. Final pH, 7.0 ± 0.2.

M34. Nitrate Broth, Enriched (CDC)

Infusion broth 25 g

KNO<sub>3</sub> (nitrite-free) 2 g

Distilled water 1 liter

Dispense 4 ml portions into 13 x 100 mm tubes with inverted Durham tubes. Autoclave 15 min at 121°C. Final pH, 7.3 ± 0.2.

M35. Oxidative-Fermentative (OF) Test Medium

Base

Peptone 2 g

NaCl 5 g

K<sub>2</sub>HPO<sub>4</sub> 0.3 g

Bromthymol blue 0.03 g

Agar 2.5 g

Distilled water 1 liter

Heat with agitation to dissolve agar. Dispense 3 ml portions into 13 x 100 mm tubes. Autoclave 15 min at 121°C. Cool to 50°C; pH, 7.1.

Carbohydrate stock solution. Dissolve 10 g carbohydrate in 90 ml distilled water. Sterilize by filtration through 0.22 µm membrane. Add 0.3 ml stock solution to 2.7 ml base in tube. Mix gently and cool at room temperature. Inoculate tubes in duplicate. Layer one tube with sterile mineral oil. Incubate 48 h at 35°C. For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

M36. Selenite Cystine Broth

Medium 1 (modification of Leifson's formulation for selenite broth)

Tryptone or polypeptone 5 g

Lactose 4 g

Sodium acid selenite (NaHSeO<sub>3</sub>) 4 g

Na<sub>2</sub>HPO<sub>4</sub> 10 g

L-Cystine 0.01 g

Distilled water 1 liter

Heat to boiling to dissolve. Dispense 10 ml portions into sterile 16 x 150 mm test tubes. Heat 10 min in flowing steam. DO NOT AUTOCLAVE. Final pH, 7.0 ± 0.2. The medium is not sterile. Use same day as prepared.

Medium 2 (North-Bartram modification)

Polypeptone 5 g

Lactose 4 g  
Sodium acid selenite (NaHSeO<sub>3</sub>) 4 g  
Na<sub>2</sub>HPO<sub>4</sub> 5.5 g  
KH<sub>2</sub>PO<sub>4</sub> 4.5 g  
L-Cystine 0.01 g  
Distilled water 1 liter

Heat with agitation to dissolve. Dispense 10 ml portions into sterile 16 x 150 mm test tubes. Heat 10 min in flowing steam. DO NOT AUTOCLAVE. Use same day as prepared.

M37. Sheep Blood Agar

Blood agar base (Oxoid No. 2) 95 ml  
Sterile sheep blood, defibrinated 5 ml

Rehydrate and sterilize base as recommended by manufacturer. Agar and blood should both be at 45-46°C before blood is added and plates are poured. Commercial pre-poured sheep blood agar plates may be used.

M38. Shigella Broth

Base  
Tryptone 20 g  
K<sub>2</sub>HPO<sub>4</sub> 2 g  
KH<sub>2</sub>PO<sub>4</sub> 2 g  
NaCl 5 g  
Glucose 1 g

Tween 80 1.5 ml

Distilled water 1 liter

Autoclave 15 min at 121°C. Final pH, 7.0 ± 0.2.

Novobiocin solution. Weigh 50 mg novobiocin into 1 liter distilled water. Sterilize by filtration through 0.45 µm membrane. Add 2.5 ml concentrate to 225 ml base.

M39. Simmons Citrate Agar

Sodium citrate\*2 g

NaCl 5 g

K<sub>2</sub>HPO<sub>4</sub> 1 g

NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> 1 g

MgSO<sub>4</sub> 0.2 g

Bromthymol blue 0.08 g

Agar 15 g

Distilled water 1 liter

Heat gently with occasional agitation. Boil 1-2 min until agar dissolves. Fill 13 x 100 or 16 x 150 mm screw-cap tubes 1/3 full. Autoclave 15 min at 121°C. Before medium solidifies, incline tubes to obtain 4-5 cm slants and 2-3 cm butts. Final pH, 6.8 ± 0.2.

M40. Sorbitol-MacConkey Agar

Peptone or gelysate 17.0 g

Protease peptone No. 3 or polypeptone 3.0 g

Sorbitol 10.0 g

Bile salts, purified 1.5 g

NaCl 5.0 g  
Agar 13.5 g  
Neutral red 0.03 g  
Crystal violet 0.001 g  
Distilled water 1 liter

Dissolve ingredients in distilled water by heating with stirring.  
Autoclave 15 min at 121°C. Final pH,  $7.1 \pm 0.2$ .

M41. Sporulation Broth (for *C. perfringens*)

Polypeptone 15 g  
Yeast extract 3 g  
Starch, soluble 3 g  
MgSO<sub>4</sub> (anhydrous) 0.1 g  
Sodium thioglycollate 1 g  
Na<sub>2</sub>HPO<sub>4</sub> 11 g  
Distilled water 1 liter

Adjust pH to  $7.8 \pm 0.1$ . Dispense 15 ml portions into 20 x 150 mm screw-cap tubes. Autoclave 15 min at 121°C.

M42. Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar

Yeast extract 5 g  
NaCl 10 g  
Peptone 10 g  
Ferric citrate 1 g  
Sucrose 20 g  
Bromthymol blue 0.04 g

Sodium thiosulfate·5H<sub>2</sub>O 10 g  
Thymol blue 0.04 g  
Sodium citrate·2H<sub>2</sub>O 10 g  
Agar 15 g  
Sodium cholate 3 g  
Distilled water 1 liter  
Oxgall 5 g

Prepare in flask at least 3 times larger than required volume of medium. Add ingredients to warm distilled water and heat to dissolve. Bring just to boil, and immediately remove from heat. DO NOT AUTOCLAVE. Cool to 50°C and pour into petri dishes. Dry the plates overnight or at 37-45°C before use.

M42. Triple Sugar Iron Agar (TSI)

Medium 1	Medium 2
Polypeptone 20 g	Beef extract 3 g
NaCl 5 g	Yeast extract 3 g
Lactose 10 g	Peptone 15 g
Sucrose 10 g	Proteose peptone 5 g
Glucose 1 g	Glucose 1 g
Fe(NH <sub>4</sub> ) <sub>2</sub> (SO <sub>4</sub> ) <sub>2</sub> ·6H <sub>2</sub> O 0.2 g	Lactose 10 g
Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> 0.2 g	Sucrose 10 g
Phenol red 0.025 g	FeSO <sub>4</sub> 0.2 g
Agar 13 g	NaCl 5 g
Distilled water 1 liter	Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> 0.3 g

Phenol red 0.024 g

Agar 12 g

Distilled water 1 liter

These two media are interchangeable for general use. Suspend ingredients of Medium 1 in distilled water, mix thoroughly, and heat with occasional agitation. Boil about 1 min to dissolve ingredients. Fill 16 x 150 mm tubes 1/3 full and cap or plug to maintain aerobic conditions. Autoclave Medium 1 for 15 min at 118°C. Prepare Medium 2 in the same manner as Medium 1, except autoclave 15 min at 121°C. Before the media solidify, incline tubes to obtain 4-5 cm slant and 2-3 cm butt. Final pH, 7.3 ± 0.2 for Medium 1 and 7.4 ± 0.2 for Medium 2.

For use with halophilic *Vibrio* spp., add NaCl to a final concentration of 2-3%.

#### M150. Trypticase Novobiocin (TN) Broth

Trypticase soy broth 30 g

Bile salts No. 3 1.5 g

Dipotassium phosphate 1.5 g

Novobiocin 20 mg

Distilled water 1 liter

Dissolve all ingredients except novobiocin by heating and stirring; autoclave at 121°C for 15 min. Prepare stock solution of novobiocin by adding 20 mg monosodium novobiocin per ml of distilled water. Filter-sterilize. Make fresh stock each time of use, or store frozen at -



10°C in the dark (compound is light-sensitive) for not more than 1 month (half-life is several months at 4°C). Add 1 ml stock solution per liter of medium.

M43. Trypticase (Tryptic) Soy Agar

Trypticase peptone 15 g

Phytone peptone 5 g

NaCl 5 g

Agar 15 g

Distilled water 1 liter

Heat with agitation to dissolve agar. Boil 1 min. Dispense into suitable tubes or flasks. Autoclave 15 min at 121°C. Final pH,  $7.3 \pm 0.2$ . For use with halophilic *Vibrio*

M44. Tryptone (Tryptophane) Broth, 1%

Tryptone or trypticase 10 g

Distilled water 1 liter

Dissolve and dispense 5 ml portions into 16 x 125 or 16 x 150 mm test tubes. Autoclave 15 min at 121°C. Final pH,  $6.9 \pm 0.2$ .

M45. Tryptone Yeast Extract Agar

Tryptone 10 g

Yeast extract 1 g

\*Carbohydrate 10 g

Bromcresol purple 0.04 g

Agar 2 g

Distilled water 1 liter

Dissolve agar with heat and gentle agitation. Adjust pH to  $7.0 \pm 0.2$ . Fill 16 x 125 mm tubes 2/3 full. Autoclave 20 min at  $115^{\circ}\text{C}$ . Before use, steam medium 10-15 min. Solidify by placing tubes in ice water.

\*Glucose and mannitol are the carbohydrates used for identification of *S. aureus*.

M46. Tryptose Phosphate Broth (TPB)(for cell culture)

Tryptose 20 g

Dextrose 2 g

NaCl 5 g

$\text{Na}_2\text{HPO}_4$  2.5 g

Distilled water 1 liter

Sterilize by filtration through  $0.20 \mu\text{m}$  membrane.

M47. Urea Broth

Urea 20 g

Yeast extract 0.1 g

$\text{Na}_2\text{HPO}_4$  9.5 g

$\text{K}_2\text{HPO}_4$  9.1 g

Phenol red 0.01 g

Distilled water 1 liter

Dissolve ingredients in distilled water. DO NOT HEAT. Sterilize by filtration through  $0.45 \mu\text{m}$  membrane. Aseptically dispense 1.5-3.0 ml portions to 13 x 100 mm sterile test tubes. Final pH,  $6.8 \pm 0.2$ .

## REAGENTS

R1. 4 M Ammonium Acetate

Ammonium acetate 308.4 g

Distilled water to make 1 liter

R2. 0.25 M Ammonium Acetate

Ammonium acetate 19.3 g

Distilled water to make 1 liter

R3. Basic Fuchsin Staining Solution

Dissolve 0.5 g basic fuchsin dye in 20 ml 95% ethanol. Dilute to 100 ml with distilled water. Filter if necessary with Whatman No. 31 filter paper to remove any undissolved dye. (TB Carbofuchsin ZN staining solution, available from Difco Laboratories, is satisfactory.)

R4. 0.1 M Bicarbonate Buffer (pH 9.6)

$\text{Na}_2\text{CO}_3$  1.59 g

$\text{NaHCO}_3$  2.93 g

Distilled water 1 liter

Store at room temperature for not more than 2 weeks.

R5. Bovine Serum Albumin (BSA) (1 mg/ml)

Nuclease-free bovine serum albumin 10 mg

Distilled water 10 ml

Place 0.5 ml portions into 1.5 ml plastic conical centrifuge tubes.  
Store frozen.

R6. 1% Bovine Serum Albumin in Cholera Toxin ELISA Buffer

Bovine serum albumin (BSA) 1 g

ELISA buffer for (cholera toxin), pH 7.4 100 ml

Dissolve BSA in ELISA buffer. Aliquot and store at -20°C.

R7. 1% Bovine Serum Albumin in PBS

Bovine serum albumin (BSA) 1 g

Phosphate-buffered saline, pH 7.4 100 ml

Dissolve BSA in PBS buffer. Aliquot and store at -20°C.

R8. Brilliant Green Dye Solution. 1%

Brilliant green dye 1 g

Distilled water (sterile) 10 ml

Dissolve 1 g dye in sterile water. Dilute to 100 ml. Before use, test all batches of dye for toxicity with known positive and negative test microorganisms.

R9. Bromcresol Purple Dye Solution. 0.2%

Bromcresol purple dye 0.2 g

Distilled water (sterile) 100 ml

Dissolve 0.2 g dye in sterile water and dilute to 100 ml.

R10. Bromthymol Blue Indicator. 0.04%

Bromthymol blue 0.2 g

0.01 N NaOH 32 ml

Dissolve bromthymol blue in NaOH. Dilute to 500 ml with distilled water.

R11. Butterfield's Phosphate-Buffered Dilution Water

Stock solution

$\text{KH}_2\text{PO}_4$  34 g

Distilled water 500 ml

Adjust pH to 7.2 with 1 N NaOH. Bring volume to 1 liter with distilled water. Sterilize 15 min at 121°C. Store in refrigerator.

Dilution blanks

Take 1.25 ml of above stock solution and bring volume to 1 liter with distilled water. Dispense into bottles to 90 or  $99 \pm 1$  ml. Sterilize 15 min at 121°C.

R12. Catalase Test

Pour 1 ml 3% hydrogen peroxide over growth on slant culture. Gas bubbles indicate positive test. Alternatively, emulsify colony in 1 drop 3% hydrogen peroxide on glass slide. Immediate bubbling is positive catalase test. If colony is taken from blood agar plate, any carry-over of red blood cells can give false-positive reaction.

R12a. Chlorine Solution, 200 ppm,

Containing 0.1% Sodium Dodecyl Sulfate

Commercial bleach (5.25% sodium hypochlorite) 8 ml  
Distilled water containing 1 g sodium dodecyl sulfate 992 ml

Dissolve 1 g sodium dodecyl sulfate in 992 ml distilled water. Add 8 ml commercial bleach and mix well. Make immediately before use.

R13. 0.05 M Citric Acid (PH 4.0)  
Citric acid (monohydrate) 10.5 g  
Double distilled water to make 1 liter

Dissolve citric acid in 900 ml distilled water. Adjust pH to 4.0 with 6 M NaOH and dilute to 1 liter. Store in refrigerator.

R14. Clark's Flagellar Stain

Solution A

Basic fuchsin, special 1.2 g  
95% ethanol 100 ml

Mix and let stand overnight at room temperature.

Solution B

Tannic acid 3.0 g  
NaCl 1.5 g  
Distilled water 200 ml

Mix solutions A and B. Adjust pH to 5.0 with 1 N NaOH or 1 N HCl, if necessary. Refrigerate 2-3 days before use. Stain is stable 1 month at 4°C or may be stored frozen indefinitely (50 ml portions). To use, thaw stain, remix, and store at 4°C. Optimum staining time for each

batch varies 5-15 min. To determine staining time (after 2-3 days refrigeration at 4°C), stain a known flagellated organism on 3 or more cleaned slides for various times (e.g., 5, 10, 15 min). Mark best staining time on all containers.

IMPORTANT: Stain will not work unless slides are clean. To clean slides, soak them 4 days at room temperature in cleaning solution (either acid dichromate or 3% concentrated HCl in 95% ethanol). Rinse 10 times in fresh tap water and twice in distilled water. Air-dry at room temperature. Store in covered container.

#### Staining Procedure

To prepare suspension, pick small amount of growth from 18-24 h plate (equivalent to 1 mm colony). Do not pick up agar. Suspend gently in 3 ml distilled water. (Flagella can be knocked off.) Suspension should be faintly opalescent.

To prepare slide, pass cleaned slide through blue part of burner flame several times to remove residual dirt. Cool slide, flamed side up, on paper towel. Mark wax line across slide to give area 2.5 x 4.5 cm. Place large loopful of suspension in center of slide adjacent to wax line. Tilt slide, letting drop run down center of slide to end. If drop does not run evenly, slide is dirty. Discard it. Air-dry slide on level surface.

R15. Coating Solution for *V. vulnificus* EIA  
Phosphate-buffered saline 100 ml  
Triton X-100 (a polyoxyethylene ether) 20 µl

Mix Triton X-100 with PBS, pH 7.4.

R16. Crystal Violet Stain (for Bacteria)

1. Crystal violet in dilute alcohol

Crystal violet (90% dye content) 2 g

Ethanol (95%) 20 ml

Distilled water 80 ml

2. Ammonium oxalate crystal violet.

Either solution is generally considered suitable as a simple stain to observe morphology.

R17. Disinfectants

(for preparation of canned foods for microbiological analysis)

1. Alcoholic solution of iodine

Potassium iodide 10 g

Iodine 10 g

Ethanol (70%) 500 ml

2. Sodium hypochlorite solution

\*Sodium hypochlorite 5.0-5.25 g

Distilled water 100 ml

\*Laundry bleach, which is 5.25% sodium hypochlorite (NaOCl), may be used.



R18. Dulbecco's Phosphate-Buffered Saline (DPBS)

NaCl 8.0 g

KCl 200 mg

Na<sub>2</sub>HPO<sub>4</sub> 1.15 g

KH<sub>2</sub>PO<sub>4</sub> 200 mg

CaCl<sub>2</sub> 100 mg

MgCl<sub>2</sub>·6H<sub>2</sub>O 100 mg

Distilled water 1 liter

Dissolve ingredients in water. Sterilize by filtration. Final pH, 7.2.

R19. 0.5 M EDTA

Na<sub>2</sub>EDTA 186.12 g

Dissolve in 800-900 ml dH<sub>2</sub>O . Adjust pH to 8.0 with 10 N NaOH.

Add distilled water to make 1 liter.

R20. EIA (*V. vulnificus*) Wash Solution

NaCl 87.65 g

Tween 20 5.0 ml

Dissolve ingredients in 10 liters of deionized water.

R21. Ethanol Solution, 70%

Ethanol, 95% 700 ml

Distilled water add to final volume of 950 ml.

R22. Ferric Chloride. 10%

FeCl<sub>3</sub> 10 g

Distilled water 90 ml

**R23. Formalinized Physiological Saline Solution**

Formaldehyde solution (36-38%) 6 ml

NaCl 8.5 ml

Distilled water 1 liter

Dissolve 8.5 g NaCl in 1 liter distilled water. Autoclave 15 min at 121°C. Cool to room temperature. Add 6 ml formaldehyde solution. Do not autoclave after addition of formaldehyde.

**R24. Gel Diffusion Agar. 1.2%**

NaCl 8.5 g

Sodium barbital 8.0 g

Merthiolate (crystalline) 0.1 g

Noble special agar (Difco) 12.0 g

Distilled water 1 liter

Dissolve NaCl, sodium barbital, and merthiolate in 900 ml distilled water. Adjust pH to 7.4 with 1 N HCl and/or 1 N NaOH. Bring volume to 1 liter. Add Noble agar. Melt agar mixture in Arnold steamer. Filter in steamer, while hot, through 2 layers of analytical grade filter paper (e.g., No. 588, Schleicher and Schuell or equivalent). Dispense in small (15-25 ml) portions into 4 oz prescription bottles. Do not remelt more than twice.

**R25. Gel-Phosphate Buffer**

Gelatin 2 g

Na<sub>2</sub>HPO<sub>4</sub> 4 g

Distilled water 1 liter

Use gentle heat to dissolve ingredients. Sterilize 20 min at 121°C.  
Final pH, 6.2.

**R26. Giemsa Stain**

Giemsa powder 1 g

Glycerol 66 ml

Methanol (absolute) 66 ml

Distilled stain in glycerol by heating 1.5-2.0 h at 55-60°C. Add methanol. Store stain in tightly stoppered bottle at 22°C for at least 2 weeks. Dilute stock solution with distilled water (1+9) before use.

**R27. Glycerin-Salt Solution (Buffered)**

Glycerin (reagent grade) 100 ml

K<sub>2</sub>HPO<sub>4</sub> (anhydrous) 12.4 g

KH<sub>2</sub>PO<sub>4</sub> (anhydrous) 4 g

NaCl 4.2 g

Distilled water 900 ml

Distilled NaCl and bring volume to 900 ml with water. Add glycerin and phosphates. Adjust pH to 7.2. Autoclave 15 min at 121°C. For double strength (20%) glycerin solution, use 200 ml glycerin and 800 ml distilled water.

**R28. Gram Stain**

(commercial staining solutions are satisfactory)

**Hucker's crystal violet**

**Solution A**

Crystal violet (90% dye content) 2 g

Ethanol, 95% 20 ml

**Solution B**

Ammonium oxalate 0.8 g

Distilled water 80 ml

Mix solutions A and B. Store 24 h and filter through coarse filter paper.

**Gram's iodine**

Iodine 1 g

Potassium iodide (KI) 2 g

Distilled water 300 ml

Place KI in mortar, add iodine, and grind with pestle for 5-10 s. Add 1 ml water and grind; then add 5 ml of water and grind, then 10 ml and grind. Pour this solution into reagent bottle. Rinse mortar and pestle with amount of water needed to bring total volume to 300 ml.

**Hucker's counterstain (stock solution)**

Safranin O (certified) 2.5

Ethanol, 95% 100 ml

Working solution: Add 10 ml stock solution to 90 ml distilled water.

**Staining Procedure**

(Gram stain) Fix air-dried films of food sample in moderate heat. Stain films 1 min with crystal violet-ammonium oxalate solution. Wash briefly in tap water and drain. Apply Gram's iodine for 1 min. Wash in tap water and drain. Decolorize with 95% ethanol until blue color is no longer released (about 30 s). Alternatively, flood slides

with ethanol, pour off immediately, and reflood with ethanol for 10 s. Wash briefly with water, drain, and apply Hucker's counterstain (safranin solution) for 10-30 s. Wash briefly with water, drain, blot or air-dry, and examine.

**R29. Endospore Stain (Schaeffer-Fulton) Solution**  
A Malachite green 10 g Distilled water 100 ml Filter to remove undissolved dye.  
Solution B Safranin O 0.25 g Distilled water 20 ml

**R30. Hippurate Solution, 1%**

Dissolve 0.1 g sodium hippurate in 10 ml distilled water. Filter-sterilize and store refrigerated or in 0.4 ml aliquots at -20°C. Commercial preparations are also available.

**R31. Horseradish Peroxidase**

(color development solution)

**Solution A (horseradish)**

HRP color development reagent 60 mg

Ice cold methanol 20 ml

Mix to dissolve. Protect from light and prepare fresh.

**Solution B**

Ice cold hydrogen peroxide, 30% 60 µl

Tris-buffered saline 100 ml

Prepare fresh before use. Mix ice cold solution A with room temperature solution B. Use immediately.

**R32. 1 N Hydrochloric Acid**

HCl (concentrated) 89 ml

Distilled water to make 1 liter

**R33. Kovacs' Reagent**

*p*-Dimethylaminobenzaldehyde 5 g

Amyl alcohol (normal only) 75 ml

HCl (concentrated) 25 ml

Dissolve *p*-dimethylaminobenzaldehyde in normal amyl alcohol. Slowly add HCl. Store at 4°C. To test for indole, add 0.2-0.3 ml reagent to 5 ml of 24 h bacteria culture in tryptone broth. Dark red color in surface layer is positive test for indole.

**R34. 0.1 N Lithium Hydroxide**

Lithium hydroxide (anhydrous) 2.395 g

Distilled water 1 liter

**R35. Lugol's Iodine Solution**

Potassium iodide (KI) 10 g

Iodine 5 g

Distilled water 100 ml

Dissolve KI in about 20-30 ml of distilled water. Add iodine and heat gently with constant mixing until iodine is dissolved. Dilute to 100 ml

with distilled water. Store in amber glass-stoppered bottle in the dark.

**R36. Methyl Red Indicator**

Methyl red 0.10 g  
Ethanol, 95% 300 ml  
Distilled water to make 500 ml

Dissolve methyl red in 300 ml ethanol. Bring volume to 500 ml with distilled water.

**R37. Methylene Blue Stain (Loeffler's)**

**Solution A**

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

**Solution B**

Diluted potassium hydroxide (0.01%) 100 ml

Mix solutions A and B.

**R38. Nitrite Detection Reagents**

**A. Sulfanilic acid reagent**

Sulfanilic acid 1 g  
5 N acetic acid 125 ml

**B. N-(1-naphthyl)ethylenediamine reagent**

N-(1-naphthyl)ethylenediamine dihydrochloride 0.25 g  
5 N acetic acid 200 ml

**C. alpha-Naphthol reagent**

alpha-Naphthol 1 g

5 N acetic acid 200 ml

To prepare 5 N acetic acid, add 28.75 ml glacial acetic acid to 71.25 ml distilled water. Store reagents in glass-stoppered brown bottles. To perform test, add 0.1-0.5 ml each of reagent A and either reagent B or reagent C (as specified in method) to culture grown in liquid or semisolid medium. Development of red-violet color with reagents A and B or orange color with reagents A and C indicates that nitrate has been reduced to nitrite. Since color produced with reagents A and B may fade or disappear within a few minutes, record reaction as soon as color appears. If no color develops, test for presence of nitrate by adding small amount of zinc dust. If color develops, nitrate has not been reduced.

**Nitrate reduction test for enteropathogenic *E. coli*.** To 3 ml of 18-24 h culture in indole-nitrite medium, add 2 drops each of reagents A and B. Red-violet color indicates that nitrate has been reduced to nitrite. Check negative tests by adding small amount of zinc dust; if red-violet color does not appear, nitrate has been reduced.

**D. Alternative test reagents.** 5-Amino-2-naphthylene sulfonic acid (Cleve's acid) and N,N-dimethyl-1-naphthylamine have been recommended as substitutes for preparation of reagent B. Absolute ethanol may be substituted for acetic acid in reagent C. However, comparative evaluations should be conducted before substitution of these alternative reagents.



**R39. Oxidase Reagent**

N,N,N',N'-Tetramethyl-p-phenylenediamine·2HCl 1 g

Distilled water 100 ml

This is the preferred reagent. Use freshly prepared. However, reagent can be used up to 7 days if stored in a dark glass bottle under refrigeration. Apply freshly prepared solution directly to young culture (24 h) on either agar plate or slant. Oxidase-positive colonies develop a pink color and progressively turn dark purple. If cultures are to be preserved, complete the transfer from plates to which reagent has been added within 3 min, since reagent is toxic to organisms.

**R40. Peptone Diluent, 0.1%**

Peptone 1 g

Distilled water 1 liter

Autoclave 15 min at 121°C. Final pH, 7.0 + 0.2.

**R41. 0.01 M Phosphate-Buffered Saline (pH 7.5) Stock solution (0.1 M)**

Na<sub>2</sub>HPO<sub>4</sub> (anhydrous) 12.0 g

NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O 2.2 g

NaCl 85.0 g

Distilled water 1 liter

Dissolve ingredients in distilled water and bring volume to 1 liter. Dilute stock solution 1+9 in double distilled water. Mix well. Adjust pH to 7.5 with 0.1 N HCl or 0.1 N NaOH if necessary.

**R42. 0.02 M Phosphate Saline Buffer (PH 7.3-7.4)**

Prepare stock solutions of 0.2 M mono- and disodium phosphate in 8.5% salt solutions and dilute 1:10 for preparation of 0.02 M phosphate saline buffer.

**Stock solution 1**

Sodium phosphate dibasic anhydrous  $\text{Na}_2\text{HPO}_4$  (anhydrous)  
(reagent grade) 28.4 g  
NaCl (reagent grade) 85.0 g  
Distilled water to make 1 liter

**Stock solution 2**

Sodium phosphate monobasic monohydrate  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$   
(monohydrate) (reagent grade) 227.6 g  
NaCl (reagent grade) 85.0 g  
Distilled water to make 1 liter

To obtain 0.02 M phosphate-buffered saline (0.85%), make 1:10 dilutions of each stock solution. For example:

Stock solution 1 50 ml    Stock solution 2 10 ml

Distilled water 450 ml    Distilled water 90 ml

Approximate pH, 8.2    Approximate pH, 5.6

Using pH meter, titer diluted solution 1 to pH 7.3-7.4 by adding about 65 ml of diluted solution 2. Use resulting 0.02 M phosphate saline buffer solution in the lysostaphin susceptibility test on *S. aureus*.

**NOTE:** Do not titer 0.2 M phosphate buffer to pH 7.3-7.4 and then dilute to 0.02 M strength. This results in a drop in pH of approximately 0.25. Addition of 0.85% salt after pH adjustment also results in a drop of approximately 0.2.

**R43. Physiological Saline Solution 0.85% (Sterile)**

NaCl 8.5 g

Distilled water 1 liter

Dissolve 8.5 g NaCl in water. Autoclave 15 min at 121°C. Cool to room temperature.

**R44 Potassium Hydroxide Solution. 40%**

KOH 40 g

Distilled water to make 100 ml

**R45. Saline Solution. 0.5% (Sterile)**

NaCl 5 g

Distilled water 1 liter

Dissolve NaCl in water. Autoclave at 121°C for 15 min.

**R46. Salts-Phosphate Buffered Saline Solution (Salts-PBS)**

NaCl 121 g

KCl 15.5 g

MgCl<sub>2</sub> 12.7 g

CaCl<sub>2</sub>·2H<sub>2</sub>O 10.2 g

NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O 2.0 g

$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  3.9 g

Distilled water 1 liter

Adjust pH to 7.4.

**R47. Slide Preserving Solution**

Prepare 1% acetic acid solution (10 ml glacial acetic acid, reagent grade + 990 ml distilled water). Add 1 ml glycerin to each 100 ml of solution.

**R48. Sodium Bicarbonate Solution. 10%**

Sodium bicarbonate 100 g

Distilled water to make 1 liter

Sterilize by filtration.

**R49. 0.2 M Sodium Chloride Solution**

NaCl 11.7 g

Distilled water to make 1 liter

Dispense in suitable containers. Autoclave 15 min at 121°C.

**R50. 1 N Sodium Hydroxide Solution**

NaOH 40 g

Distilled water to make 1 liter

Use for adjusting pH of culture media.

**R51. 10 N Sodium Hydroxide Solution**

NaOH 400 g

Distilled water to make 1 liter

**R52. Standard Saline Citrate (SSC) Solution (20%)**

NaCl (reagent grade) 175.3 g

Sodium citrate 88.2 g

Dissolve in 800 ml deionized water and adjust to pH 7 with 10 N NaOH. Bring volume to 1 liter.

**6X SSC**

NaCl (reagent grade) 52.6 g

Sodium citrate 26.5 g

Dissolve in 800 ml deionized water and adjust to pH 7 with 10 N NaOH. Bring volume to 1 liter.

**3X SSC**

6X SSC 500 ml

Deionized water 500 ml

**2X SSC**

6X SSC 333 ml

Deionized water 667 ml

**R53. Tris-Buffered Saline (TBS) (PH 7.5)**

Tris 2.42 g

NaCl 29.24 g

Double distilled water to make 1 liter

Dissolve ingredients. Adjust pH to 7.5 with HCl and bring volume to 1 liter.

**R54. Tris-Buffered Saline (TBS), with Gelatin**

**1% solution**

Gelatin 1 g

TBS, pH 7.5 100 ml

**3% solution**

Gelatin 3 g

TBS, pH 7.5 100 ml

Add gelatin to TBS at 40°C. Stir to dissolve. Cool to room temperature before use.

**R55. Tris-Buffered Saline (TBS)-Tween**

Tween 2050 F1 TBS, pH 7.5 100 ml

Dissolve Tween 20 in TBS.

**R56. Tris-Buffered Saline (TABS), 1% or 3% Gelatin, or Tween 20**

Tris 2.42 g

NaCl 29.24 g

Distilled water 1 liter

Dissolve ingredients in distilled water by heating and stirring. Adjust pH to 7.5 with HCl. Autoclave 15 min at 121°C.

**For 1% and 3% Gelatin-TABS**, add 10 g and 30 g gelatin, respectively, to ingredients before autoclaving. Adjust final pH to 7.5 with HCl.

**For Tween-TABS**, add 0.5 ml Tween 20 to ingredient and adjust pH to 7.5 before autoclaving.

### **R57. Voges-Proskauer (VP) Test Reagents**

#### **Solution 1**

alpha-Naphthol 5 g

Alcohol (absolute) 100 ml

#### **Solution 2**

Potassium hydroxide 40 g

Distilled water to make 100 ml

**Voges-Proskauer (VP) test.** Transfer 1 ml of 48 h culture to test tube and add 0.6 ml solution 1 and 0.2 ml solution. Shake after adding each solution. To intensify and speed reaction, add a few creatine crystals to mixture. Let stand at room temperature. Read results 4 h after adding reagents. Development of eosin pink color is positive test.

## GLOSSARY

**Asexual reproduction:** Reproduction in which sex cells are not involved; as by binary fission or budding.

**Acute:** Having rapid onset, severe symptoms and a short course.

**Antigen:** Foreign substance when gets into the body induces immune response.

**Antibody:** Endogenous glycoprotein, which reacts with antigen.

**Blood Brain Barrier:**

**Chronic:** Of long duration; denoting a disease with slow progression.

**Convulsion:** Paroxysms of involuntary muscular contraction and relaxation.

**Congenital:** Present at birth.

**Dehydration:** a condition resulting from loss of excessive body fluid.

**Disease:** Pathological condition of the body that presents with group of clinical symptoms and signs; and abnormal laboratory findings.

**DNA:** A nucleic acid consisting of deoxyribose, phosphoric acid and bases.

It is present in chromosomes of the nuclei of cells, is the chemical basis of heredity and the carrier of genetic information for living cells.

**Endogenous:** Produced or originating from within a cell or organism.

**Endoplasmic reticulum:** Net work of membranous tubules within a cell and involved in transport of proteins synthesized on the ribosomes; and synthesis of lipids.

**Electrolyte:** An ionized salt in blood, tissue fluids and cells.



**Fastidious:** Requiring precise nutritional and environmental conditions for growth and survival.

**Genome:**

**Hematogenous:** Through the blood stream.

**Histone:** Positively charged protein that is part of chromatin in eukaryotic cells.

**Hydrocephalus:** Excessive fluid in the brain ventricles.

**Iatrogenic:** Any adverse mental or physical condition induced in a patient through the effects of treatment by a physician or surgeon.

**Incubation Period:** The time interval between exposure and development of disease.

**Infertility:** The inability or diminished ability to produce offspring.

**Lysosome:** Cell organelle that is part of the intracellular digestive system.

**Microscopic:** Can not be observed with naked eye.

**Macroscopic:** Can be observed with naked eye.

**Microscope:** Optical instrument that greatly magnifies minute objects.

**Microorganism:** Minute living body not seen with naked eye.

**Mitochondria:** Oval shaped cell organelles that contain the enzymes for aerobic stages of cell respiration and thus the site of ATP synthesis.

**Microtubule/Microfilament:** Tubular structures present in an eukaryotic cell and are important for maintaining rigidity; transporting substances in different directions within a cell.

**Nuclear membrane:** A membrane enveloping nucleus of a living cell.

**Nucleolus:** Structure in the nucleus of a cell made of DNA, RNA, and protein.

It is the site of synthesis of ribosomal RNA(rRNA).

**Purulent:** Full of pus

**Postulate:** A supposition or view, usually self-evident that is assumed with out proof.

**Pleural effusion:** Fluid accumulated in pleural cavity.

**Primary stain:** The dye applied first in differential staining procedures.

**Counter stain:** The dye which stains the micro-organism or part of it after decolorization of the primary stain.

**Mordant:** It is a substance which facilitates the reaction of the primary stain with the material to be stained. It combines with the stain and then facilitate the reaction. Basic mordant reacts with acidic stain and acidic mordant react swith basic stain.

**Decolorizer :** It is a chemical added in differential staining procedure to selectively remove the stain from the materials that are not intended to be stained.

**Pathogen :** Organism that causes disease

**Virulence :** Degree of pathogenicity in causing disease which depends on toxin production and invasiveness.

**Invasiveness :** The ability to penetrate in to the tissues, overcome the host defense, multiply and disseminate widely.

**Toxicity:** The capacity to damage the tissues.

**Opportunistic :** Normally harmless organism causing disease during lowered host resistance.

**Infection:** The result of breakdown in the host-parasite relationship and follows when the balance is tipped in favor of the parasite.



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