# Shree H. N. Shukla Institute of Pharmaceutical Education and Research,Rajkot

## B. Pharm
## Semester-5

## STUDY MATERIAL

**Subject Name:Pharmaceutical Microbiology**
**Subject Code: BP504TP**

**CHAPTER 2** : Staining and sterility

CONTAINS:
Identification of bacteria using staining techniques (simple, Gram's &Acid fast staining) and biochemical tests (IMViC). Study of principle, procedure, merits, demerits and applications of physical, chemical gaseous,radiation and mechanical method of sterilization. Evaluation of the efficiency of sterilization methods. Equipment employed in large scale sterilization. Sterility indicators.

# Staining Techniques

In their natural state, most of the cells and microorganisms that we observe under the microscope lack color and contrast. This makes it difficult, if not impossible, to detect important cellular structures and their distinguishing characteristics without artificially treating specimens. We have already alluded to certain techniques involving stains and fluorescent dyes, and in this section we will discuss specific techniques for sample preparation in greater detail. Indeed, numerous methods have been developed to identify specific microbes, cellular structures, DNA sequences, or indicators of infection in tissue samples, under the microscope. Here, we will focus on the most clinically relevant techniques.

## Preparing Specimens for Light Microscopy

In clinical settings, light microscopes are the most commonly used microscopes. There are two basic types of preparation used to view specimens with a light microscope: wet mounts and fixed specimens.

The simplest type of preparation is the **wet mount**, in which the specimen is placed on the slide in a drop of liquid. Some specimens, such as a drop of urine, are already in a liquid form and can be deposited on the slide using a dropper. Solid specimens, such as a skin scraping, can be placed on the slide before adding a drop of liquid to prepare the wet mount. Sometimes the liquid used is simply water, but often stains are added to enhance contrast. Once the liquid has been added to the slide, a coverslip is placed on top and the specimen is ready for examination under the microscope.

The second method of preparing specimens for light microscopy is **fixation**. The "fixing" of a sample refers to the process of attaching cells to a slide. Fixation is often achieved either by heating (**heat fixing**) or chemically treating the specimen. In addition to attaching the specimen to the slide, fixation also kills microorganisms in the specimen, stopping their movement and metabolism while preserving the integrity of their cellular components for observation.

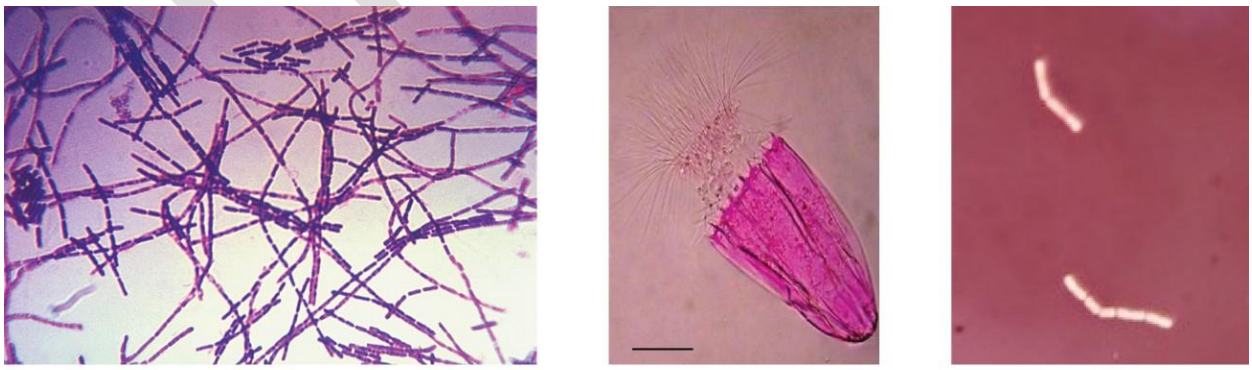
To heat-fix a sample, a thin layer of the specimen is spread on the slide (called a **smear**), and the slide is then briefly heated over a heat source (Figure 1b). **Chemical fixatives** are often preferable to heat for tissue specimens. Chemical agents such as acetic acid, ethanol, methanol, formaldehyde (formalin), and glutaraldehyde can denature proteins, stop biochemical reactions, and stabilize cell structures in tissue samples (Figure 1c).


(a) (b) (c)

Figure 1. (a) A specimen can be heat-fixed by using a slide warmer like this one. (b) Another method for heat-fixing a specimen is to hold a slide with a smear over a microincinerator. (c) This tissue sample is being fixed in a solution of formalin (also known as formaldehyde). Chemical fixation kills microorganisms in the specimen, stopping degradation of the tissues and preserving their structure so that they can be examined later under the microscope. (credit a: modification of work by Nina Parker; credit b: modification of work by Nina Parker; credit c: modification of work by "University of Bristol"/YouTube)

In addition to fixation, **staining** is almost always applied to color certain features of a specimen before examining it under a light microscope. Stains, or dyes, contain salts made up of a positive ion and a negative ion. Depending on the type of dye, the positive or the negative ion may be the chromophore (the colored ion); the other, uncolored ion is called the counterion. If the chromophore is the positively charged ion, the stain is classified as a **basic dye**; if the negative ion is the chromophore, the stain is considered an **acidic dye**.

Dyes are selected for staining based on the chemical properties of the dye and the specimen being observed, which determine how the dye will interact with the specimen. In most cases, it is preferable to use a **positive stain**, a dye that will be absorbed by the cells or organisms being observed, adding color to objects of interest to make them stand out against the background. However, there are scenarios in which it is advantageous to use a **negative stain**, which is absorbed by the background but not by the cells or organisms in the specimen. Negative staining produces an outline or silhouette of the organisms against a colorful background (Figure 2).



(a) (b) (c)

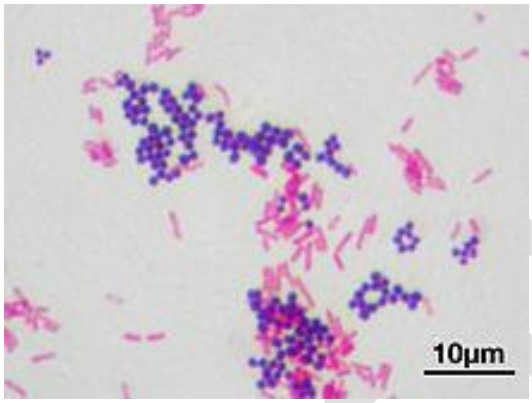
Figure 2. (a) These *Bacillus anthracis* cells have absorbed crystal violet, a basic positive stain. (b) This specimen of *Spinoloricus*, a microscopic marine organism, has been stained with rose bengal, a positive acidic stain. (c) These *B. megaterium* appear to be white because they have not absorbed the negative red stain applied to the slide. (credit a: modification of work by Centers for Disease Control and Prevention; credit b: modification of work by Roberto Danovaro, Antonio Pusceddu, Cristina Gambi, IbenHeiner, Reinhardt MobjergKristensen; credit c: modification of work by Anh-Hue Tu)

Because cells typically have negatively charged cell walls, the positive chromophores in basic dyes tend to stick to the cell walls, making them positive stains. Thus, commonly used basic dyes such as **basic fuchsin**, **crystal violet**, **malachite green**, **methylene blue**, and **safranin** typically serve as positive stains. On the other hand, the negatively charged chromophores in acidic dyes are repelled by negatively charged cell walls, making them negative stains. Commonly used acidic dyes include **acid fuchsin**, **eosin**, and **rose bengal**. Table 2 provides more detail.

Some staining techniques involve the application of only one dye to the sample; others require more than one dye. In **simple staining**, a single dye is used to emphasize particular structures in the specimen. A simple stain will generally make all of the organisms in a sample appear to be the same color, even if the sample contains more than one type of organism. In contrast, **differential staining** distinguishes organisms based on their interactions with multiple stains. In other words, two organisms in a differentially stained sample may appear to be different colors. Differential staining techniques commonly used in clinical settings include Gram staining, acid-fast staining, endospore staining, flagella staining, and capsule staining.

## Gram's technique

In 1884, physician Hans Christian Gram was studying the etiology (cause) of respiratory diseases such as pneumonia. He developed a staining procedure that allowed him to identify a bacterium in lung tissue




taken from deceased patients as the etiologic agent of a fatal type of pneumonia. Although it did little in the way of treatment for the disease, the Gram stain method made it much easier to diagnose the cause of a person's death at autopsy. Today we use Gram's staining techniques to aid in the identification of bacteria, beginning with a preliminary classification into one of two groups: **Gram positive** or **Gram negative**.

Figure 5. Bacteria stained with Gram stain.

The differential nature of the Gram stain is based on the ability of some bacterial cells to retain a primary stain (crystal violet) by resisting a decolorization process. Gram staining involves four steps. First cells are stained with crystal violet, followed by the addition of a setting agent for the stain (iodine). Then alcohol is applied, which selectively removes the stain from only the Gram negative cells. Finally, a secondary stain, safranin, is added, which counterstains the decolorized cells pink.

Although Gram didn't know it at the time, the main difference between these two types of bacterial cells is their cell walls. Gram negative cell walls have an outer membrane (also called the envelope) that dissolves during the alcohol wash. This permits the crystal violet dye to escape. Only the decolorized cells take up the pink dye safranin, which explains the difference in color between the two types of cells. At the conclusion of the Gram stain procedure, Gram positive cells appear purple, and Gram negative cells appear pink.

When you interpret a Gram stained smear, you should also describe the morphology (shape) of the cells, and their arrangement. In Figure 5, there are two distinct types of bacteria, distinguishable by Gram stain reaction, and also by their shape and arrangement.

# Biochemical tests (IMViC)

The ' tests are a group of individual tests used in microbiology lab testing to identify an organism in the coliform group. A coliform is a gram negative, aerobic anaerobic rod which produces gas from lactose within 48 hours. The presence of some coliforms indicate fecal contamination.

The term "IMViC" is an acronym for each of these tests. "I" is for indole test; "M" is for methyl red test; "V" is for Voges-Proskauer test, and "C" is for citrate test. The lower case "i" is merely for "in" as the Citrate test requires coliform samples to be placed "in Citrate". Triple sugar iron test (TSI) is also included in this test.

These tests are useful in distinguishing members of Enterobacteriaceae.

## Indole test

In this test, the organism under consideration is grown in peptone water broth. It contains tryptophan, which under the action of enzyme tryptophanase is converted to an Indole molecule, pyruvate and ammonium. The indole is then extracted from the broth by means of xylene. To test the broth for indole production, Kovac's reagent is added after incubation. A positive result is indicated by a pink/red layer forming on top of the liquid.

## Methyl red and Voges–Proskauer test

These tests both use the same broth for bacterial growth. The broth is called MRVP broth. After growth, the broth is separated into two different tubes, one for the methyl red (MR) test and one for the Voges-Proskauer (VP) test.

The methyl red test detects production of acids formed during metabolism using mixed acid fermentation pathway using pyruvate as a substrate. The pH indicator Methyl Red is added to one tube and a red color appears at pH's lower than 4.2, indicating a positive test (mixed acid fermentation is used). The solution remaining yellow (pH = 6.2 or above) indicates a negative test, meaning the butanediol fermentation is used.

The VP test uses alpha-naphthol and potassium hydroxide to test for the presence of acetylmethylcarbinol (acetoin), an intermediate of the 2,3-butanediol fermentation pathway. After adding both reagents, the tube is shaken vigorously then allowed to sit for 5-10 minutes. A pinkish-red color indicates a positive test, meaning the 2,3-butanediol fermentation pathway is used.

## Citrate test

This test uses Simmon's citrate agar to determine the ability of a microorganism to use citrate as its sole carbon source. The agar contains citrate and ammonium ions (nitrogen source) and bromothymol blue as an indicator. The citrate agar is green before inoculation, and turns blue as a positive test indicator, meaning citrate is utilized.

# Sterilization methods

## Physical method: -

a.Dry heat sterilization:

1.Flaming: in this method the articles should be sterilized on the hot flame of Bunsen burner.

2. Hot air oven: - It is also called dry heat sterilizer. The articles should be sterilized at the temperature 150c-170c for 1hour.

Glass wear like test tube, pipette, flask and glass syringes etc. can be sterilized by this method. Fixed oil glycerin, liquid paraffin, propylene glycol and zinc oxide etc also can be sterilized by this method.

b.Moist heat sterilization: -

Moist heat sterilization is also known as steam sterilization. It is done is an auto clave but on small scale of pressure cooker can be used.

1. Auto clave: - Auto cleaving is the process of moist heating in an autoclave, in which destroys the micro-organism at the process of moist heating in an autoclave, in which destroys the micro-organism at the temperature of 120c on 1510 pressure for 20 minutes. Time is recorded from the temperature at 121c. the sterilization is done by steam heat under pressure.

Use: - Glass wear, surgical dressing, rubber, gloves, gurgical instrument are sterilized by this method (Autoclaved)

Solution packed in sealed container or seated ampoules are sterilized by this method. If the solution is below 100ml auto calved for 30minute. More than 100ml on hour period is needed.

2. Horizontal large scale autoclave: - It is used pharmaceutical industry ad big hospital.

3.Sterilization by boiling water: - The boiling water bath or electric boiling sterilizer is useful for sterilizing instruments like syringe, needles, knives blades, scissor, scalpel and other surgical instrument. Temperature at the 100c (boiling point) for 20 minutes.

4. Other method of sterilization: -

Pasteurization.

a. Holding method: 62.8c and held the temperature for 30min.

b. Flash method or high temperature short time: in this method at the temperature 161F for 25 second.

## Chemical method: -

a. Gaseous sterilization: - It is especial type of chemical sterilization in which chemical reused as gas or vapor and not the liquid but solid dissolved in a solution many gas like sulfur dioxide, chlorine

formaldehyde, ozone, ethylene oxide, have bactericidal effect. Now day ethylene oxide widely used as gaseous sterilizing agent.

b. Sterilization by disinfectant: - Disinfectant are mainly used for sterilizing the surfeit for aseptic wash. In emergency. They may be used for sterilizing for the sugars instrument, iodine, chloride, caracole soap solution, phenol an formaldehyde are commonly used disinfectant.

## Radiation

UV, x-rays and gamma rays are all types of electromagnetic radiation that have profoundly damaging effects on DNA, so make excellent tools for sterilization.

The main difference between them, in terms of their effectiveness, is their penetration.

UV has limited penetration in air so sterilisation only occurs in a fairly small area around the lamp. However, it is relatively safe and is quite useful for sterilising small areas, like laminar flow hoods.

X-rays and gamma rays are far more penetrating, which makes them more dangerous but very effective for large scale cold sterilization of plastic items (e.g. syringes) during manufacturing.

## Mechanical

**Mechanical sterilization removes** but**does not destroy** microorganisms and clarifies solutions by eliminating particulate matter. For **solutions** rendered unstable by thermal, chemical, or radiation sterilization, filtration is the preferred method .

A **depth filter** or **screen filter** may be used :

**Depth filter** usually consist of fritted glass or unglazed porcelain (i.e., substances that trap particles in channels ).

**Screen (membrane) filters** are films measuring 1-200 mm thick made of cellulose esters, microfilament, polycarbonate , synthetics polymers, silver, or stainless steel. **A mesh** of millions of microcapillary pores of identical size filter the solution by a process of physical sieving .

**Types of screen filters**

- Particulate filters

Particulate Filters remove particles of glass, plastic, rubber, and other contaminants.These filter also are used to reduce the risk of phlebitis associated with administration of reconstituted powders. filtration removes any undissolved powder particles that may cause venous inflammation. The pore size of standard particulate filters ranges from 0.45-5mm .

Special particulate filters are required to filter blood, emulsion (e.g., fat emulsion), or colloidal dispersions or suspensions because these preparation have a larger particle size.

- Microbial filters

With a pore size of 0.22 mm or smaller, ensure complete microbial removal and sterilization .

This is referred to as cold sterilization

- Final filter

Which may be either particulate or microbial , are often included as part of the tubing used in drug administration . They are referred to as in – line filters and are used to remove particulates or microorganisms from an intravenous (IV) solution during infusion .

# Equipment used for large scale sterilization

## Autoclave

Is a device to sterilize equipment and suppliesby subjecting them to high pressure saturatedsteam .

TYPES

- Portable autoclave (Bench autoclave)
- Stationary autoclave (Large sterilizer)

Main Features

- Lid(door) fitted with clamps and asbestosjacket , stationary autoclave may be doubledoors at both ends one for loading and onefor unloading.
- Pressure gauge
- Thermocouple for measurement of temp
- Air vent to remove air before sterilization.
- Safety valve to permit escape of excesssteam to prevent explosion .
- Modern autoclaves are recording (recordpressure, temp during the whole process )supplied with timer and are automaticallycontrolled .

## Hot air oven

Is a device to sterilize subject and supplies bysubjecting them to direct heat

It is appropriate for the materials that can notwithstand steam sterilization (e.g. oily materials& powders)

This method can be used only if higher temperature can be used, lessexposure time is required.

Main Features

- Door fitted with the clamps and asbestosjacket has the single door
- Regulator for temperature control
- Fan attached inside for air circulation
- Perforated shelf for keeping subject inside

## Radiation

Non ionizing radiations (UV)

◦ UV in region of 2537 A° has been shown toposses the greatest activity in destroying MO

◦ Commonly employed in reduction of air-borne contamination in the maintenance ofaseptic areas & rooms

◦ Source of artificial UV radiation s is UV lamps(generally called sterilizing lamp or germicidallamp)

◦ UV light is absorbed by the nucleic acid ofthe cell where it does the greatest damage


## Filtration

This is a non-thermal method of sterilizationused widely in the p'ceutical industry whereheat labile solutions are to be sterilized. This is useful for large volume solutions, eyedrops, antibiotic solutions, sera &carbohydrate solutions. This also useful for separation ofbacteriophages & bacterial toxins frombacteria for the isolation of MO which arescanty in fluids

3 main stages involved in filtration

1. Passage of the solution through apreviously sterilized bacteria-proof filterunite
2. Aseptic transference of filtrate to sterilecontainers then sealed aseptically

3. Testing of sample for sterility

### Gaseous (Ethylene Oxide)

This type of sterilization is done by putting the specimen in large chambers and then creating vacuum in those chambers. Once vacuum has been generated, the chamber is filled with the sterilization gas, like Ethylene Oxide, etc. and then subjected to temperature and pressure as per requirement.

## Sterility Indicators

Changing appearances in color or pattern, the sterilization indicators visually show if cleaning conditions are passing or procedures have been completed. Eliminating any confusion or possibility instruments will not be sterile, indicators are used routinely in clinical and research environments where contamination elimination is crucial. With the temperature resistance required to endure the purification, the sterilization indicators are available in different forms such as tapes, ampoules, and sticks.

They can be broadly be divided into 2 types:

### Biological Indicators for Sterilization

Sterilization indicators, such as spore strips and indicator tape, enable routine monitoring, qualification,and load monitoring of the steam sterilization process. They indicate whether the conditions during a steam autoclave cycle were adequate to achieve a defined level of microbial inactivation.

**Bacterial Spores:**

- Bacillus atrophaeus (BA)
- Geobacillusstearothermophilus (GS)

### Chemical Indicators for Sterilization

Chemical indicators are designed to respond to one or more of the physical conditions within the sterilizing chamber. Use them to routinely monitor the process parameters of your sterilization process to ensure that they meet your quality assurance goals.

**Popular Applications:**

- Pre-vacuum sterilizers
- Dry heat sterilization
- Steam sterilization processes
- Ethylene oxide (EO) sterilization processes
- VHP Biodecontamination Units