PHARMACEUTICAL MICROBIOLOGY AND BIOTECHNOLOGY

Sterilization Methods and Principles

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Introduction

Sterilization can be defined as any process that effectively kills or eliminates transmissible agents (such as fungi, bacteria, viruses and prions) from a surface, equipment, foods, medications, or biological culture medium. In practice sterility is achieved by exposure of the object to be sterilized to chemical or physical agent for a specified time. Various agents used as steriliants are: elevated temperature, ionizing radiation, chemical liquids or gases etc. The success of the process depends upon the choice of the method adopted for sterilization.

Pharmaceutical Importance of Sterilization

• Moist heat sterilization is the most efficient biocidal agent. In the pharmaceutical industry it is used for: Surgical dressings, Sheets, Surgical and diagnostic equipment, Containers, Closures, Aqueous injections, Ophthalmic preparations and Irrigation fluids etc.

• Dry heat sterilization can only be used for thermo stable, moisture sensitive or moisture impermeable pharmaceutical and medicinal. These include products like; Dry powdered drugs, Suspensions of drug in non aqueous solvents, Oils, fats waxes, soft hard paraffin silicone, Oily injections, implants, ophthalmic ointments and ointment bases etc.

• Gaseous sterilization is used for sterilizing thermolabile substances like; hormones, proteins, various heat sensitive drugs etc.

• U.V light is perhaps the most lethal component in ordinary sunlight used in sanitation of garments or utensils.

• Gamma-rays from Cobalt 60 are used to sterilize antibiotic, hormones, sutures, plastics and catheters etc.

• Filtration sterilizations are used in the treatment of heat sensitive injections and ophthalmic solutions, biological products, air and other gases for supply to aseptic areas. They are also used in industry as part of the venting systems on fermentors, centrifuges, autoclaves and freeze driers. Membrane filters are used for sterility testing.

Variables that affect sterilization include:

1. The dryness of devices to be processed
2. The temperature and humidity of the processing area
3. Whether or not the devices were properly prepared and loaded into the sterilizer
4. Whether or not the sterilizing agent is properly delivered into the system
5. The sterilizer's condition and maintenance protocol
6. Whether or not the correct sterilization method and cycle were used

Terms commonly used

Survivor curves

They are plots of the logarithm of the fraction of survivors (microorganisms which retain viability following a sterilization process) against the exposure time or dose.

Expression of resistance

D-value
D-value is indicative of the resistance of any organism to a sterilizing agent. For radiation and heat treatment, D-value is the time taken at a fixed temperature or the radiation dose required to achieve a 90% reduction in viable count.

Z-value
Z-value represents the increase in temperature needed to reduce the D-value of an organism by 90%.

Methods of Sterilization
The various methods of sterilization are:

1. Physical Method
    a. Thermal (Heat) methods
    b. Radiation method
    c. Filtration method
2. Chemical Method
    a. Gaseous method

1. Heat Sterilization
Heat sterilization is the most widely used and reliable method of sterilization, involving destruction of enzymes and other essential cell constituents. The process is more effective in hydrated state where under conditions of high humidity, hydrolysis and denaturation occur, thus lower heat input is required. Under dry state, oxidative changes take place, and higher heat input is required.

This method of sterilization can be applied only to the thermostable products, but it can be used for moisture-sensitive materials for which dry heat (160-180^{0}C) sterilization, and for moisture-resistant materials for which moist heat (121-134^{0}C) sterilization is used.

The efficiency with which heat is able to inactivate microorganisms is dependent upon the degree of heat, the exposure time and the presence of water. The action of heat will be due to induction of lethal chemical events mediated through the action of water and oxygen. In the presence of water much lower temperature time exposures are required to kill microbe than in the absence of water. In this processes both dry and moist heat are used for sterilization.

a. **Dry Heat Sterilization:** Examples of Dry heat sterilization are:

1. Incineration
2. Red heat
3. Flaming
4. Hot air oven

It employs higher temperatures in the range of 160-180^{0}C and requires exposures time up to 2 hours, depending upon the temperature employed. The benefit of dry heat includes good penetrability and non-corrosive nature which makes it applicable for sterilizing glasswares and

metal surgical instruments. It is also used for sterilizing non-aqueous thermostable liquids and thermostable powders. Dry heat destroys bacterial endotoxins (or pyrogens) which are difficult to eliminate by other means and this property makes it applicable for sterilizing glass bottles which are to be filled aseptically.

Hot-air oven

Dry heat sterilization is usually carried out in a hot air oven, which consists of the following:

i) An insulated chamber surrounded by an outer case containing electric heaters.
ii) A fan
iii) Shelves
iv) Thermocouples
v) Temperature sensor
vi) Door locking controls.

Operation

i) Articles to be sterilized are first wrapped or enclosed in containers of cardboard, paper or aluminum.
ii) Then, the materials are arranged to ensure uninterrupted air flow.
iii) Oven may be pre-heated for materials with poor heat conductivity.
iv) The temperature is allowed to fall to 40^0C, prior to removal of sterilized material.

b.**Moist Heat Sterilization:** Moist heat may be used in three forms to achieve microbial inactivation

1. Dry saturated steam – Autoclaving
2. Boiling water/ steam at atmospheric pressure
3. Hot water below boiling point

Moist heat sterilization involves the use of steam in the range of 121-134^{0}C. Steam under pressure is used to generate high temperature needed for sterilization. Saturated steam (steam in thermal equilibrium with water from which it is derived) acts as an effective sterilizing agent. Steam for sterilization can be either wet saturated steam (containing entrained water droplets) or dry saturated steam (no entrained water droplets).

Autoclaves use pressurized steam to destroy microorganisms, and are the most dependable systems available for the decontamination of laboratory waste and the sterilization of laboratory glassware, media, and reagents. For efficient heat transfer, steam must flush the air out of the autoclave chamber. Before using the autoclave, check the drain screen at the bottom of the chamber and clean if blocked. If the sieve is blocked with debris, a layer of air may form at the bottom of the autoclave, preventing efficient operation. Autoclaves should be tested periodically with biological indicators like cultures of *Bacillus stearothermophilus* to ensure proper function. This method of sterilization works well for many metal and glass items but is not acceptable for rubber, plastics, and equipment that would be damaged by high temperatures (Figure 1).

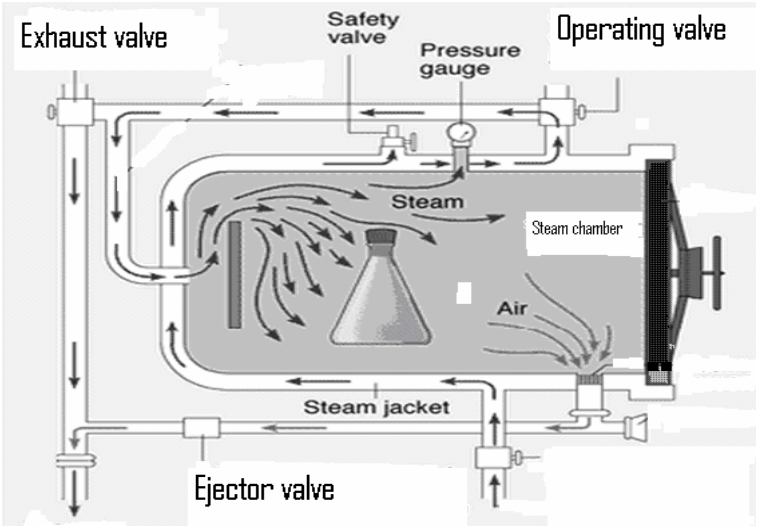

Fig. 1: An Autoclave

Autoclaves, or steam sterilizers essentially consist of following:

i) A cylindrical or rectangular chamber, with capacities ranging from 400 to 800 liters.
ii) Water heating system or steam generating system
iii) Steam outlet and inlet valves
iv) Single or double doors with locking mechanism.
v) Thermometer or temperature gauge
vi) Pressure gauges

Operation

For porous loads (dressings) sterilizers are generally operated at a minimum temperature of 134^0C, and for bottled fluid, sterilizers employing a minimum temperature of 121^0C are used. Ensure that there should be sufficient water in the autoclave to produce the steam. The stages of operation of autoclaves include air removal, steam admission and sterilization cycle (includes heating up, holding/exposure, and cooling stages).

Gaseous Sterilization

The chemically reactive gases such as formaldehyde, (methanol, H.CHO) and ethylene oxide $(CH_2)_2O$ possess biocidal activity. Ethylene oxide is a colorless, odorless, and flammable gas.

The mechanism of antimicrobial action of the two gases is assumed to be through alkylations of sulphydryl, amino, hydroxyl and carboxyl groups on proteins and amino groups of nucleic acids. The concentration ranges (weight of gas per unit chamber volume) are usually in range of 800-1200 mg/L for ethylene oxide and 15-100 mg/L for formaldehyde with operating temperatures of 45-63°C and 70-75°C respectively.

Both of these gases being alkylating agents are potentially mutagenic and carcinogenic. They also produce acute toxicity including irritation of the skin, conjunctiva and nasal mucosa.

a. Ethylene oxide sterilizer: An ethylene oxide sterilizer consists of a chamber of 100-300-Litre capacity and surrounded by a water jacket. Air is removed from sterilizer by evacuation, humidification and conditioning of the load is done by passing sub-atmospheric pressure steam, then evacuation is done again and preheated vaporized ethylene oxide is passed. After treatment, the gases are evacuated either directly to the outside atmosphere or through a special exhaust system.

Ethylene oxide gas has been used widely to process heat-sensitive devices, but the aeration times needed at the end of the cycle to eliminate the gas made this method slow.

b. Low temperature steam formaldehyde (LTSF) sterilizer: An LTSF sterilizer operates with sub atmospheric pressure steam. At first, air is removed by evacuation and steam is admitted to the chamber.

Liquid Sterilization

a. Peracetic Acid liquid sterilization: Peracetic acid was found to be sporicidal at low concentrations. It was also found to be water soluble, and left no residue after rinsing. It was also shown to have no harmful health or environmental effects. It disrupts bonds in proteins and enzymes and may also interfere with cell membrane transportation through the rupture of cell walls and may oxidize essential enzymes and impair vital biochemical pathways.

In a low-temperature liquid chemical sterile processing system, several steps must be followed for effective sterilization:
1. Pre-cleaning of the devices is necessary because many devices have small connected lumens.

2. Leak testing is done to ensure there are no leaks that could allow fluid to enter/leak the ampoules/vials and cause damage.

3. The appropriate tray/container must then be selected, and if the device has lumens, the appropriate connector attached.

4. The sterilant concentrate is provided in a sealed single- use cup and requires no pre-mixing or dilution.

The disadvantages of this method of sterilization are that the devices must be immersible, must fit in the appropriate tray, and must be able to withstand the 55°C temperature the process uses.

b. Hydrogen Peroxide Sterilization: This method disperses a hydrogen peroxide solution in a vacuum chamber, creating a plasma cloud. This agent sterilizes by oxidizing key cellular components, which inactivates the microorganisms. The plasma cloud exists only while the energy source is turned on. When the energy source is turned off, water vapor and oxygen are formed, resulting in no toxic residues and harmful emissions. The temperature of this sterilization method is maintained in the 40-50°C range, which makes it particularly well-suited for use with heat-sensitive and moisture-sensitive medical devices. The instruments are wrapped prior to sterilization, and can either be stored or used immediately.

There are five phases of the hydrogen peroxide processing cycle:

1. A vacuum phase creates a vacuum in the chamber and the pressure drops to less than one pound per square inch. This phase lasts about 20 minutes.

2. In the injection phase, the aqueous hydrogen peroxide is introduced into the vacuum chamber and is vaporized into a gas, which creates a rise in pressure due to the increase of molecules.

3. During the diffusion phase the hydrogen peroxide vapor spreads throughout the chamber and the increased pressure drives the sterilant into the packs, exposing the instrument surfaces to the sterilant and killing the microorganisms.

4. During the plasma phase the radio frequency energy is applied, stripping the electrons from some of the molecules and producing a low-temperature plasma cloud. Following this reaction, the activated compounds lose their high energy and recombine to form oxygen and water.

5. The purpose of the venting phase is to introduce filtered air into the chamber and return the chamber to atmospheric pressure so that the door can be opened. It lasts about one minute.

Radiation Sterilization

Many types of radiation are used for sterilization like electromagnetic radiation (e.g. gamma rays and UV light), particulate radiation (e.g. accelerated electrons).The major target for these radiation is microbial DNA. Gamma rays and electrons cause ionization and free radical production while UV light causes excitation.

Radiation sterilization with high energy gamma rays or accelerated electrons has proven to be a useful method for the industrial sterilization of heat sensitive products. But some undesirable changes occur in irradiated products, an example is aqueous solution where radiolysis of water occurs.

Radiation sterilization is generally applied to articles in the dry state; including surgical instruments, sutures, prostheses, unit dose ointments, plastic syringes and dry pharmaceutical products. UV light, with its much lower energy, and poor penetrability finds uses in the sterilization of air, for surface sterilization of aseptic work areas, for treatment of manufacturing grade water, but is not suitable for sterilization of pharmaceutical dosage forms.

a. Gamma ray Sterilizer: Gamma rays for sterilization are usually derived from cobalt-60 source, the isotope is held as pellets packed in metal rods, each rod carefully arranged within the source and containing 20 KCi of activity. This source is housed within a reinforced concrete building with 2 m thick walls. Articles being sterilized are passed through the irradiation chamber on a conveyor belt and move around the raised source.

Ultraviolet Irradiation: The optimum wavelength for UV sterilization is 260 nm. A mercury lamp giving peak emission at 254 nm is the suitable source of UV light in this region.

Electron Accelerator

There are two types of electron accelerator machines, the electrostatic accelerator which produces electrons with maximum energies of 5 MeV, and the microwave linear accelerator which produces electrons with maximum energies of 10 MeV. Higher energies cause better penetration into the product but there is a risk of induced radiation.

A high energy electron beam is generated by accelerating electrons from a hot filament down an evacuated tube under high potential difference, and then additional energy is imparted to this beam in a pulsed manner by a synchronized traveling microwave. Articles to be sterilized are arranged on a horizontal conveyor belt and are irradiated from one or both sides.

Filtration Sterilization

Filtration process does not destroy but removes the microorganisms. It is used for both the clarification and sterilization of liquids and gases as it is capable of preventing the passage of both viable and non viable particles.

The major mechanisms of filtration are sieving, adsorption and trapping within the matrix of the filter material. Sterilizing grade filters are used in the treatment of heat sensitive injections and ophthalmic solutions, biological products and air and other gases for supply to aseptic areas. They are also used in industry as part of the venting systems on fermentors, centrifuges, autoclaves and freeze driers. Membrane filters are used for sterility testing.

Application of filtration for sterilization of gases: HEPA (High efficiency particulate air) filters can remove up to 99.97% of particles >0.3 micrometer in diameter. Air is first passed through prefilters to remove larger particles and then passed through HEPA filters. The performance of HEPA filter is monitored by pressure differential and airflow rate measurements.

There are two types of filters used in filtration sterilization

(a) Depth filters: Consist of fibrous or granular materials so packed as to form twisted channels of minute dimensions. They are made of diatomaceous earth, unglazed porcelain filter, sintered glass or asbestos.

(b) Membrane filters: These are porous membrane about 0.1 mm thick, made of cellulose acetate, cellulose nitrate, polycarbonate, and polyvinylidene fluoride, or some other synthetic material.The membranes are supported on a frame and held in special holders. Fluids are made to transverse membranes by positive or negative pressure or by centrifugation.

Application of filtration for sterilization of liquids: Membrane filters of 0.22 micrometer nominal pore diameter are generally used, but sintered filters are used for corrosive liquids, viscous fluids and organic solvents. The factors which affects the performance of filter is the titre reduction value, which is the ratio of the number of organism challenging the filter under defined conditions to the number of organism penetrating it. The other factors are the depth of the membrane, its charge and the tortuosity of the channels.

The merits, demerits and applications of different methods of sterilization are given in Table 1.

Table 1: Merits, Demerits and Applications of Different Methods of Sterilization

Methods	Mechanism	Merits	Demerits	Applications
Heat sterilization	Destroys bacterial endotoxins	Most widely used and reliable method of sterilization, involving destruction of enzymes and other essential cell constituents.	Can be applied only to the thermostable products	Dry heat is applicable for sterilizing glasswares and metal surgical instruments and moist heat is the most dependable method for decontamination of laboratory waste and the sterilization of laboratory glassware, media, and reagents.
Gaseous sterilization	Alkylation	Penetrating ability of gases	Gases being alkylating agents are potentially mutagenic and carcinogenic	Ethylene oxide gas has been used widely to process heat-sensitive devices.
Radiation sterilization	Ionization of nucleic acids	It is a useful method for the industrial sterilization of heat sensitive products.	Undesirable changes occur in irradiated products,an example is aqueous solution where radiolysis of water occurs.	Radiation sterilization is generally applied to articles in the dry state; including surgical instruments, sutures, prostheses, unit dose ointments, plastics
Filtration sterilization	Does not destroy but removes the microorganisms	It is used for both the clarification and sterilization of liquids and gases as it is capable of preventing the passage of both viable and non viable particles.	Does not differentiate between viable and non viable particles	This method is Sterilizing grade filters are used in the treatment of heat sensitive injections and ophthalmic solutions, biological products and air and other gases for supply to aseptic areas.

Tests for Sterility
Tests for sterility are carried out by two methods:

(a) Membrane Filtration Method
(b) Direct Transfer / Inoculation Method.

The Membrane Filtration Method is used as the method of choice wherever feasible.

Media used in Sterility Testing
Fluid Thioglycollate Medium (Medium 1) and Soybean-Casein Digest Medium (Medium 2) are the two media generally used for tests for sterility.

Medium 1 (Fluid Thioglycollate Medium)
Composition:
Pancreatic Digest of Casein----15.0 g
Yeast Extract (water-soluble)----5.0 g
Glucose monohydrate/anhydrous-----5.5 g/5.0 g
Sodium chloride------2.5 g
L-Cystine-------0.5 g
Sodium thioglycollate------ 0.5 g
0.1% Resazurin Sodium Solution (freshly prepared)---1.0 mL
Granulated Agar (moisture not more than 15%) ----0.75 g
Purified Water------1000 mL
Polysorbate 80-----5.0 mL
pH after sterilization (measured at room temperature): 7.1± 0.2

Method of Preparation: The pancreatic digest of casein, yeast extract, glucose, sodium chloride, L-cystine, agar and water are mixed in the proportions given above and heat until dissolved. Sodium thioglycollate is dissolved in the solution. The specified quantity of Polysorbate 80 is added if this ingredient is to be included. If necessary, 1 M sodium hydroxide or 1 M hydrochloric acid is added so that after the solution is sterilized its pH will be 7.1± 0.2. If the solution is not clear, mixture is heated to boiling and filtered while hot through moistened filter paper. Resazurin sodium solution is added and mix.

Medium 2 (Soybean-Casein Digest Medium)
Composition
Pancreatic Digest of Casein----17.0 g
Papain Digest of Soybean Meal----3.0 g
Glucose monohydrate/anhydrous--2.5 g /2.3 g
Sodium chloride----5.0 g
Dipotassium hydrogen phosphate (K_2HPO_4) -----2.5 g
Purified Water----1000 mL
Polysorbate 80----5.0 mL
pH after sterilization (measured at room temperature): 7.3±0.2

Method of Preparation: The ingredients are mixed in the proportions given above with slight warming. The solution is cooled to room temperature. The specified quantity of Polysorbate 80 is added if this ingredient is to be included. If necessary, sufficient 1 M sodium hydroxide or 1M

hydrochloric acid so that after the solution is sterilized its pH will be 7.3± 0.2. If the solution is not clear it is filtered through moistened filter paper.

Alternative media types may be appropriate where the nature of the product or method of manufacture can result in the presence of fastidious organisms (e.sg vaccines, blood products). Validation studies should indicate that alternative media are capable of supporting the growth of a wide range of micro-organisms in the presence of the product.

Method of Membrane Filtration

Procedure

The filter should be a membrane filter disc of cellulose esters or other suitable plastics, having a nominal average pore diameter not exceeding 0.45 μm. The membrane should be held firmly in a filtration unit which consists of a supporting base for the membrane, a receptacle for the fluid to be tested, a collecting reservoir for the filtered fluid, and the necessary tubes or connections. The apparatus is so designed that the solution to be filtered can be introduced and filtered under aseptic conditions. It permits the aseptic removal of the membrane for transfer to medium or it is suitable for carrying out the incubation after adding the medium to the apparatus itself.

Cellulose nitrate filters are recommended for aqueous, oily and weakly alcoholic solutions and cellulose acetate filters for strongly alcoholic solutions. The entire unit should be sterilized by appropriate means with the membrane filter and sterile airways in place. The method of sterilization should not be deleterious to the membrane, eg, weaken it or change the nominal average pore diameter. The sterile airways should provide free access to the sterilizing agent. After sterilization, the apparatus should be free of leaks to the atmosphere except through the sterile airways.

Method of Direct Transfer

Procedures

Liquids and soluble or dispersible solids: Appropriate quantities of the preparation to be examined are added directly into Medium 1 and Medium 2. Approximately equal quantities of the preparation should be added to each vessel of medium. The test vessels of Medium 1 is incubated at 30 - 35°C and the vessels of Medium 2 is incubated at 20- 25°C.

The volume of Medium 1 should be such that the air space above the medium in the container is minimized. The volume of Medium 2 should be such that sufficient air space is left above the medium to provide conditions that permit the growth of obligate aerobes. Unless otherwise prescribed, in no case should the volume of material under test be greater than 10% of the volume of the medium alone, i.e, 90% medium and 10% product. If a large volume of product is to be tested it may be preferable to use concentrated media, prepared so as to take the subsequent dilution into account. Where appropriate the concentrated medium may be added directly to the product in its container. Wherever possible solid articles such as devices should be tested by immersion in or filling with culture media. Immerse all parts of each article in sufficient medium contained in one vessel to completely cover all parts. The volume of Medium 1 should be such that the air space above the medium in the container is minimized. The volume of Medium 2 should be such that sufficient air space is left above the medium to provide conditions that permit the growth of obligate aerobes. Place half the articles into Medium 1 and the remaining half into

Medium 2. Incubate the test vessels of Medium 1 at 30 - 35°C and the vessels of Medium 2 at 20 - 25°C.

Ointments and oily preparations: Ointments and oily preparations may be tested by the method of Direct Transfer if testing by the method of Membrane Filtration is not feasible, i.e. when a suitable solvent is not available

Incubation and examination of sterility tests: All test vessels of Medium 1 are incubated at 30 - 35°C. The vessels of Medium 2 are incubated at 20 - 25°C. All test and control vessels, other than the subcultured vessels referred to below, must be incubated for at least 14 days unless microbial contamination is detected at an earlier time.

If turbidity, precipitate, or other evidence of microbial growth during incubation is seen: the suspected growth is examined microscopically by Gram stain; attempts are made to grow single colonies using appropriate microbiological methods; colonies of each type of micro-organism present are examined for colonial morphology and cellular morphology by Gram stain; attempts are made to identify the isolates, as far as the genus, and preferably species.

Interpretation of the test results: If microbial growth is not evident in any of the vessels inoculated with the product, the sample tested complies with the test for sterility, if microbial growth is evident the product does not comply with the test for sterility unless it can be clearly demonstrated that the test was invalid for causes unrelated to the product being examined. If the test is declared to be invalid it may be repeated with the same number of units as in the original test. If there is no evidence of growth in any vessels inoculated with the product during the repeat test the product passes the test for sterility. This interpretation applies even if growth occurs in negative product control vessels. If there is evidence of growth in the test vessels the product fails the test for sterility. Further testing is not permitted under any circumstances.

Evaluation of Sterilization Method

Sterile products possess several unique properties, such as freedom from microorganism, pyrogens, particulates and high standards of purity and quality. This ultimate goal in the manufacture of sterile products can be attained by evaluation of sterilization procedure. The sterilization processes are likely to be subjected to the most detailed and complex validation procedures.

The judgment of sterility has relied on official sterility test. A validated manufacturing procedure is one which has been proved to do what it purports to do. The proof of evaluation is obtained through the collection and evaluation of data, preferably beginning, from the process development phase and continuing through the production phase. Evaluation of processing includes equipments, process, personnel, material etc.

The principle involve in the evaluation of sterilization process are:

i. To build sterility into product.
ii. Perform a maximum level of probability.
iii. Establish specification and performance characteristic.
iv. To provide greater assurance of support of the result.

v. Specific methodology, process and equipment.
vi. Final product testing using validated analytical method and
vii. Verification, calibration and maintenance of equipments used in the processes.

Evaluation of sterilization methods are done to ensure that the product produce by design process should be of best quality. The process control and finished product testing alone are not sufficient to assure product quality. When testing a specified portion of the total product and if the specified portion passes the test of sterility, it cannot assure that the total product is sterile.

Evaluation of sterilization methods provides a high degree of assurance which indicates a specific process will consistently produce a product that will meets it predetermined specifications and quality assurance. So this action proves that any procedure, process, equipments, material activity or system actually leads to the expected result and produce quality product. This concept of evaluation has been expended to encompass a wide range of activities from analytical methods used for quality control of drug substance and drug products.

The purpose of evaluation of any material equipment is achieved by means of a validation protocol which details the test to be carried out; frequency of testing and results expected that is the acceptance criteria.

Process of Microbial Destruction

Microbial destruction methods such as heat, chemical, and radiation sterilization are used. Upon exposure of such treatment, microorganisms die according to logarithmic relationship between concentration or population of the living cells and the time exposure or radiation dose. The relationship between microbial population and time may be linear or non linear.

The D value or time required or dose required for one log reduction in microbial population may be calculated from these plots.

D value

It is the rate of killing of micro organism. It determines the time required to reduce the microbial population by one decimal point i.e. it is the time required for 90% reduction in the microbial population. Hence the time or dose it takes to reduce thousand microbial cells to hundred cells is the D value.

D value is important in the validation of sterilization process for several reasons.

i. It is specific for each microorganism in environment subjected to specific sterilizing agent or condition.
ii. The knowledge of D value at different temperature in heat sterilization is necessary for the calculation of Z value.
iii. The D value is used in the calculation of biological factor F.
iv. Extra-polation of D value predicts number of log reduction of microbial population.

D value is affected by several parameters which are as follows.

i. The type of microorganism used as biological indicator

ii. The formulation component and characteristics
iii. The surface on which the microorganism is exposed
iv. The temperature, gas concentration and radiation dose

D value is determined by

i. Survival curve method: The survival curve method is based on plotting the log number of the surviving organism verses independent variable such as time, gas concentration or radiation dose
ii. Fraction negative method: In this method, sample containing similar spore population are treated in an identical environment and the number of sample still showing microbial growth after treatment and incubation are determined.

Data obtained by survival curve method are plotted semi logarithmically. Data points are connected by least square analysis.

$$\text{Log } N = a + bt$$

Where N is number of surviving organism, t is time, a is γ intercept and b is slope of line as determined by linear regression.

D value is the reciprocal of linear slope

$$D = 1/b$$

Z value

This term is exclusively used in the validation of heat sterilization process. The Z value is the reciprocal of slope resulting from the plot of the logarithm of D value verses the temperature at which the D value was obtained. The Z value may be defined as the temperature required for one log reduction in the D value.

The accepted standard (Z value) for steam sterilization of Bacillus stearothermophilus spores and dried heat sterilization for Bacillus subtilis are 10˚C and 22˚C respectively. These plots are important because one can determine D value of the indicator micro organism at any temperature of interest. The magnitude of slope indicates the relative degree of lethality as temperature is increased or decreased.

F value

The F value measures equivalent time, not clock time that a monitored article is exposed to the desired temperature e.g. 121^{o} C.

F value is calculated from following equation.

$$F = \Delta t \sum 10^{(T-To)/Z}$$

Where; Δt is the time interval for the measurement of product temperature t
T is reference temperature
To is 121^{o}C for steam sterilization.

Evaluation and In Process Monitoring of Sterilization Procedures

Dry Heat Sterilization

Physical indicator: In this process temperature record chart is made of each sterilization cycle with dry heat sterilization. This chart forms the batch documentation and is compared against a master temperature records. The temperature should be taken as the coolest part of the loaded sterilizer, further information on heat distribution and penetration within sterilizer can be gained by the use of thermocouple place at selected site in the chamber or injected into test packs or bottles.

Chemical indicator: It is based on the ability of heat to alter the chemical or physical characteristics of variety of chemical substances. This change should take place only when satisfactory condition for sterilization prevails. Thus conforming that sterilization cycle has been successfully completed. Chemical indicators generally under go melting or color change.

Biological indicator: The biological indicators are the standardized bacterial spore preparations which are usually in the form of suspension in water or culture medium or of spore dried on paper or plastic carriers, they are placed in sterilizer.

After the sterilization process the aqueous suspension /spores are on carriers are aseptically transferred to an appropriate nutrient medium, which is then incubated and occasionally seen for the growth. Clostridium species is generally used for dry heat sterilization indicator (Table 2).

Table 2 : Dry Heat Sterilization

Indicators	Sterilization Methods	Principle	Device	Parameter monitored
Physical	Dry heat	Temperature recording charts	Temperature recording charts	Temperature
Chemical	Dry heat	Temperature sensitive coloured solution	Browne's tube	Temperature, Time
		Temperature sensitive chemical	A temperature sensitive white wax concealing a black marked	Temperature
Biological	Dry heat	Temperature sensitive microbes	Bacillus subtilis	D value

Moist Heat Sterilization

Physical Indicator: In this process temperature record chart is made of each sterilization cycle with dry heat sterilization. This chart of the batch documentation is compared against a master temperature records. The temperature should be taken as the coolest part of the loaded sterilizer, further information on heat distribution and penetration within sterilizer can be gained by the use of thermocouple place at selected site in the chamber or injected into test packs or bottles.

Chemical Indicator: It is based on the ability of heat to alter the chemical or physical characteristics of variety of chemical substances. This change should take place only when satisfactory condition for sterilization prevails. Thus conforming that sterilization cycle has been successfully completed chemical indicator generally under go melting or color change.

Biological Indicator: Spores of *B. Steareothermophylus* in sealed ampoules of culture medium are used for moist heat sterilization monitoring and these may be incubated directly at 55 ^{0}C, thus may eliminate the need of aseptic transfer (Table 3).

Aseptic transfer is also avoided by use of self contained units where the spores strip and the nutrient medium are present in the same device ready for mixing after use.

The bacterial spores should have following qualities

i. It should be non pathogenic
ii. Should posses above average resistant to the particular sterilization process.

Table 3: Moist Heat Sterilization

Indicators	**Sterilization Methods**	**Principle**	**Device**	**Parameter monitored**
Physical	Moist heat	Temperature recording charts	Temperature recording charts	Temperature
Chemical	Moist heat	Temperature sensitive coloured solution	Browne's tube	Temperature, Time
		Steam sensitive chemical	A device which is impregnated into a carrier material.	Saturated steam
Biological	Moist heat	Temperature sensitive microbes	*Bacillus Stearothermophilus*	D value

Gaseous Sterilization

Physical Indicator: Gas concentration is measured independently of pressure rise, often by reference to weight of gas used.

Chemical Indicator: The chemical indicator used here are Royach Sacket, the indicator paper impregnated with reactive chemical which undergoes a distinct colour change on reaction. Chemical indicators are valuable monitors of the condition prevailing at the coolest of most in accessible part of a sterilizer.

Biological Indicator: As with chemical indicator they are usually packed in dummy packs located at strategic sites in the sterilizer. Alternatively for gaseous sterilization, these may also be placed in tubular helix device. The species of bacteria generally used for gaseous sterilization are *B.subtilis var.niger* and *B.subtilis var.golbigii*

One of the longstanding criticisms of biological indicator is that the incubation period required is very long in order to find satisfactory results (Table 4).

Table 4: Gaseous Sterilization

Indicators	Sterilization methods	Principle	Device	Parameter monitored
Physical	Gaseous	Temperature recording charts	Temperature recording charts	Temperature
Chemical	Gaseous	Reactive chemical	Indicator paper impregnated with reactive chemical.	Gas concentration, Temperature, Time
		Capillary principle	Based on same migration along wick principle	Gas concentration, Temperature, Time
		Temperature sensitive chemical	A temperature sensitive white wax concealing a black marked	Temperature
Biological	Gaseous	Temperature sensitive microbes	Bacillus subtilis	D value

Radiation Sterilization

Physical Indicator: In radiation sterilization a plastic or perspex dosimeter which gradually darkens in proportion to the radiation it absorbs give an accurate measure of the radiation dose and is considered to be the best technique currently available for the radiation sterilization process.

Chemical Indicator: Chemical dosimeter acidified with cerric ammonium sulphate or cerric sulphate solution .These responds to irradiation by dose change in the applied density. Those are considered best and accurately measure relation dose.

Biological Indicator: These are consist of standardized bacterial spore preparation which are usually in the form of suspension in water or culture medium or of spore dried on paper or plastic carriers , they are placed in sterilizer.

After the sterilization process the aqueous suspension /spores are on carriers are aseptic ally transferred to an appropriate nutrient medium, which is then incubated and periodically observed for the growth. *Clostridium* species is generally used for dry heat sterilization indicator (Table 5).

Filtration Sterilization

Physical Indicator: Sterilizing filters are subjected to a bubble point pressure test. This is a technique for determining the pore size of a filter, and may also be used to check the integrity of certain types of filters. The principle of the test is that the wetted filter in its assembled unit is subjected to an increasing air or nitrogen gas pressure difference. The pressure difference recorded when the first bubble of gas breaks away from the filter is related to maximum pore size. When the gas pressure is further increased slowly there is general eruption of bubble over the entire surface. The pressure difference here is related to the mean pore size. Pressure difference below the expected value would signify a damage or faulty filter.

Table 5: Radiation Sterilization

Indicators	Sterilization Methods	Principle	Device	Parameter monitored
Physical	Radiation	Recording charts	Recording charts	Radiation Dose
Chemical	Radiation	Radio chromic chemicals	Plastic device impregnated with radio sensitive chemicals which undergo colour changes at relative low radiation doses	Only indicate exposure to radiation
		Dosimeter device	Acidified ferric ammonium sulphate solutions responds to irradiation by dose related changes in their optical density	Accurately measures radiation doses
Biological	Radiation	Radiation sensitive microbes	*Bacillus pumilus*	D value

Biological Indicator: Filtration sterilization require a different approach from biological monitoring, the test effectively measure in the ability of a filter to produce a sterile filtrate from a culture of suitable organism *S.marcesence*, a small gram negative rod shape bacterium. *B.diminuta* used as a biological indicator having a dimension 0.5 micrometer and 0.3 micrometr respectively has been used for filters of 0.45 micrometer and 0.22 micrometer. The extent of the passage of this organism through membrane filter is enhanced by increasing the filtration pressure. Thus successful sterile filtration depends markedly on the challenge condition. Such test are used as the part of filter manufacture characterization and quality assurance process, and users initial validation procedure. They are not employed as a test of filter performance in use (Table 6).

Table 6: Filtration Sterilization

Indicators	Sterilization methods	Principle	Device	Parameter monitored
Physical	Filtration sterlization	Forcibly passing of solution through the membrane.	Bubble point pressure test	Pressure
Biological	Filtration sterlization	Retention of bacteria	*P. diminuta*	Size of microorganism

Examples of Materials Sterilized by Different Methods

Different techniques which are used for the sterilization of different materials are discussed in the tabular form (Table 7).

Table 7: List of Materials Sterilized by different Methods

Materials		Methods of Sterilization /Preferred Methods
Injections	**Intravenous infusions** a. Isotonic solution of sodium chloride/Glucose b. Blood Products and Plasma substitutes e.g. Dextran and degraded gelatin	Filtration sterilization Terminal sterilization a. Autoclaving for thermostables b. Radiation for thermolabiles
	Intravenous Additives e.g. Potassium Chloride, Lignocaine,Heparin, certain Vitamins, Antibiotics	Physical methods(Freeze Thaw Method)
	Total Parenteral Nutrition(TPN)	Filtration Sterilization
	Small Volume Injections e.g. **Vaccines:** Influenza Vaccines, Vaccinea, Polio Vaccines, Rabies Vaccines **Antibiotics:** Benzyl penicillin, Streptomycin Sulphate, Zinc Bacitracin, polymixin Sulphate, Dihydrostreptomycin Sulphate **Vitamins:** Ascorbic acid, Vitamin A, Vitamin E **Freeze Dried Products:** Few hormones, several Vitamins, Vaccines **Miscellaneous:** Diazepam Inj., Insulin Inj., Promethazine HCl Inj.	Radiation Sterilization (using Gamma radiation) Radiation Sterilization (using Gamma radiation) Radiation Sterilization (using Gamma radiation) Filtration sterilization Radiation Sterilization (using Gamma radiation)
Non injectable sterile fluids	Non injectable waters Urological irrigation solution Peritoneal dialysis and heamodialysis solution Inhaler solution	Filtration sterilization / Terminally sterilization by Autoclaving

Ophthalmic preparation	Eye Drops e.g. Cholramphenicolo eye drops, Timolol eye drops,Pilocarpin eye drops, brominidine eye drops, Atropine eye drops	Thermostables by Autoclaving at 121˚C for 15 minutes Thermolabiles by Filtration Sterilization
	Eye lotions e.g. Cholramphenicolo eye lotions, Timolol eye lotions,Pilocarpin eye lotions, brominidine eye lotions, Atropine eye lotions	Thermostables by Autoclaving at 121˚C for 15 minutes Thermolabiles by Filtration Sterilization
	Eye ointment e.g. Simple eye ointment BP	Dry heat sterilization at 160˚C for 2 hours
	Contact lens solutions e.g. wetting solution, cleansing solution, soaking solutions	Thermostables by autoclaving at 121˚C for 15 minutes and thermolabiles by Filtration Sterilization
Dressings	Chlorhexidine gauze dressing	Any combination of dry heat, ethylene oxide and gamma radiation
	Framycetin gauze dressing	
	Knitted viscous primary dressing	
	Paraffin gauze dressing	
	Perforated film absorbent dressing	
	Polyurethane foam dressing	
	Semi permeable adhesive dressing	
	Sodium fusidate gauze dressing	
	Absorbent cotton wool	Any methods
	Elastic adhesive dressing	Ethylene oxide or gamma radiation
	Plastic wound dressing	Ethylene oxide or gamma radiation
	Absorbent cotton gauze	Any methods
	Gauze pads	Any methods
	Adhésive viscose wadding	Any methods
Implants	Steroid implants Hormonal implants	Dry heat sterlization
Absorbable heamostate	Oxidized cellulose	Gaseous sterilization (using ethylene oxide and formaldehyde)
	Absorbable gelatin foam	Dry heat sterilization at 150 ˚C for 1 hour

Absorbable heamostate	Human fibrin foam	Dry heat sterilization at 130 ˚C for 3 hours
	Calcium alginate	Moist heat sterilization by autoclaving
Surgical ligatures and sutures	Catgut	Dry heat sterilization at 160 ˚C for 2 hours/ gamma radiation
	Non absorbable type e.g. nylons, silk and polypropylene	Moist heat sterilization (Autoclaving)/ gamma radiation
Instruments and equipments	Syringes (glass) Syringes(glass),dismantled Syringes (disposable) Needles (all metal) Needle (disposable)	Dry heat using Gamma radiation
	Metal instruments	Dry heat
	Disposable instruments Rubber gloves Administration sets	Gamma radiation
	Respiratory parts	Dry heat sterilization
	Dialysis machines Fragile heat sensitive equipment	Chemical sterilization using formalin and ethylene oxides
Miscellaneous	Dry bulk drugs	Dry heat sterilization
	Porcelain	Dry heat sterilization
	Food products	Radiation sterilization or gaseous sterilization
	Culture medium	Gaseous sterilization
	Mouths of culture tubes and bottles	Dry heat sterilization
	Air sterilization in hospitals, manufacturing house, Schools etc.	Radiation sterilization