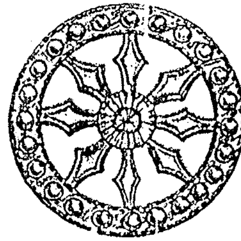


CHAPTER -IV

CONTROL OF MICROORGANISMS

- STERILISATION
- DISINFECTION

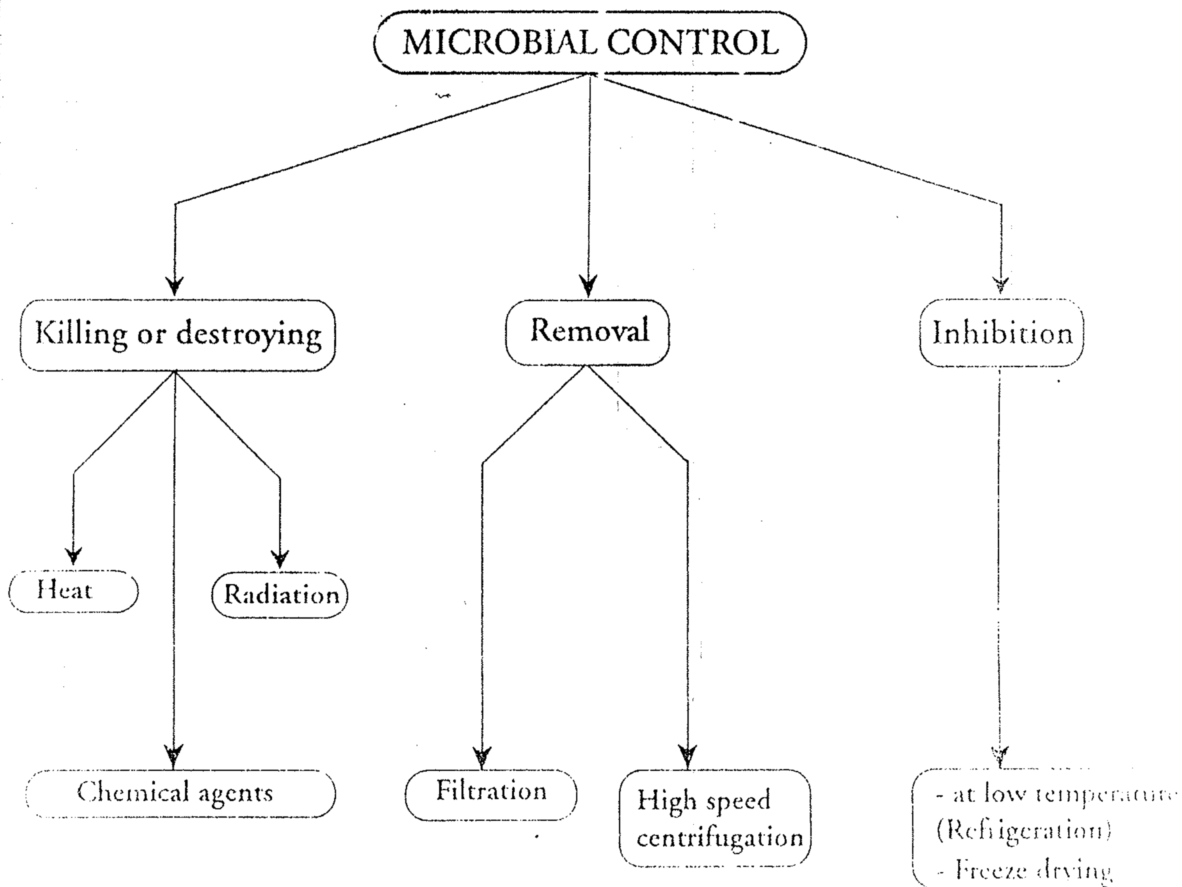
CONTROL OF MICROORGANISMS

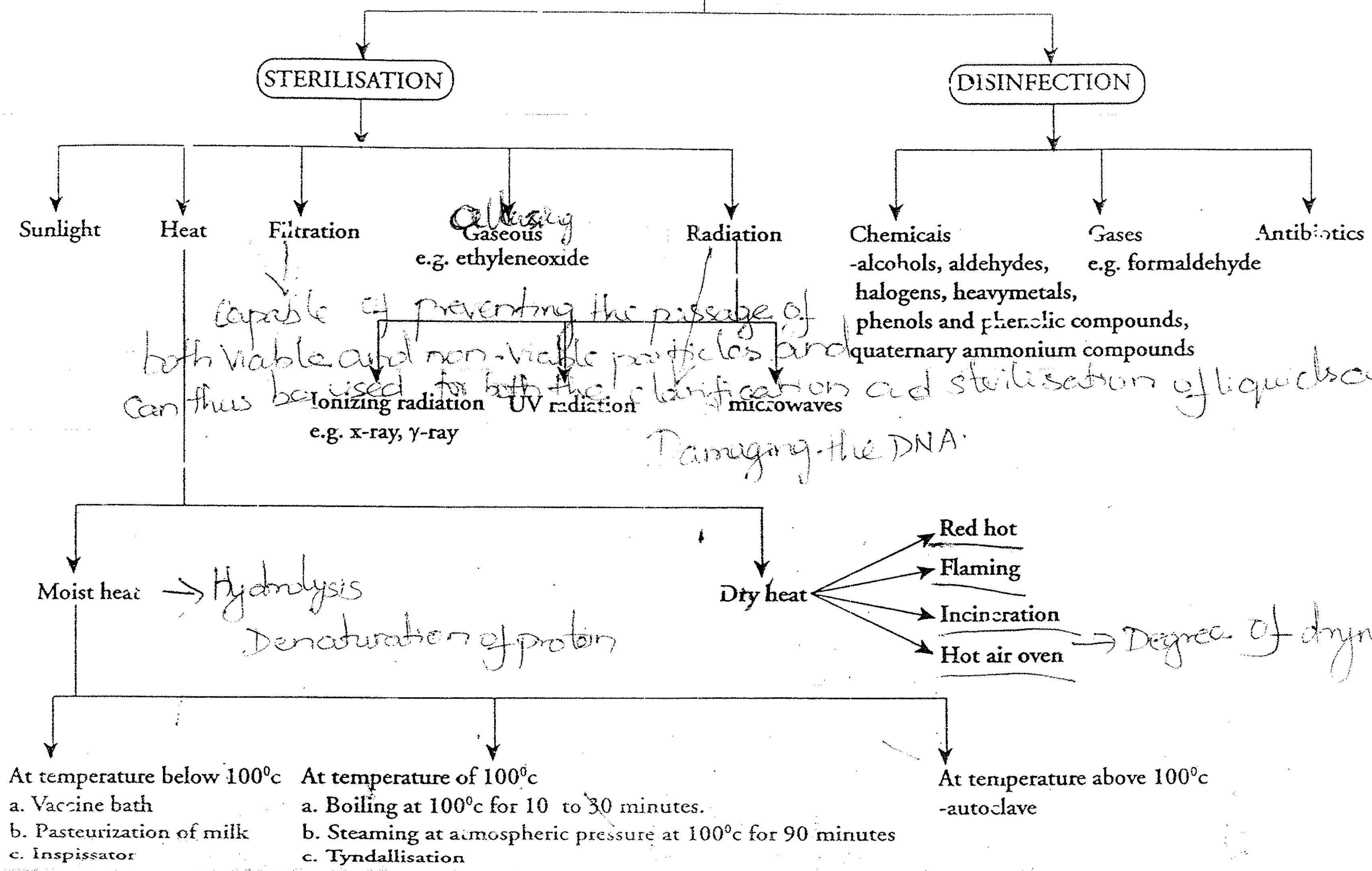
> *"I shall guard my asepsis as a girl should guard her virginity"*
>
> \- ARNOLD. K. HENRY

Objectives

1. To prevent infection of human beings.
2. To prevent spoilage of food.
3. To prevent contamination of materials used in pure culture work in laboratories, diagnosis, research and industry.
4. To prevent high death rate due to postoperative infection in hospitals.

GENERAL PRINCIPLES OF MICROBIAL CONTROL




METHODS OF CONTROL
STERILISATION
DISINFECTION
Sunlight
Heat
Filtration
Gaseous
e.g. ethyleneoxide
Radiation
Chemicals
-alcohols, aldehydes,
halogens, heavymetals,
phenols and phenolic compounds,
quaternary ammonium compounds
Gases
e.g. formaldehyde
Antibiotics
Ionizing radiation
e.g. x-ray, γ-ray
UV radiation
microwaves
Moist heat
Dry heat
Red hot
Flaming
Incineration
Hot air oven
At temperature below 100°c
a. Vaccine bath
b. Pasteurization of milk
c. Inspissator
At temperature of 100°c
a. Boiling at 100°c for 10 to 30 minutes.
b. Steaming at atmospheric pressure at 100°c for 90 minutes
c. Tyndallisation
At temperature above 100°c
-autoclave

STERILISATION

Sterilisation is a process of destroying the vegetative or spore state of all microorganisms in medium or material.

Sunlight

Direct and continuous exposure to sunlight is destructive to many pathogens because sunlight possesses bactericidal activity, which plays an important role in spontaneous sterilisation under natural conditions. This is mainly due to the presence of uv light. Sunlight sterilises the water in tanks, rivers and lakes. Articles such as linen, bedding and furniture may be sterilised by exposure to sunlight for several hours.

Heat

Heat is the most efficient method of sterilisation. There are various factors, which influence the heat sterilisation they are

I. Nature of heat
II. Temperature and time
III. Nature of organisms and spores present in the article
IV. Type of material.

I. Nature of heat

There are two fundamental processes

- Moist heat sterilisation
- Dry heat sterilisation

I Moist heat sterilisation

The moist heat kills the bacteria by coagulating or denaturing their proteins and enzymes. Microorganisms exhibit differences in their resistance to moist heat. For e.g. vegetative form of bacteria killed at 60°c to 70°c, whereas spores require temperature above 100°c for destruction.

Moist heat sterilisation can be divided into three namely,

a. At temperature below 100°c.
b. At temperature of 100°c.
c. At temperature above 100°c.

a. At temperature below 100°c

1. Vaccine bath

This is a special water bath used to sterilise the bacterial vaccine at 60°c for one hour as most vegetative bacteria are killed at this temperature and time. Apart from bacterial vaccine, serum or body fluids sterilised at 56°c at one hour.

2. Pasteurization of milk

The pasteurization process, reducing total bacterial count of the milk by 97% to 99%, is effective because, the common milk borne pathogens are tubercle bacillus, salmonella, streptococcus, coxiellaburnetii and brucella. They do not form spores and are quite sensitive to heat.

There are two methods namely,

i. Holder method
ii. Flash method

i. Holder method

This is done by maintaining a temperature of 63°c for 30 minutes.

ii. Flash method

This is done by maintaining a temperature of 72°c for 20 seconds.

Note:

Generally, all vegetative bacteria are rapidly killed in temperature of 60-65°c except coxiella burnetii, a relatively heat resistant bacteria which may not be killed in holder method and can be killed in flash method.

3. Inspissator

It is a copper box, which surrounded by a water jacket. To this box thermostat is attached for automatic regulation of temperature. The medium is kept in the chamber and closed with glass lid.

Temperature for sterilisation

80 - 85°c.

Duration

3 days.

Use

Sterilisation of Loeffler's serum medium, Dorset's egg medium, L.J medium.

b. At temperature of 100°c.

i. Boiling at 100°c

It is not efficient method for killing bacteria. Eventhough boiling for 10 to 30 minutes kills most vegetative forms of bacteria. However, the spores of bacteria withstand boiling for a considerable time.

Use

It is used to sterilise glass syringes, tubes, rubber, stoppers and small surgical instruments.

2. Steam at atmospheric pressure at 100°c for 90 minutes.

Certain bacteriological media such as sugar media will decompose if subjected to the higher temperature of auto clave. For this, Arnold or Koch steriliser is used. The articles to be sterilised are kept on a perforated plate through which steam passes and escapes through an opening at the top.

Duration

90 minute.

Temperature

100°c.

In this method, most vegetative organisms are destroyed except thermophiles.

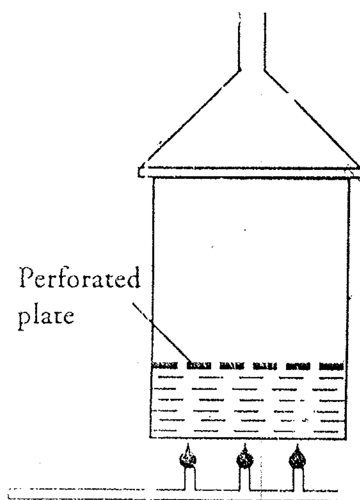


Fig 4.1 Arnold steriliser

3. Tyndallization

Tyndallization is a process, which involves heating the material at 100°c on three consecutive days.

Use

This is used for sterilising egg, serum or sugar containing media and gelatin media, which will damaged at higher temperature of autoclave.

c. At temperature above 100°c

This can be successfully carried out by autoclave. Here water boils in a closed, airtight container under increased pressure; the temperature rises above 100°c, which destroys the vegetative cells and thermo resistant bacterial spores.

Autoclave

1. It is a modified pressure cooker consists of a vertical or horizontal cylinder. It is made up of stainless steel or gunmetal.
2. Autoclaving in the process of sterilisation by saturated steam under high pressure above 100°c.

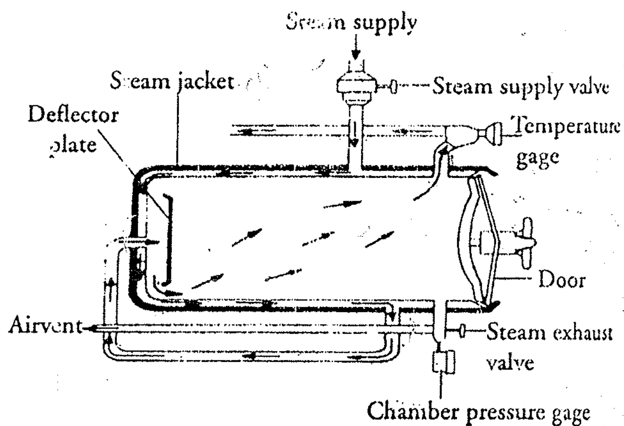


Fig 4.2 Autoclave

The steam circulates with in the jacket is supplied under high pressure to the closed ...er chamber, where goods are kept for ...ilisation.

1/5th part of the cylinder is filled with ...er and the materials to be sterilised are placed ...ide.

The lid is closed, which is attached with ...charge tap on it and open.

The heater is put on; safety valve is ...justed to required pressure.

For sometimes, after boiling of water ...side the chamber, the steam is allowed to escape ...til the cylinder becomes air free. Then the ...scharge tap is closed.

Sterilisation time

Temperature	*Pressure*	*Time*
121°c	15 pounds	15-20 minutes
126°c	20 pounds	10 minutes
133°c	30 pounds	3 minutes.

Dry heat sterilisation

1. Redhot

Certain articles like inoculation wire loops can be sterilised instantly in the flame of Bunsen burner to get red hot. This is done before and after doing experiments.

2. Flaming

Here the articles are not allowed to get red hot but passed over the flame. It is used to sterilise glass slides, scalpels, needles, cotton-wool plugs.

3. Incineration

This method is employed for destruction of infective materials like sputum and stools.

4. Hot air oven

It is widely used steriliser by dryheat. The sterilisation temperature is about 160°c for 2 hours or 180°c for 30 minutes.

Uses

1. Glass wares - Petridishes, syringes, test tubes, pipettes, flasks.
2. Surgical instruments-forceps, scalpel, scissors.
3. Powders can be sterilized to avoid clumping.

Precautions

- Any inflammable, volatile substance and thermolabile substance like rubber must not be sterilised by this method.
- The sterilising articles must be absolutely dry.
- The instrument must not be over loaded.
- After sterilisation, the oven must be allowed to cool before opening the doors to prevent cracking of glasswares.

II. Temperature and time

The time required for killing bacteria is inversely proportional to the temperature. For e.g.

a. In moist heat sterilisation

Temperature	*Sterilisation time*
100°c	20 hours
121°c	15 to 20 minutes
126°c	10 minutes
133°c	3 minutes

b. In dry heat sterilisation

Temperature	*Sterilisation time*
160°c	2 hours
170°c	1 hour
180°c	30 minutes

III. Nature of microorganisms and spores present in the article.

Nature of organism	*Moist heat temperature*	*Time*
Vegetative form of bacteria, yeast and fungi	60-65°c	30 minutes
Spore form of actinomycetes, yeasts and fungi	70°c	5 minutes
Bacterial spores	121°c	15 minutes

IV. Type of materials

Sterilisation is greatly depends on the type of material. For e.g. proteins, sugars and fat offer some degree of protection to the effect of heat. The following table shows the sterilisation time, temperature and biological indicators.

Method	*Temp (°C)*	*Time (minutes)*	*Biological indicator*
Moist heat	121 126 134	15 10 3	B. Stearo thermophilus
[illegible]	160 170 180	120 60 30	B.Subtilis varniger

Advantage of moist heat over dry heat

Penetration power is more, so that it kills microorganisms and spores at lower temperature in shorter duration.

GASEOUS STERILIZATION

1.Ethyleneoxide

Ethyleneoxide is colourless, flammable, sterilant gas at ordinary room temperature. It is an alkalizing agent and exhibit lethal effect on bacteria and it's spores. It has good degree of penetration power, eventhough plastics. The penetration power can be increased by increasing the temperature.

Use

It is used to sterilize plastic goods, polyethylene tubes, artificial artery, vaccines and culture media.

2. Formaldehyde gas

It is an alkylating agent destroys the microorganisms by alkylation process of the cell constituents.

It is an irritant and toxic gas when inhaled. This toxic effect can be neutralized by exposure to ammonia vapour when disinfection process completed.

Use

It is used for sterilizing instruments, and fumigating operation theatre, sick rooms, warts and laboratories.

FILTRATION

This method is used to remove microorganisms physically. Certain thermolabile substance like antibiotic solution, sera, carbohydrate can be sterilized by this technique. By this technique, we can obtain bacteria free filtrates of toxins and bacteriophages.

Types of filters

1. Candle type filters.
   e.g. Barkfeld, chamberland.
2. Asbestos disc filters. e.g. seitz.
3. Sintered glass filters.
4. Collodion or membranous filters.
5. Slow sand filters.

1. Candle type filters

Both barkfeld and chamberland filters were used in olden days to sterilise pharmaceutical products. The main disadvantage of these filters is low flow rate, difficulty in cleaning and media passes through the filter.

* *Barkfeld filter*

These filters are made up of kieselguhr. They are available in three grades of porosity namely,

V	-	the coarest
W	-	the finest
N	-	intermediate.

* *Chamber land filter*

These filters are made up of unglanzed porcelain filters. The chamberland filters varies are made up of porosities, which are graded L1,L1a, L2, L3, L5 L7,L9 and L11.

2. Asbestos disc filter

e.g. Seitz filter

The filter is made up of magnesium silicate. The filtrate is supported on a metal mount, which is attached to vacuum flask through a rubber cork.

3. Membrane filters

This filters is most efficient than others. It is made up of biological inert cellulose esters, polyvinylidiene fluoride and polytetrafluro ethylene.

4. Slow sand type filters

It is used to filter the water.

RADIATION

Radiations are of two types

* Ionising radiation.
* Non ionising radiation.

Ionising radiation

Ionising radiation include gamma rays, x-ray and accelerated electrons. These are more effective than non ionising radiation.

Use

It is used to sterilise thermolabile substances like antibiotics, rubber, steroids, hormones and surgical material such as surgical blade, catgut and sutures.

Non ionising radiation

U.V radiation, I.R radiation are the non ionising radiation.

U.V rays

U.V rays with 240 to 280 nm have bactericidal activity, which act by

1. Denaturation of bacterial proteins.
2. Damage of DNA.
3. Inhibition of DNA replication, formation of hydrogen peroxide and other toxic products in the culture medium.

When compared to Gram negative bacteria Gram positive bacteria show a slightly greater resistant to U.V radiation.

Application

It is mainly used in disinfection of bacteriological laboratories and operation theatre.

Infrared radiation

This is very convenient method for sterilising syringes.

DISINFECTION

It is a process of destruction of vegetative forms of pathogenic organisms, which are capable of producing infection.

Disinfectants

Those agents, which are used for disinfection process, called disinfectants.

Types of disinfectant

1. Acid and alkali

Generally, strong acids and alkali kill the bacteria but weak organic acids inhibit their growth. Mycobacterium tuberculosis shows resistant to acid and alkali than other bacteria.

2. Alcohols

Ethyl alcohol and isopropylalcohol show appreciable action on vegetative form of bacteria but not in spores or viruses. They are used as topical antiseptics. They kill the bacteria by denaturing the proteins. Isopropyl alcohol is mainly preferred than ethyl alcohol because it is better fat solvent, more bactericidal and less volatile methyl alcohol is effective against fungal spores, but it is toxic and inflammable.

3. Glutaraldehyde

It is a dialdehyde used as 2% aqueous solution, which acts as sporicidal and tuberculocidal in action. It is used to disinfect rubber, facemasks, metal instruments and polyethylene tube.

4. Dyes

Acridine and aniline dyes are used extensively as skin and wound antiseptics. They are more active against gram positive than gram negative organisms. Both dyes are bacaterio static in action.

Examples

Aniline dyes

Brilliant green, malachyte green and crystal violet.

Acridine dyes

Proflavine, acriflavine, euflavine and aminacrine.

5. Halogens

Chlorine and iodine are commonly used as water and skin disinfectants respectively. They are bactericidal substances.

Chlorine is used in the form of bleaching powder, sodium hypochlorite and chloramine.

Iodine is used as tincture of iodine.

6. Chlorinated lime

This is a cheap, powerful disinfectant used to disinfect feces, urine and utensils.

7. Chloroform

It is either liquid or strong vapour kills the vegetative form of bacteria.

8. Phenol and its derivatives

Phenol is chief products obtained by distillation of coal tar between the temperature of 170°c and 270°c and pocessesses wide spectrum of action. Phenol (1%) has bactericidal action, but it causes toxicity so that the use of phenol is restricted. Certain derivatives of phenols like cresols, chloroxylenols, chlorhexidine and hexa chlorophane are used.

a. Cresol

It is used as solutions of cresols in soaps(lysol). It is cheaper but toxic to skin or tissues and mainly used for preliminary sterilisation of infected glass-wares in laboratory, disinfection of excreta, cleaning floors of wards and operation theatre in hospitals.

b. Chloroxylenol

It is an active ingredient of dettol and most significantly effective against Gram negative bacteria.

c. Chlorhexidine

It has appreciable bactericidal action. It is less toxic so that it is used as topical application.

e.g.

Savalon is the combination of chlorhexidine and cetrimide. This is widely used in burns, wounds, for surgical instrument and pre operative disinfection of skin.

d. **Hexa chlorophane**

It is a very good bacteriostatic used in soap. It is more effective against Gram positive than Gram negative bacteria and is applied on skin as prophylaxis against staphylococcal infection.

e. **Black fluids and white fluids**

These are cheaper used for the disinfection of drainage, agricultural and horticultural equipments etc.

Black fluids are homogenous solution of coal tar acids or crude petroleum acids, where as white fluids are finely dispersed emulsions, not solutions of coal tar or crude petroleum acids. Generally, white fluids are more stable than black fluids.

9. Surface active compounds

There are four basic type of compound, they are

a. **Anionic compounds**
e.g. Soaps (Sodium stearate) slightly antibacterial.

b. **Cationic compounds**

They are quatery ammonium compounds are incompatible with soaps.
e.g. cetrimide, cetylpyridinium chloride, benzalkonium chloride.

c. **Amphoteric or ampholytic compounds**

These compounds contain both cationic and anionic groups in the same molecule. They have the detergent properties of anionic surfactants combined with the disinfectant properties of cationic surfactants. e.g. Tego compounds.

8. Salts

Salts of heavy metals such as mercuric chloride and silver nitrate have toxic effect on bacteria due to affinity of certain bacterial proteins.

Merthiolate is a common laboratory preservative and it is used in a dilution of 1 in 10,000 for preservation of antisera and other laboratory specimens like serum and urine.

9. Formaldehyde

It is lethal to all types of bacteria and fungus including spores. It is used as aqueous solution and in gaseous form.

Formalin

Commercial formalin contains 40% formaldehyde gas in water to which 10% methanol has been added to prevent polymerisation.

Formalin is used for killing bacteria cultures and suspensions, cleaning contaminated surface, preservation of tissues for histological examination and used to preserve dead bodies.

It is also used to sterilise bacterial vaccine and in preparation of toxoid from toxin.

Formaldehyde gas

This mainly used to disinfect

1. Woolen blankerts
2. Foot wears of person with fungal infection.
3. Microbiological lab, hospital wards and operation theatre by fumigation processes.

Evaluation of disinfectants

For evaluating the disinfectants, the culture must be standardized.

Standardization of culture

Standardization can be found [illegible] colony forming unit(C.F.U).

Take 24 hours broth bacterial culture and diluted serially from 10^{-1} to 10^{-10} with distilled water.

Leave first five dilutions, take 10^{-6} to [illegible] and plate the dilution by using spread plate technique. Find out the concentration, where the visible, colonies are more, choose that concentration for the experiment.

Chemical agents & it's dilution | Presence of growth of Substance | |
---|---|---|---
 | 5 min | 10 min | 15 min
Phenol 1:80 | + | + | +
1:90 | + | – | –
1:100 | – | – | –
Test chemical 1:400 | + | + | +
1:500 | + | + | –
1:600 | – | – | –

Phenol coefficient test chemical

$= \frac{\text{Reciprocal of test chemical dilution}}{\text{Reciprocal of phenol dilution}}$

$\frac{500/1}{90/1} = 5.5$

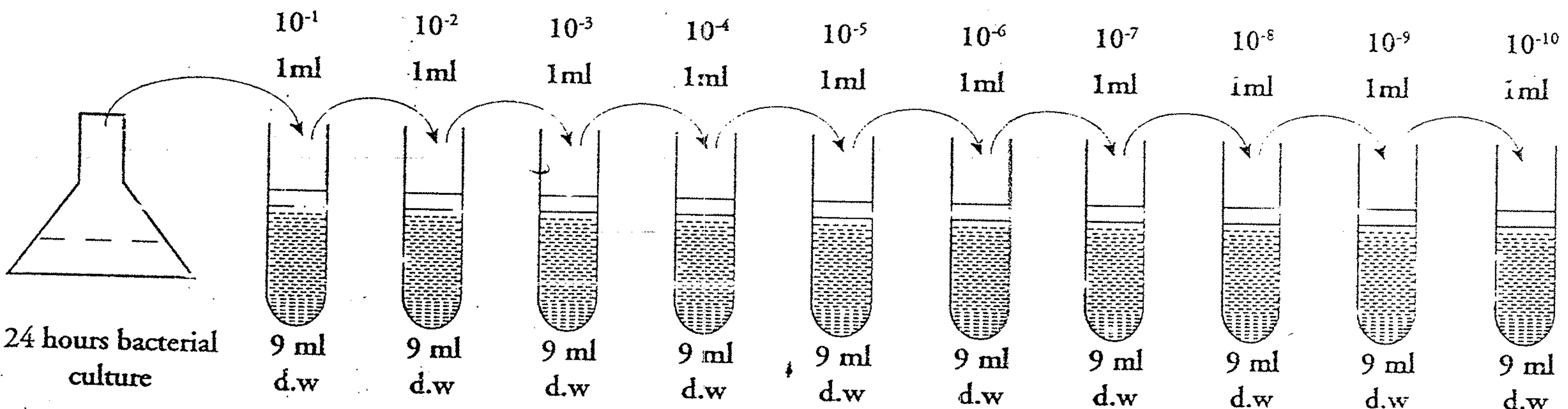


Fig 4.3 Serial dilution of 24 hours bacterial culture

Turbidimetry

This is find out by the formation of turbidity. It is used to find out the minimum inhibitory concentration.

For turbidimetry assay specialized tubes are used called **assay tubes**. Concentration of 24 hours broth culture can be find out by serial dilution. Suitable concentration of culture was selected. The selected concentration of culture is called seed broth.

0.2ml of drug is mixed with 0.8ml of seed broth, which is serially diluted. Find out, where the turbidity forms in the highest dilution, that concentration is called **minimum inhibitory concentration** (MIC).

Agar diffusion method

This method depends on the diffusion of drugs through the solid agar media. There are various types. They are,

a. Cup plate or cylinder plate method

The nutrient agar is melted, cooled suitably, poured into petridish. Spread 0.2ml of known concentration of inoculum on the surface of the solidified agar(spread plate technique). Cups or cavities are made by using a sterile borrer. Now 0.2ml of drug is poured into the cups of agar plate and then incubated at 37°c for 24 hours. If the drug has any antibacterial effect, it will show the zone of inhibition.

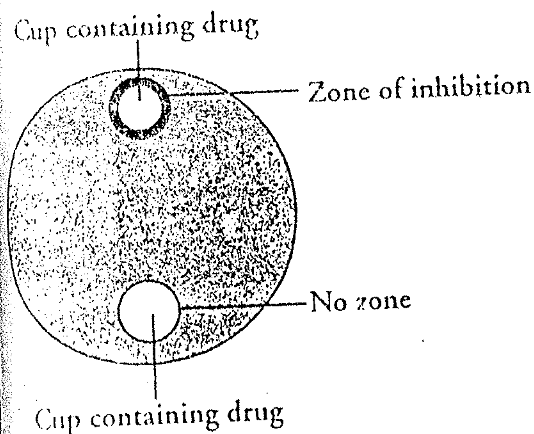


Fig. 4.4 Schematic representation of cup plate technique

b. Disc plate method

The nutrient agar is melted, cooled suitably, poured into petridishes. Spread 0.2 ml of known concentration of inoculum on the surface of the solidified agar (spread plate technique). The antibiotic discs are placed by using a sterile forceps over the agar plate atleast 15mm from the edge of the plate. Now the disc is gently pressed to give better contact with agar. The plates were incubated at 37°c for 24 hours. Antibiotic potency is based on the formation of zone of inhibition.

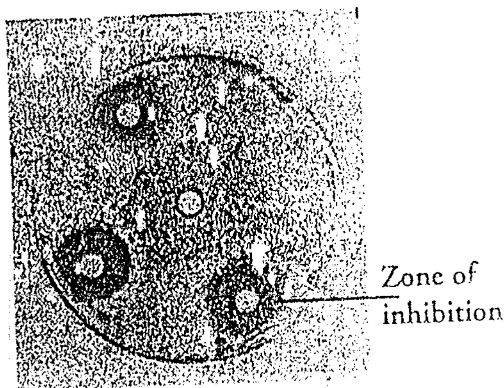


Fig 4.5 Disc plate technique

c. Ditch plate technique

The nutrient agar is melted, cooled suitably and poured into petridishes. The solidified media is cut with a sterile blade to make a ditch. The drug is poured very carefully into the ditch. Various microorganisms are streaked on the sides of the ditch. This method is used to find out potency of drug against various micro organisms by means of inhibition of growth on streaked area.

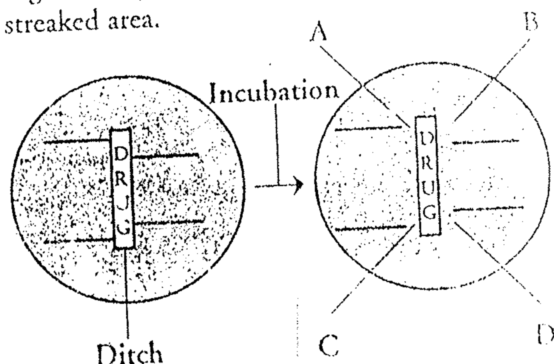


Fig 4.6 Schematic representation of ditch plate technique

d. Gradient plate technique

This technique is used to isolate the resistant mutants. The petridish is kept in a slanting position; a sufficient amount of melted nutrient agar is poured and solidified in the slanting position. Another layer of agar is poured over it, which contains antibiotic solution and solidified it. After solidification, 0.2 ml of bacterial culture was spreaded over the solid surface and incubated it at 37°c for 24 hours to 48 hours. The organisms will grow, where the concentration of the drug is below the critical level. The antibiotics get diluted on the lower layer and a gradient of concentration will be produced. Thus, the resistant mutants can be isolated.

ASSESSMENT OF BACTERICIDAL ACTIVITY

RIDEAL - WALKER TEST

1. This is a phenol coefficient test compares the antimicrobial activity of a chemical compound to that of phenol under standardized environmental conditions.
2. For assessment, standard Redial-Walker broth is used.
3. *Reaction mixture*
   a. The 24 hours culture of salmonella typhi is standardized by serial dilution.
   b. For control, phenol is diluted as 1 in 105.
   c. The test disinfection also diluted in such a way, 1 in 1000, 1 in 1100, 1 in 1200, 1 in 1300.
   d. Maintain this mixture at temperature about 17.5 ± 0.5.
4. Dilutions of test disinfectant and phenol are inoculated with 0.2ml of 24 hours standardized salmonella typhi culture.
5. Sub culture of reaction mixture are taken and transferred to broth after 2.5, 5, 7.5 and 10 minutes. The broth tubes are incubated at 37°c for 48 hours to 72 hours and are examined for the presence or absence of growth.

$$\text{R.W. Coefficient} = \frac{\text{Dilution of test disinfectant killing in } 7\tfrac{1}{2} \text{ but not in 5 minutes.}}{\text{Dilution of phenol killing in } 7\tfrac{1}{2} \text{ but not in 5 minutes.}}$$

Results

Dilutions of test disinfectants	*Time (minutes)*			
	2.5	5	7.5	10
1 in 1000	+	-	-	-
1 in 1100	+	+	-	-
1 in 1200	+	+	+	-
1 in 1300	+	+	+	+
Phenol 1in105	+	+	-	-

$$\textit{R.W Coefficient} = \frac{1100}{105} = 10.47$$

Examples for R.W.Coefficient

Phenol 1% in water	-	1
Lysol	-	3 - 4
Roxenol	-	5 - 5.5
White fluids	-	10 - 11
Black fluid	-	14 - 15

CHICK MARTIN TEST

1. Test organism

Saccharomyces cerevisiae

2. Standard disinfectant

Phenol.

The temperature of reaction mixture is ...ined at 20°c, the exposure time is 30 ...es.

The chick martin coefficient is the average ...highest concentration of phenol showing ...in both tubes and the lowest concentration ...ing growth, divided by the same average for the test disinfectant, for e.g.

Phenol	*Tubes*		*Test disinfectant*	*Tubes*	
%	1	2	%	1	2
3	-	-	0.52	-	-
2	-	-	0.49	-	-
1.8	+	-	0.39	+	+
1.6	+	+	0.32	+	+

$$\text{Chickmartin coefficient} = \frac{\text{Highest concentration of phenol showing growth} + \text{lowest concentration of phenol showing growth}}{2} \div \frac{\text{Highest concentration of test showing growth} + \text{lowest concentration of test showing growth}}{2}$$

$$= \frac{1.6 + 2}{2} \div \frac{0.39 + 0.49}{2} = 1.8 \div 0.44$$

$$= 4.09$$

FACTORS AFFECTING DISINFECTANT ACTION

...me of Contact

When the bacterial population is ...ed to lethal environment will give death. ...time of contact is increased, the lethal effect ...increase. This can be explained by first order ...tion.

$$k = \frac{1}{t} \log \frac{B}{b}$$

k – Velocity constant

t- Time for the viable counts to fall from B to b.

B- initial number of organisms.

b- Final number of organisms.

2. Concentration of Disinfectant

The lethal effect of bacterial population is increased by increasing the concentration of disinfectant. The relation between concentration,

time taken to kill at a given temperature can be expressed by

$C^n t$ - a constant

C - Concentration

n - Concentration exponent or dilution coefficient for the disinfectant

t - time

Take log

n log + log t = Constant

$$n = \frac{\log t_2 - \log t_1}{\log C_1 - \log C_2}$$

t_1 = death time of disinfectant concentration C_1

t_2 = death time of disinfectant concentration C_2

3. Temperature

The lethal effect on bacterial population can be increased by increasing the temperature. The effect of temperature on bactericidal activity may be expressed quantitatively by means of temperature coefficient.

$$(T_2 - T_1) = \frac{t_1}{t_2}$$

t_1 - extinction time at T_1

t_2 - extinction time at T_2

The temperature coefficient may be expressed by 10^0 rise. This is denoted by Q_{10}.

Q_{10} values may be calculated easily by determining the extinction time between two temperature differing 10^0c.

$$Q_{10} = \frac{\text{Time to kill at } T^0 c}{\text{Time to kill at } (T + 10^0)}$$

e.g. The Q_{10} value for phenol is 4.

4. Chemical composition

The chemical composition of micro organism will influence greatly the disinfection process.

e.g. The presence of lipids reduces the disinfecting ability of phenolics.

5. p^H

The p^H may affect the disinfection process.

Generally, the optimum pH of bacterial growth is 6 - 8 . If any alternation take place, the rate of growth declines.