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

B. Pharm
Semester-5

STUDY MATERIAL

Subject Name:Pharmaceutical Microbiology
Subject Code: BP504TP

HNSIPER

CHAPTER 3 Fungi and virus :

CONTAINS:Study of morphology, classification, reproduction/replication and cultivation of Fungi and Viruses. Classification and mode of action of disinfectants Factors influencing disinfection, antiseptics and their evaluation. For bacteriostatic and bactericidal actions. Evaluation of bactericidal & Bacteriostatic. Sterility testing of products (solids, liquids, ophthalmic and other sterile products) according to IP, BP and USP.

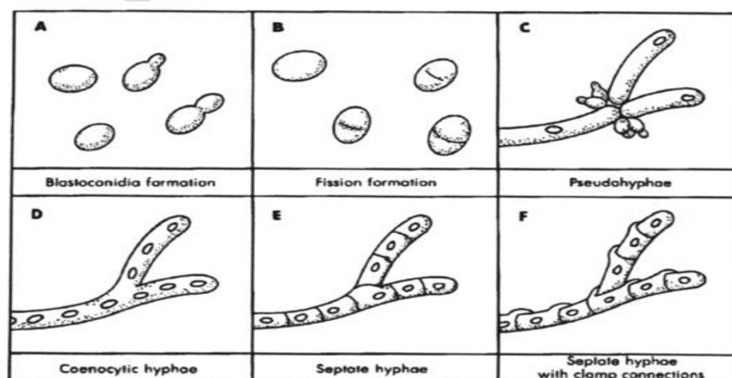
Fungi

A **fungus** (plural: **fungi**) is a kind of living organism: yeasts, moulds and mushrooms are types of **fungi**. The **fungi** are a separate kingdom of living things, different from animals and plants. **Fungi** have cells with nuclei. Their cell walls contain chitin, unlike the cell walls of plants, which contain cellulose.

Morphology of fungi

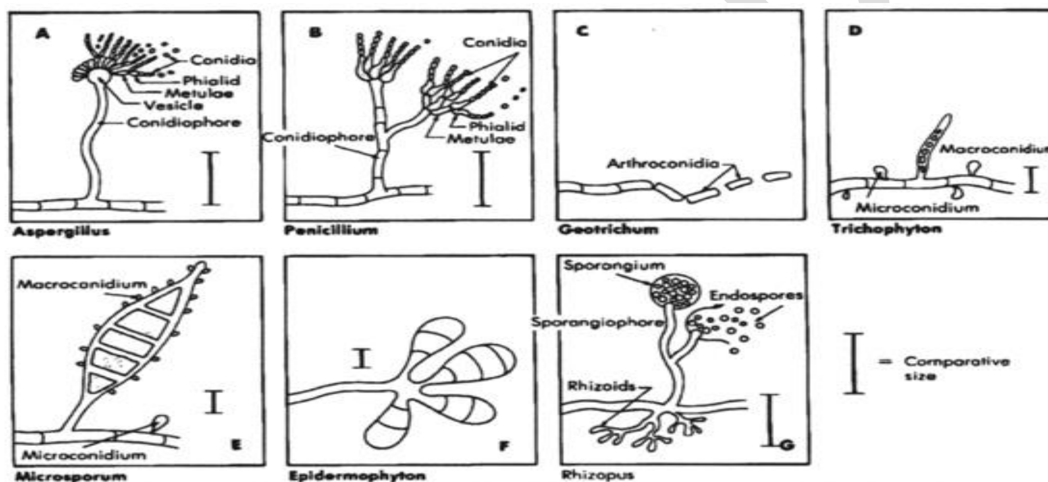
The fungi are more evolutionarily advanced forms of microorganisms, as compared to the prokaryotes (prions, viruses, bacteria). They are classified as **eukaryotes**, i.e., they have a diploid number of chromosomes and a nuclear membrane and have sterols in their plasma membrane. Genetic complexity allows morphologic complexity and thus these organisms have complex structural features that are used in speciation.

Fungi can be divided into two basic morphological forms, yeasts and hyphae. **Yeasts**are unicellular fungi which reproduce asexually by **blastoconidia** formation (budding) or fission. **Hyphae**are multi-cellular fungi which reproduce asexually and/or sexually. **Dimorphism** is the condition where by a fungus can exhibit either the yeast form or the hyphal form, depending on growth conditions. Very few fungi exhibit dimorphism. Most fungi occur in the hyphae form as branching, threadlike tubular filaments. These filamentous structures either lack cross walls **(coenocytic)** or have cross walls (**septate**) depending on the species. In some cases septate hyphae develop **clamp connections** at the septa which connect the hyphal elements.

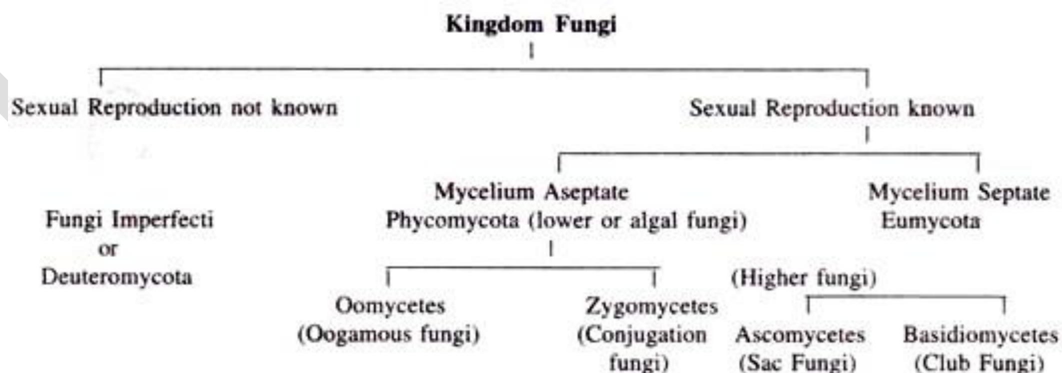


A. Yeast cells reproducing by blastoconidia formation; B. Yeast dividing by fission; C. Pseudohyphal development; D. Coenocytic hyphae; E. Septate hyphae; F. Septate hyphae with clamp connections From *Medical Microbiology, 1990*, Murray, et al., p. 299, Fig. 28-1. Reproduced with permission.

A mass of hyphal elements is termed the **mycelium** (synonymous with **mold**). Aerial hyphae often produce asexual reproduction propagules termed **conidia**(synonymous with **spores**). Relatively large and complex conidia are termed **macroconidia** while the smaller and more simple conidia are termed **microconidia**. When the conidia are enclosed in a sac (the sporangium), they are called **endospores**. The presence/absence of conidia and their size, shape and location are major features used in the laboratory to identify the species of fungus in clinical specimens.



A. Aspergillus; B. Penicillium; C. Geotrichum; D. Trichophyton; E. Microsporum; F. Epidermophyton and G. Rhizopus.



Classification of fungi

Oomycetes – The Oogamous Fungi:

Some of the important points of Oomycetes are listed below:

1. The mycelium is coenocyte (multinucleate and aseptate).

2. Hyphal wall contains cellulose and other glucans in many members. In some cases chitin or fungus cellulose is also present.

3. Asexual reproduction involves the formation of spore containing sacs or sporangia. In aquatic conditions the sporangia produce zoospores. In terrestrial conditions the sporangia often behave as spores, equivalent to conidia. Because of it, the sporangia are often called conidiosporangia.

4. Zoospores are generally biflagellate with heterokont flagellation in which one flagellum is smooth while the other is of tinsel type (having fine surface outgrowths Called mastigonemes).

5. Gametes are usually non-flagellate.

6. Sexual reproduction is by gametangial contact in which the male sex organs or antheridium passes its product into the female sex organ or oogonium through a fertilization tube.

7. The product of sexual reproduction is oospore.

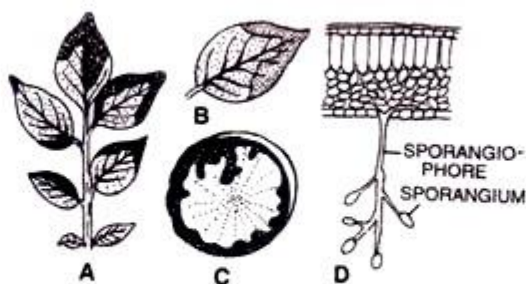


Fig. 2.45. *Phytophthora infestans*— Late blight of Potato. A, infected leaf. B, infected leaflet. C, section of infected tuber. D, sporangiophore coming out of leaflet.

Examples:

1. Late Blight:

Phytophthora infestans causes late blight of Potato and occasionally of Tomato as well. Blight is the appearance of brownish to black dead areas.

They are first formed on the margins and tips of leaflets. Later on the whole foliage becomes blighted. Tuber yield is reduced. The surface of the tubers also shows blighting. Irish famine of 1845- 1847 was caused by late blight of Potato.

2. White Rust:

It occurs in crucifers and is characterised by the appearance irregular white blisters containing, conidiosporangia on the leaves and stems. White rust is caused by Albugo Candida (= Cystopus Candidas: Fig. (2.46).

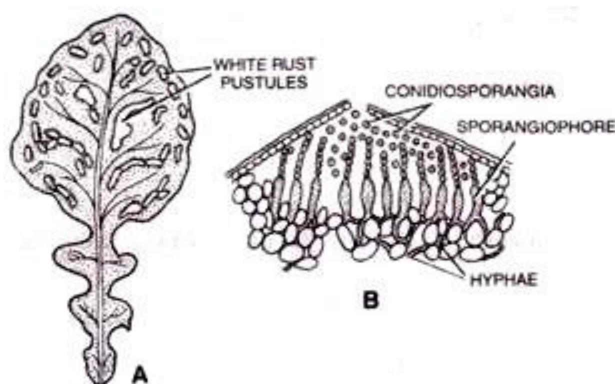


Fig. 2.46. *Albugo candida* (*Cystopus candidus*)— White Rust. A, infected leaf; B, T.S. infected leaf.

3. Damping off:

Pythium debaryanum kills seedlings of a number of plants through collapse of stem just above the ground level.

4. Downy Mildew:

The pathogen produces a cottony or wooly bloom on the surface of the host. Sclerospora graminicola spreads downy mildew in cereals and green ear disease of Pennisetum typhoides (vem. Bajra). Peronospora parasitica causes downy mildew in a number of plants, e.g., Pea, Mustard, Spinach, Onion, etc..

Zygomycetes – The Conjugation Fungi:

1. It is a class of terrestrial fungi which are mostly saprotrophic, rarely parasitic.

2. The mycelium is coenocytic (multinucleate, aseptate).

3. Hyphal wall contains chitin or fungus cellulose.

4. Motile cells (zoospores and planogametes) are absent.

5. Mito-spores are non-motile. They are called sporangiospores as the spores are formed inside sporangia borne at the tips of special hyphae called sporangiophores.

6. Sexual reproduction occurs through gametangial copulation or conjugation. Because of it, zygomycetes are also called conjugation fungi.

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7. The gametes are multinucleate and are called coenogametes.

8. Sexual reproduction produces a resting diploid spore called zygospore. Because of the presence of zygospore, the group of fungi is called zygomycetes. Zygospore differs from oospore in that during its formation a distinct large food laden non-motile female gamete is not produced.

9. Zygospore does not give rise to new mycelium directly. Instead it produces a new sporangium called germ sporangium (previously called zygospo-rangium). Germ sporangium forms meiospores called germ spores.

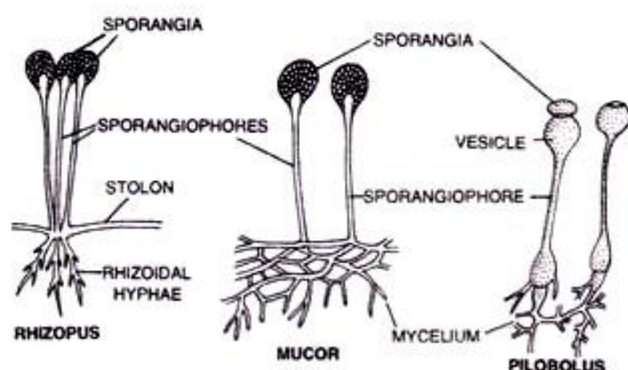

Fig. 2.47. Three common zygomycetes.

Examples:

1. Squirting Fungus:

Pilobolus crystallinus is a coprophilous or dung mould in which mature sporangia are thrown away up to a distance of 2m.

2. Rhizopus and Mucor:

Rhizopus stolonifer (= R. nigricans) is popularly known as black bread mould. Mucor caninus or M. mucedo is coprophilous. It is also called dung mould. Rhizopus and Mucor are the common saprotrophic fungi that attack a variety of food stuffs.

Soft rot or leak disease of Strawberry, Apple, Sweet Potato, etc. is due to Rhizopus. Mucor pusillus causes infection of internal organs in human beings. Absidia corymbifera causes bronchomycosis. Both Rhizopus and Mucor species (e.g., Rhizopus oryzae, Mucor javanicus) are used in alcoholic fermentation. The two also produce a number of organic acids like citric acid, lactic acid and fumaric acid.

Ascomycetes – The Sac Fungi:

Some of the important points of Ascomycetes are listed below:

1. Ascomycetes (askos- sac, mykes- fungus) is a class of diverse fungi numbering over 30,000 species. They include pigmented moulds (brown, green, blue, pink), powdery mildews, yeasts, cup fungi, morels and truffles. Nutritionally they are saprotrophic, decomposers, coprophilous or parasitic.

2. The mycelium consists of septate hyphae. Yeasts are an exception in that they are basically unicellular. They may, however, form short temporary filamentous structure called pseudo-mycelium.

3. The septa possess central pores called septal pores. The pores allow communication between adjacent cells. Septal pores show plugging of different types.

4. Cell wall contains chitin or fungus cellulose.

5. Motile structures do not occur in the life cycle.

6. In yeasts, asexual reproduction occurs through budding and fission. Oidia stage, similar to yeast, is found. In some other ascomycetes as well.

7. In majority of ascomycetes, the common mode of asexual reproduction is through the formation of conidia (singular-conidium). Conidia are non-motile fungal mitospores which are produced exogenously from the tips and sides of hyphae called conidiophores.

Conidia are often coloured brown, green, blue or pink. They provide colouration to the fungus. Greenish and bluish growth on bread, citrus fruits and old leather is due to moulds belonging to ascomycetes e.g., Penicillium, Aspergillus.

8. Conidiophores may be branched or un-branched, scattered or aggregated to form structures like acervulus, synnema, sporodochium, etc.

9. Sexual reproduction takes place through fusion of sex cells, somatic cells, gametangial contact between an antheridium and ascogonium, and autogamy.

10. Fertilization occurs in two steps, plasmogamy and karyogamy. Karyogamy is delayed after plasmogamy. A new transitional phase appears in the life cycle. It is called dikaryophase. The cells of dikaryophase are called dikaryotic cells. Each such cell possesses two nuclei (n+n).

11. Some dikaryotic cells function as ascus mother cells. The latter act as the seats of both karyogamy and meiosis. This converts the cells into asci (singular- ascus).

12. Ascus is a sporangial sac peculiar to ascomycetes. 4-8 haploid meiospores named ascospores are produced internally in each ascus. In most of the cases half the number of ascospores belong to one mating type while the other half belong to the second mating type.

13. The asci may occur freely or get aggregated with dikaryotic mycelium to form fructifications called ascocarps. Ascocarps are of many types: cup-like (apothecium, e.g., Peziza), flask-shaped (perithecium

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e.g., Neurospora), elongated with a slit (hysterothecium) or closed (cleistothecium e.g., Penicillium). The fructifications of some ascomycetes are edible and considered delicacies, e.g., morels, truffles.

Examples:

1. Yeasts:

Yeasts are a group of non-mycelial or pseudomycelial ascomycetes which multiply asexually by budding or fission and where asci are not organised into ascocarps.

Depending upon the mode of asexual reproduction, yeasts are of three types— budding yeasts e.g. Saccharomyces), fission yeasts (e.g., Schizosaccharomyces) and halobial yeasts (both budding and fission, e.g., Saccharomycoides). Yeasts in which ascus formation is known are named as true yeasts.

Related forms which resemble yeasts in most characteristics but where ascus formation is not reported are called false yeasts, e.g., Candida, Mycoderma, and Cryptococcus. They are otherwise included amongst deuteromycetes.

Economic Importance:

(i) Brewing Industry:

Under anaerobic conditions sugary solutions inoculated with yeasts are converted into alcoholic beverages, e.g., beer, wine, cider, toddy. They are concentrated further to produce rum and whisky. The two common yeasts used by brewing industry are Saccharomyces cerevisiae (Beer or Baker's yeast) and S. ellipsoidens (Wine Yeast),

(ii) Baking Industry:

Kneaded flour is inoculated with Saccharomyces cerevisiae (Baker's Yeast). It produces carbon dioxide and alcohol. The two evaporate during baking, making the dough soft and spongy,

(iii) Vitaminised Food:

Yeast used in brewing industry is regularly harvested and used as vitaminised food,

(iv) Curing:

Yeasts are used in curing cocoa beans,

(v) Spoilage of Food:

Being saprotrophic, yeasts attack various food stuffs including tomato products, foods having lactic acid and carbonated beverages,

(vi) Silk Industry:

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Some yeast reduces the yield of silk industry by attacking silkworms,

(vii) Plant Diseases:

Species of Nematospora attack Cotton, Tomato and Beans,

(viii) Human Diseases:

Candida albicans causes thrush and inflammation of genitalia. Cryptococcus neoformans attacks nervous system producing lesions, meningitis and brain tumour. Torula produces skin nodules and lesions of viscera.

2. Aspergillus:

It is a common green smoky mould which not only contaminates laboratory cultures (hence weed of laboratory) but also various food stuffs including bread, butter, cheese, syrups, jams, jellies, textile and leather goods. It causes rotting of dates, figs, pomegranates, cigars and tobacco.

Some lung (pulmonary aspergillosis) and ear infections are caused by Aspergillus species. Fermentation effected by Aspergillus yields alcohol (Sake of Japan), citric acid, gluconic acid, glycerol, B-complex vitamins, enzymes and antibiotics.

3. Penicillium:

P. chrysogenum yields the antibiotic penicillin. The latter was the first commercial antibiotic. It was formerly obtained from P. notatum. P. griseofulvum produces antifungal drug griseofulvin.

The fungus is employed in ripening of cheese (camembert and roquefort varieties) and production of organic acids. The fungus is otherwise known to spoil food, citrus fruits, apple, grape, paper, wood and ensilage. The blue-green mould appearing on citrus fruits is Penicillium.

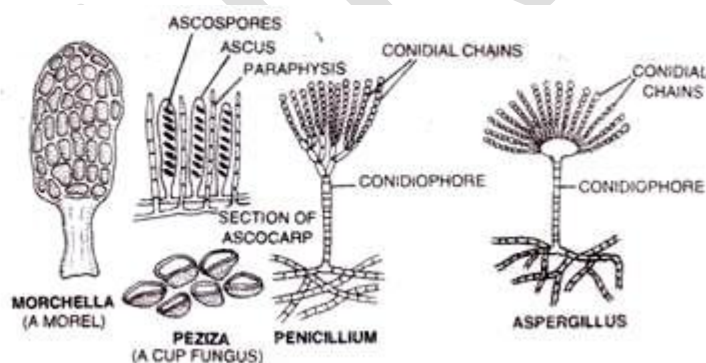

Fig. 2.48. Some ascomycetes.

4. Neurospora (Pink Bread Mould):

N. crassa is often employed in studies conducted in experimental genetics. It is often called “Drosophila of plant kingdom”.

5. Erysiphe:

The fungus produces powdery mildew (fungal disease in which pathogen results in a powdery coating on the surface of the host), e.g., Erysiphe graminicola (E. graminis, on cereals like Wheat, Barley), E. polygoni (on legumes like Pea).

6. Claviceps:

Claviceps purpurea produces ergot of rye and other cereals in which ears come to have sclerotia of the fungus. Eating of infected cereals produces ergotism. Ergotism is of two types, gangrenous and spasmodic. The sclerotia contain a number of alkaloids, the most important being lysergic acid.

Ergot is used as a medicine to control migraine, enlarged prostate glands and uterine haemorrhage after child birth. These days lysergic acid is prepared through fermentation activity of C. paspali. LSD, a hallucinogen, is D-lysergic acid diethylamide-15.

7. Sclerotinia:

S. fruticola causes brown rot of Peach, Plum and Pear.

8. Cup Fungi:

The ascocarp is cup-shaped, e.g., Peziza.

9. Morels:

Morels are ascomycetes with edible ascocarps that have fleshy sponge-like conical cap or pileus and a stalk like stipe, e.g., Morchella esculenta (vern. Gucchi), M. crassipes, M. deliciosia.

10. Truffles:

They are edible ascomycetes with tuber-like subterranean ascocarps that are often dug out with the help of trained dogs and pigs, e.g., Tuber uncinatum, T. aestivum.

Basidiomycetes – The Club Fungi:

1. Basidiomycetes (Gk. basidium- small base, mykes- fungus) are the most advanced and most commonly seen fungi as their fructifications are often large and conspicuous, e.g., mushrooms (gill fungi), toadstools, puff balls, bracket fungi, etc.

2. The class contains about 25,000 species.

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3. Basidiomycetes are among the best decomposers of wood. Only a few insects can compete with basidiomycetes in decomposing hard woods and woody structures of trees. Basidiomycetes are able to decompose both cellulose and lignin. Lignin is not metabolised by most other fungi and even bacteria.

For decomposing wood, these fungi secrete cellulose and lignin digesting enzymes. The enzymes create spaces in the wood for hyphae to pass inwardly. It is because of this that we sometimes observe toadstools and mushrooms to come out of wooden structures. Ganoderma species causes decay of wood even of standing trees.

4. Motile structures or cells are absent.

5. Mycelia are of two types, primary and secondary. Primary mycelium contains monokaryotic cells, that is, cells with single haploid nuclei (n).

6. Monokaryotic phase or primary mycelium may multiply by oidia, conidia-like spores and pycniospores. Dikaryotic mycelium does not multiply by asexual spores.

7. There is often differentiation of two mating types, (+) and (-).

8. Sexual reproduction does not involve sex organs. Instead plasmogamy (fusion of protoplasts without fusion of their nuclei) occurs by fusion between basidiospores and other monokaryotic spores, between a spore or spermatium and a hypha or between two hyphal cells of primary mycelia.

9. Karyogamy is delayed for long. The intervening phase is called dikaryophase. It produces a new mycelium called secondary mycelium which is dikaryotic (n+n).

10. Secondary mycelium is long lived. It consists of profusely branched septate hyphae.

11. Septa possess dolipores or central pores with barrel-shaped outgrowths.

12. Hook-shaped outgrowths are found on the sides of septa. They are called clamp connections. Clamp connections are meant for proper distribution of dikaryons at the time of cell division.

13. Secondary mycelium can perennate in the soil or wood by means of sclerotia (often rounded or ellipsoid firm masses of hyphae) or rhizomorphs (root-like aggregation of hyphae with well defined apical meristems).

14. Dikaryophase or secondary mycelium may multiply by different types of spores— chlamydospores, aecidiospores, uredospores, teleutospores, etc.

15. Karyogamy and meiosis occur in club-shaped structures known as basidia (singular— basidium). The name of the class is based after them. A basidium may be aseptate (holobasidium) or septate vertically or transversely (phragmobasidium).

16. A basidium commonly produces four meiospores or basidiospores exogenously at the tips of fine outgrowths called sterigmata.

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17. The fungi may or may not produce fructifications called basidiocarps. The basidiocarps vary from microscopic forms to large macroscopic structures. Some puff balls and brackets can be over 50 cm in diameter.

Examples:

1. Rusts:

They are characterised by the formation of rusty pustules containing the spores. A basidiocarp is absent, (i) Puccinia graminis tritici — black rust of wheat, (ii) Puccinia glumarum — yellow rust of wheat, (iii) Hemileia vastatrix — leaf rust of coffee.

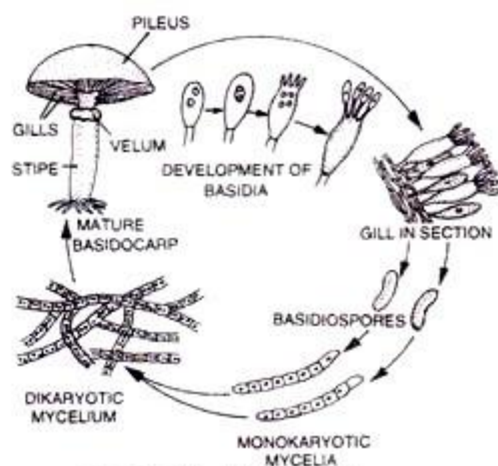

Fig. 2.49. Life history of mushroom *Agaricus campestris*.

2. Smuts:

They produce thick-walled black-coloured resting spores called smut spores (= teleutospores = chlamydospores). Smuts are of two types, covered and loose. In covered smuts the spore mass remains within the host till the latter is set free, e.g. Ustilago maydis (smut of corn), Tilletia tritici (bunt or stinking smut of wheat). In loose smut the spores are exposed while attached to the host, e.g. Ustilago tritici (loose smut of wheat).

3. Mushrooms:

They are edible and nonedible agaric ales which possess umbrella like basidiocarp the edible mushrooms generally possess coloured basidiospores. Common examples are Agaricus campestris, Agaricus brunnescens (= A. bisporus), Volvariella volvacea (Paddy Straw Mushroom), Lentinus edodes (Shiitake Mushroom).

4. Toadstools:

Toadstools are nonedible, often poisonous mushrooms which generally have white spores. Amanita caesarea (Caesaer's Mushroom) was used in poisoning Roman emperor Caesar. The other toadstools are Amanita phalloides (Death Cap) and A. muscaria (Fly Agaric).

5. Bracket Fungi (Shelf Fungi):

The basidiocarps or fructifications appear on tree trunks, logs, lumber, etc. just as brackets or shelves, e.g. Fomes applanatus (perennial), Polyporus sulphureus (annual).

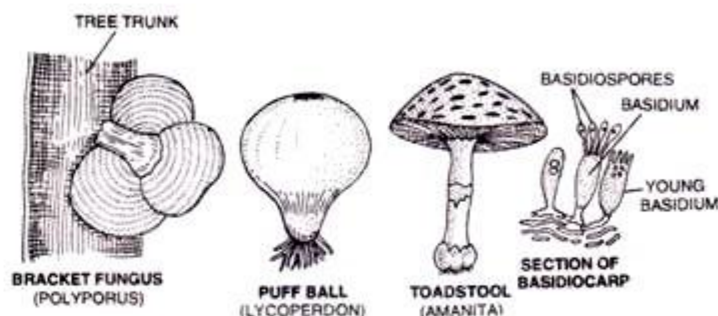

Fig. 2.50. Some basidiomycetes.

6. Puffballs:

The basidiocarp is a stalked rounded structure which on ripening sends out puffs of spores. The fructification may grow above or below the substratum. Puffballs are odoriferous. They are edible in the young state, e.g. Lycoperdon oblongisporum, L. giganteum.

7. Armillariella (= Armillana):

A. mellea (Honey Mushroom) is an edible mushroom which is a serious root parasite of both hardwoods and conifers. The fungus sends rhizomorphs into the phloem of the host and hence block the food supply.

8. Hallucinogens:

Psilocybe mexicana (Sacred Mushroom) has hallucinating properties similar to LSD. It is used by Mexican Indians for certain religious ceremonies.

Deuteromycetes – The Fungi Imperfecti:

Some of the important points of Deuteromycetes are listed below:

1. Deuteromycetes is an artificial class of fungi which has been created to include all those fungi in which sexual stage is either absent or not known.

2. Some of the deuteromycetes are unicellular like yeasts. They are often studied along with the latter.

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3. The mycelium is usually septate. Coenocytic forms are not known. Clamp connections, typical of basidiomycetes, are absent.

4. Asexual reproduction often occurs by conidia along with some other types of spores. In some cases even asexual spores are absent.

5. It is believed that most members of deuteromycetes are actually ascomycetes in which sexual reproduction is either absent or yet to be discovered.

Examples:

1. Red Rot (Fig. 2.51):

Colletotrichum falcatum produces red rot of sugarcane which is conspicuous on leaf midribs as well as in canes. It reduces juice content of canes and brings about withering of leaves. The fungus develops sickle-shaped conidia. The perfect stage is Glomerella tucumanensis.

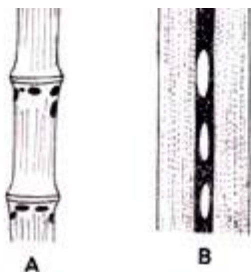

Fig. 2.51. Red rot disease of Sugarcane. A, infected cane. B, part of infected leaf.

2. Helminthosporium (Fig. 2.52):

Helminthosporium oryzae causes leaf spot disease of rice commonly called sesame or brown leaf spot of rice. It caused Bengal famine of 1942-43 and similar conditions in Krishna-Godavari area in 1989-1990. The perfect stage of the fungus is Cochliobolus miyabeanus. The conidia are 5-10 septate.

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Fig. 2.52. Sesame or brown leaf spot disease of rice. A, infected leaf. B, with infected grains.

3. Early Blight:

Alternaria sotani causes early blight of Potato and Tomato. The leaves develop small oval brown spots with concentric rings. The leaves as well as the branches wither and fall down. The conidia are beaked bottle-like multi-septate with a number of transverse and a few longitudinal septa.

4. Tikka Disease:

Circular necrotic dark brown or blackish leaf spots develop in groundnut due to Cercospora (e.g. C. personata). The conidia are septate and filamentous. The perfect stage is Mycosphaerella (e.g., M. berkeleyii).

5. Wilts:

Many economically important plants (e.g., Potato, Tomato, Cotton, Banana, Flax, Pigeon Pea) show sudden signs of wilting due to blockage of tracheary elements by growth of fungus Fusarium especially F. oxysporum. The fungus shows three types of spores — chlamydospores, micro conidia and macro conidia.

6. Gibberellins:

They were first discovered in the extracts of Fusarium moniliformae growing on rice (bakane or foolish disease of rice). The perfect stage of fungus is Gibberella fujikuroi. Gibberellins are natural plant growth hormones.

7. Trichoderma (Fig. 2.53).

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It is a soil fungus used in biological control of other fungi as it produces allelochemics against them. If the fungus happens to pass into human alimentary canal it produces leucopenia called alimentary canal aleukia.

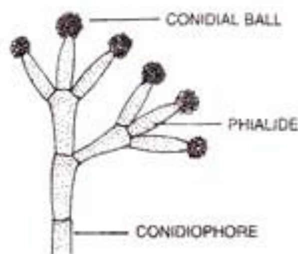

Fig. 2.53. Conidiophore of *Trichoderma*.

Reproduction of fungi

Fungi can reproduce asexually by fragmentation, budding, or producing spores, or sexually with homothallic or heterothallic mycelia.

Key Terms

homothallic: male and female reproductive structures are present in the same plant or fungal mycelium

gametangium: an organ or cell in which gametes are produced that is found in many multicellular protists, algae, fungi, and the gametophytes of plants

spore: a reproductive particle, usually a single cell, released by a fungus, alga, or plant that may germinate into another

sporangium: a case, capsule, or container in which spores are produced by an organism

karyogamy: the fusion of two nuclei within a cell

plasmogamy: stage of sexual reproduction joining the cytoplasm of two parent mycelia without the fusion of nuclei

Fungi reproduce sexually and/or asexually. Perfect fungi reproduce both sexually and asexually, while imperfect fungi reproduce only asexually (by mitosis).

In both sexual and asexual reproduction, fungi produce spores that disperse from the parent organism by either floating on the wind or hitching a ride on an animal. Fungal spores are smaller and lighter than plant seeds. The giant puffball mushroom bursts open and releases trillions of spores. The huge number of spores released increases the likelihood of landing in an environment that will support growth.

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(a)

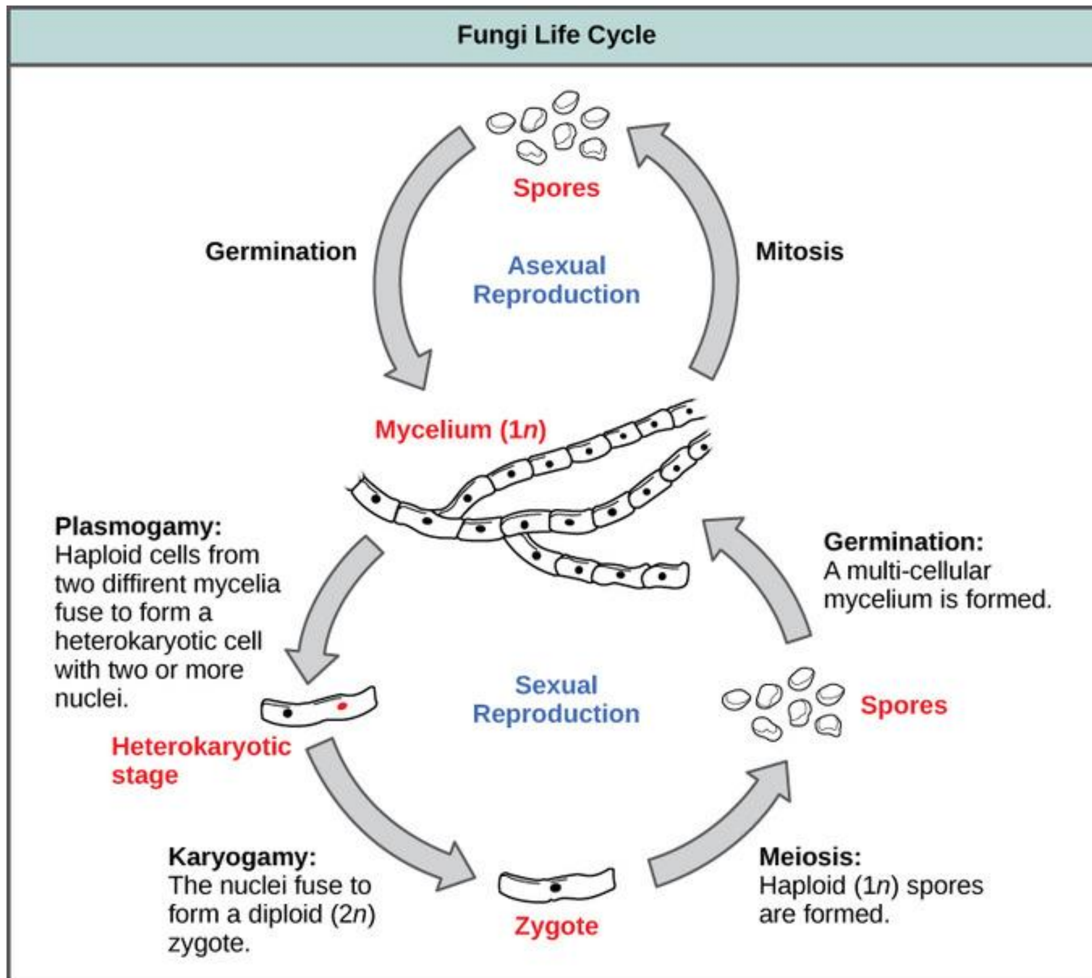
(b)

The release of fungal spores: The (a) giant puff ball mushroom releases (b) a cloud of spores when it reaches maturity.

Asexual Reproduction

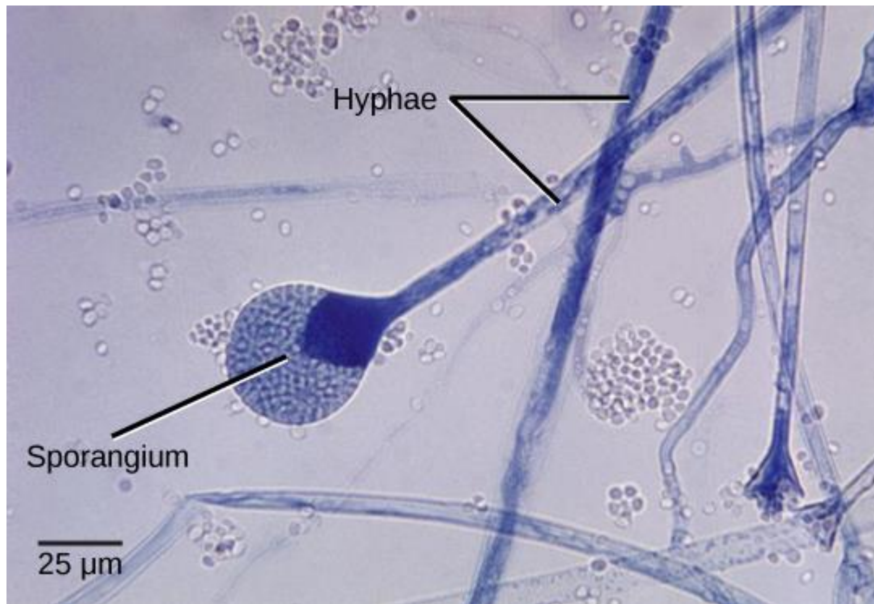
Fungi reproduce asexually by fragmentation, budding, or producing spores. Fragments of hyphae can grow new colonies. Mycelial fragmentation occurs when a fungal mycelium separates into pieces with each component growing into a separate mycelium. Somatic cells in yeast form buds. During budding (a type of cytokinesis), a bulge forms on the side of the cell, the nucleus divides mitotically, and the bud ultimately detaches itself from the mother cell.

The most common mode of asexual reproduction is through the formation of asexual spores, which are produced by one parent only (through mitosis) and are genetically identical to that parent. Spores allow fungi to expand their distribution and colonize new environments. They may be released from the parent thallus, either outside or within a special reproductive sac called a sporangium.



Types of fungal reproduction: Fungi may utilize both asexual and sexual stages of reproduction; sexual reproduction often occurs in response to adverse environmental conditions.

There are many types of asexual spores. Conidiospores are unicellular or multicellular spores that are released directly from the tip or side of the hypha. Other asexual spores originate in the fragmentation of a hypha to form single cells that are released as spores; some of these have a thick wall surrounding the fragment. Yet others bud off the vegetative parent cell. Sporangiospores are produced in a sporangium.



Release of spores from a sporangium: This bright field light micrograph shows the release of spores from a sporangium at the end of a hypha called a sporangiophore. The organism depicted is a Mucor sp. fungus: a mold often found indoors.

Sexual Reproduction

Sexual reproduction introduces genetic variation into a population of fungi. In fungi, sexual reproduction often occurs in response to adverse environmental conditions. Two mating types are produced. When both mating types are present in the same mycelium, it is called homothallic, or self-fertile. Heterothallic mycelia require two different, but compatible, mycelia to reproduce sexually.

Although there are many variations in fungal sexual reproduction, all include the following three stages. First, during plasmogamy (literally, “marriage or union of cytoplasm”), two haploid cells fuse, leading to a dikaryotic stage where two haploid nuclei coexist in a single cell. During karyogamy (“nuclear marriage”), the haploid nuclei fuse to form a diploid zygote nucleus. Finally, meiosis takes place in the gametangia (singular, gametangium) organs, in which gametes of different mating types are generated. At this stage, spores are disseminated into the environment.

Cultivation of fungi

The media described below have been chosen because they represent a sample of the variety of types used by mycologists. There are, however, many additional media; persons interested in pursuing this matter further should consult the more specialized literature.

SYNTHETIC

Czapek's Solution Agar	
Sucrose	30 g
$NaNO_3$	3.0 g
K_2HPO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
$FeSO_4 \cdot 7H_2O$	0.01 g
Agar	15 g
Distilled water	1000 ml

Czapek's Solution Agar is a synthetic medium widely used in mycological laboratories, particularly for the identification of species of *Aspergillus* and *Penicillium*. Many moulds produce very characteristic colonies on it and may also exude pigmented substances. Aerial growth is often suppressed and sporulation may be enhanced. Some moulds, however, grow poorly on this medium and may even fail to sporulate altogether, often because of their inability to synthesize vitamins. Many members of the Zygomycota are unable to process sucrose or nitrates and will do very poorly on Czapek's. The high glucose level may also cause problems. As noted above, the addition of agar to this medium makes it, in reality, a semi-synthetic one.

***Penicillium* Reference Medium**	
Glucose	9.1 g
Tris buffer, adjusted to pH 8.0 with HCl	606 mg
KNO_3	425 mg
KCl	485 mg
$MgSO_4 \cdot 7H_2O$	493 mg

$CaCl_2 \cdot 2H_2O$	44 mg
$NaH_2PO_4 \cdot H_2O$	28 mg
$FeCl_3 \cdot 6H_2O$ chelated with 8.5 mg of disodium salt of EDTA	6.2 mg
H_3BO_3	6.11 mg
$MnCl_2 \cdot 6\ H_2O$	366 Ã•g
$ZnSO_4 \cdot 7\ H_2O$	461 Ã•g
$Na_2MoO_4 \cdot 2H_2O$	14.5 Ã•g
$CoCl_2 \cdot 6H_2O$	23.8 Ã•g
$CuSO_4 \cdot 5H_2O$	20 Ã•g
Thiamine chloride	20 Ã•g
Biotin	1 Ã•g
Vitamin B_{12}	1 Ã•g
Agar	15 g
Distilled water	1000 ml

The medium is prepared as three stock solutions that can be stored and later combined to form the final mixture. The solutions are:

1. Major salts stock: 4.85 g KCl, 4.93 g $MgSO_4.7H_2O$, 0.441 g $CaCl_2.2H_2O$, 0.88 g NaCl in 1 litre

2. Buffer stock : 60.6 g/l Tris adjusted to pH 7.8 with HCl.

3. Micronutrients + vitamins stock: One litre stock prepared by mixing 5 ml each of 54.4 g/l $NaH_2PO_4.H_2O$, 12.6 g/l $FeCl_3.6H_2O$ chelated with 17 g/l of disodium salt of EDTA, 12.2 g/l H_3BO_3, 732 mg/l $MnCl.6H_2O$, 922 mg/l $ZnSO_4.7H_2O$, 29 mg/l $Na_2MoO_4.2H_2O$, 47.6 mg/l $CoCl_2.6H_2O$, 40 mg $CuSO_4.5H_2O$, 40 mg/l thiamine chloride, 2 mg/l biotin, 2 mg/l vitamin B_{12}.

The complete mixture contains in a final volume of l litre: 15 g agar, 100 ml each of major salts and micronutrient stocks, and 10 ml of buffer stock.

This medium was formulated by Dr. I. Ahmad for the cultivation of *Penicillium* species. Although it appears at first glance to be a complicated and difficult medium it is usually prepared from three stock solutions and does not take as long as one might expect. It can be modified in several ways to assess the physiological activities of moulds. Most commonly different sources of carbon are substituted for glucose and different nitrogen sources for nitrate. As formulated above,with nitrate as a nitrogen source, it may be unsuitable for certain fungi, such as many Zygomycota, unable to utilize nitrates. In such cases it may be better to use ammonium salts, although this may result in dramatic plunges in pH. Again, to be truly "synthetic", the medium should be used without agar.

SEMI-SYNTHETIC

Modified Leonian's Agar	
Maltose	6.25 g
Malt extract	6.25 g
KH_2PO_4	1.25 g
Yeast extract	1.0 g
$MgSO_4 \cdot 7H_2O$	0.625 g
Peptone	0.625 g
Agar	20 g
Distilled water	1000 ml

Leonian's medium was devised by the American mycologist L.H. Leonian, and was designed to promote sporulation in certain moulds. Later, at the University of Toronto, R.F. Cain found it to be more suitable for ascomycetes if it contained a little yeast extract; hence the term "modified" in its name. It is a good general purpose medium that will yield good growth with most fungi. Certain fungi, such as many mycorrhizal forms, will not grow on Leonian's Agar because they are unable to use maltose as an energy source. For these fungi we use Modified Melin-Norkran's medium, which has a glucose energy component.

Potato Dextrose Agar	
Thinly sliced, peeled white potatoes	500 g
Glucose	20 g
Agar	15 g
Distilled water	1000 ml

Heat potatoes at 60â–' C for 1 hour and filter through cheesecloth. Make up volume to 1000 ml and add other ingredients. Cook 1 hour and then sterilize.

Potato Dextrose Agar, or PDA, as it is usually called, is an old formula used by plant pathologists and many mycologists for general laboratory use.

Sabouraud's Agar	
Glucose	40 g
Peptone	10 g
Agar	15 g
Distilled water	1000 ml

This is the standard medium used in medical mycology. It is probably no better for these moulds than Leonian's or PDA but is simply the traditional choice and thus the medium that must be used if colonies are to be compared with those described by medical workers. It can be used as a general laboratory medium in place of Leonian's or PDA, although the amount of glucose is rather high and may suppress sporulation in some fungi.

Martin's Rose Bengal Agar	
Glucose	10 g
Peptone	5 g

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KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
Streptomycin	30 mg
Rose bengal	30 mg
Agar	15 g
Distilled water	1000 ml

Ten millilitres of a 3.3 g/l stock solution of rose bengal is added to the medium after the other ingredients (except the streptomycin) have been dissolved. After sterilization, the streptomycin is added to the cooled medium.

This medium is useful in plating techniques (see below) when the aim is to slow down the growth of colonies in a mixed culture. It discourages the growth of bacteria and certain other organisms so that they will not swamp isolates from natural materials.

Dextrose-peptone-yeast extract Agar (DPYA)	
Glucose (dextrose)	5.0 g
Peptone	1.0 g
Yeast extract	2.0 g
NH_4NO_3	1.0 g
K_2HPO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$FeCl_3 \cdot 6H_2O$	0.01 g
Oxgall	5.0 g
Sodium propionate	1.0 g

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Chlortetracycline	30 mg
Streptomycin	30 mg
Agar	20 g
Distilled water	1000 ml

DPYA is an excellent medium for isolating fungi from soil, dung and other natural substrates. The oxgall and sodium propionate restrict the growth of some rapidly spreading fungi, while the chlortetracycline and streptomycin discourage bacteria. It can also be prepared as a more general-use medium by omitting these four inhibitory substances.

NATURAL MEDIA

V-8 Agar	
V-8 vegetable juice	200 ml
$CaCO_3$	3 g
Agar	20 g
Distilled water	1000 ml

The V-8 juice used in this medium probably contains many nutrients that fungi can use, but we have little idea what they may be. It is a medium that is used routinely in plant pathology and seems to be a good complement to Leonian's or PDA. Moulds that fail to sporulate on those media often sporulate heavily on V-8, or vice versa.

Weitzman and Silva-Hutner's Agar	
Alphacel cellulose powder	20 g
Pablum baby oatmeal	10 g
Hunt's tomato paste	10 g
KH_2PO_4	1.5 g

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$MgSO_4$	1.0 g
$NaNO_3$	1.0 g
Agar	20 g
Distilled water	1000 ml

Weitzman and Silva-Hutner's medium was designed to enhance sporulation in certain medically important fungi, but is useful for a great many other moulds as well. I have found it to be a good (and often better) substitute for V-8 Agar and use it routinely in my laboratory. I grow most of my moulds on both Weitzman and Silva-Hutner's and Leonian's Agar and find very few that fail to sporulate on one or the other. Although the original formula calls for a final pH adjustment, I seldom do this and yet get good results.

PREPARATION OF MEDIA

Mixing

Culture media are not hard to prepare, even under relatively primitive conditions. It is often easiest to dissolve the agar separately in hot water and then add the other ingredients. The agar burns easily when it is heated, so it is best to avoid putting it directly on the stove or hotplate. I usually put the agar in a flask of water and then immerse this in a boiling water bath. After an hour or so the agar will be dissolved and the solution will be clear. Some people do not worry very much about whether the agar is completely dissolved or not, but to avoid an uneven batch it is best to be patient.

Once the agar is dissolved the rest of the ingredients can be stirred in; they must be completely dissolved as well. Some ingredients, of course, do not dissolve and must be mixed in suspension as thoroughly as possible.

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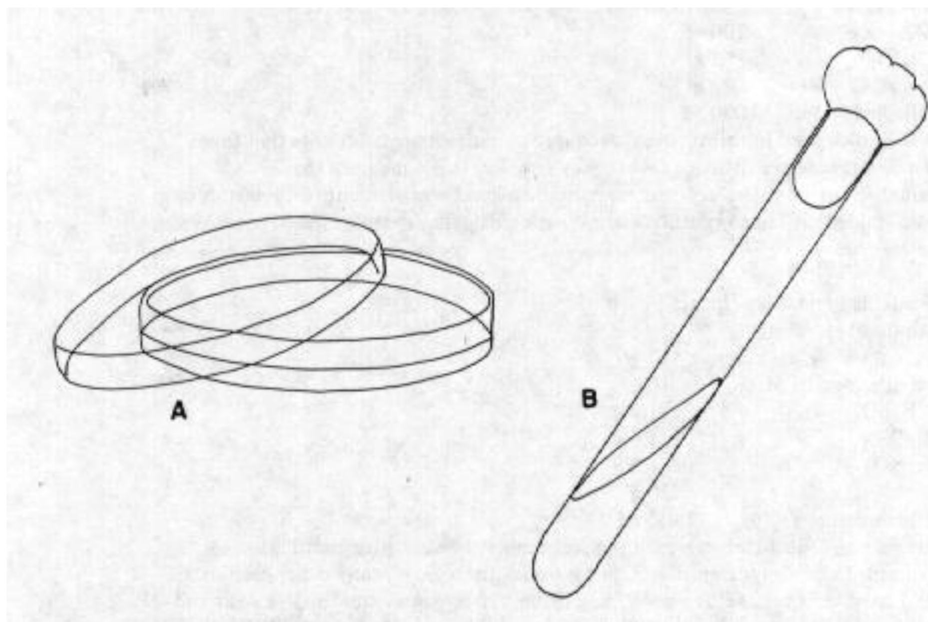

Figure 9. Glassware for cultivating moulds. A: Petri dish with lid partly removed. B: test-tube with agar slant.

When the medium is completely mixed it must then be prepared for sterilization. Here one must stop and decide what use is to be made of the finished product. If fresh isolates are to be made from natural materials or some mould we already have is to be studied, we will probably want to use *Petri dishes* (Figure 9A). Petri dishes are small, round dishes, most commonly 9 cm in diameter, that have a bottom and a loosely fitting top that will admit air but discourage entry of foreign spores. They are the most commonly used vessels for growing moulds and are available either in permanent glass or disposable plastic form. The plastic plates are already sterile when bought, but the glass ones must be heated in the oven at 230°C (450°F) for 60 minutes, to kill all foreign organisms and spores, before they can be used. But more about Petri dishes later.

It is often necessary to save cultures for later use, or for mailing, in a way that they are less vulnerable to contamination by airborne spores. The conventional method is to use test-tube or bottle cultures that have only a narrow opening (Figure 9B). The culture medium is poured into the tubes, sterilized, and allowed to solidify at about a 30°angle. When cool, the agar inside has a smooth slanting surface for the colony to occupy. The tube or bottle is capped with a cotton plug that is just tight enough for the tube to be lifted without it coming out. In place of cotton a screw cap or one of several tube-capping devices can be used.

With the use of medium in mind, one can now proceed to prepare it for sterilization. If it is to be used in Petri plates it should be transferred into smaller containers that will make it easy to pour. It is impractical to sterilize filled Petri plates, so we pour them after sterilization. If the medium is to be used in tubes, they should be filled before sterilization and slanted afterwards. Sometimes it is advantageous to save sterilized medium in larger bottles and melt it down later to pour plates.

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Sterilization

Sterilization is most often done in some kind of apparatus that will allow steaming at high pressures. In the laboratory we usually use a large sterilizer called an *autoclave*, but a simple pressure cooker works well, provided it has a pressure gauge. Most fungi and other organisms can be killed by boiling water, but there are a few, notably bacteria, that have highly resistant spores requiring sterner means. To be sure of killing *everything*, we sterilize at 121°C (250°F) for 20 minutes. To obtain this temperature the pressure must be raised to 1 kg/cm^2 (15 lb per $in.^2$ or 1 atmosphere).

When medium is sterilized in an autoclave it should not completely fill its container. Attempting to sterilize 1 litre of medium in a 1-litre flask will usually result in sudden and rather explosive boiling when it is removed from the autoclave. It is best not to fill the container more than half full. Inexperienced mycologists often assume that the 121°C for 20 minutes rule applies in all situations. However, not all materials will heat up enough to be sterilized in 20 minutes. It is an unpleasant surprise when a litre of medium becomes contaminated with bacteria because the person doing the sterilization attempted to do the whole batch in one container and barely managed to heat it to 100°C before the time was up.

After sterilization the medium is allowed to cool and solidify. Petri plates must be poured *before* the medium solidifies but while it is fairly cool. We usually test the medium by holding it against the inner side of the forearm; if it is cool enough to be poured it will not feel too hot. Beware, though of letting it cool too far, lest it solidify in the bottle! Pouring the medium when it is too hot results in unwanted condensation on the lid of the Petri dish.

Tubes, as stated above, should be left to solidify on a slant. Any agar medium that has solidified can be melted by heating in a boiling water bath. Melting of a 1/2-litre bottle of solidified medium will take about an hour if it is submerged well in boiling water. Petri plates dry up rather quickly, so it is often convenient to keep a number of bottles of solid sterilized medium that can be melted and poured when needed. When Petri plates are poured from melted agar, the neck of the bottle should always be heated over a flame first, either alcohol or gas, to kill any stray spore that may be on it.

Cultivation

Sterile technique

Once some Petri plates or tubes of sterile agar have been prepared, cultivation of one's moulds can begin. For the sake of simplicity, let us first suppose that a mould colony is already growing in a Petri dish and must be transferred to another. The essential thing to remember is that the air and all implements exposed to it are contaminated with mould spores and that these would germinate and

grow if they got into the sterile plate. The steps we take to avoid this contamination constitute what is called *sterile technique*.

To transfer the culture we do the following:

1.Take an inoculating needle, usually a thin needle or wire at the end of a long pencil-like handle, and heat it in an alcohol or gas flame until it glows bright red (Figure 10A).

2.Allow the needle to cool for about 15 seconds. (A hot needle will kill the mould that is to be transferred).

3.Open the Petri dish containing the culture just wide enough to allow entry of the needle.

4.With the heat-sterilized needle, cut out a small portion of the colony margin. Hyphal tip transfers work best as they are usually the most active parts of the culture; in addition, transfers from the heavily sporulating central portions will result in spores being spread into the air. Especially in medical work, hyphal tip transfers are essential. The excised colony margin should be only about 1 mm square (Figure 10B).

5.Transfer the square of colony margin to the sterile plate, making sure that the lid is opened only wide enough to admit the needle and make the transfer. Place the block at the centre, withdraw the needle and flame it until it is red hot, to kill all adhering spores and hyphae (Figure 10C,D).

6.Close the lid; label the plate with a marking pen, including name of culture and date. We usually wrap a thin strip of paraffin film around the sides of the plate to cover the opening, but this is not absolutely necessary; just a couple of pieces of masking tape to hold the lid down will do.

7.Leave the culture to grow in a protected place that has as little air movement as possible.

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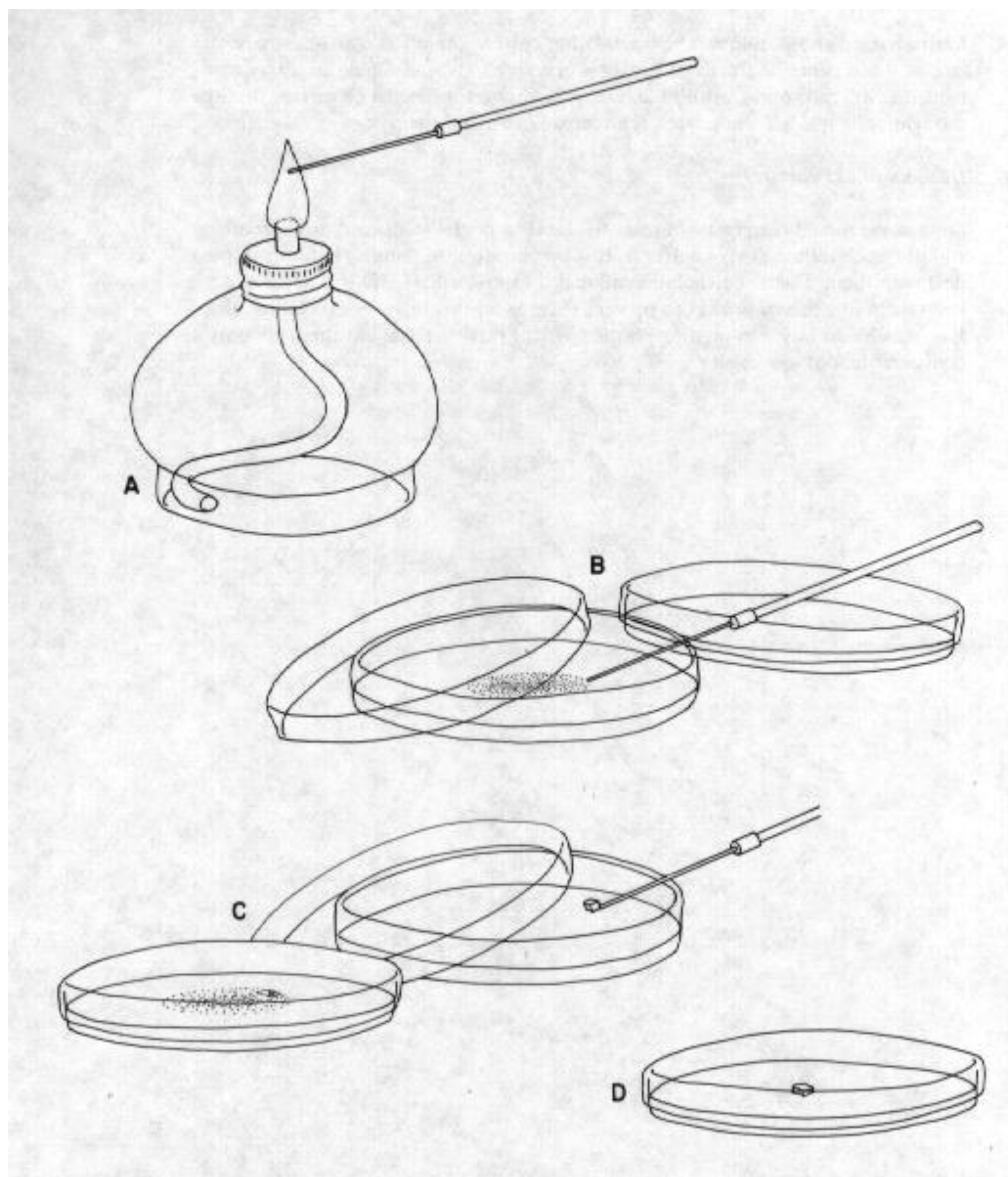

Figure 10. Steps in sterile technique. Inoculating needle is heated in an alcohol flame (A). Small piece of colony is removed from Petri dish (B) and transferred to a new dish of agar (C), yielding a plate containing a piece of the old culture at its centre (D).

Transferring from plates to tubes, tubes to plates, or tubes to tubes is done in a similar manner. When using tubes, always flame the mouth to kill any spores of airborne moulds. Never put the cotton plugs or lids of tubes on the table as they will pick up contamination.

The table itself should be clean and can be washed with water, alcohol, chlorine bleach, or other disinfectants. Some spores can survive long immersion in these substances, however, so one cannot thus expect to kill all spores on the table.

Many laboratories are now equipped with special inoculation chambers. Some, such as laminar flow chambers, have a layer of sterile filtered air flowing over the culture that excludes contaminating spores. Some models of such chambers, however, carry the air over the culture and into the worker's face. Such a device is a health hazard and should not be used. Other culture chambers consist only of an enclosed box, open at the front for the worker's hands, containing an ultraviolet light that kills all spores within the box. It is important here to be certain that the ultraviolet light is off while work is under way to avoid the risk of eye damage.

Virus

Viruses are infectious agents with both living and nonliving characteristics.

Living characteristics of viruses include the ability to reproduce – but only in living host cells – and the ability to mutate.

Nonliving characteristics include the fact that they are not cells, have no cytoplasm or cellular organelles, and carry out no metabolism on their own and therefore must replicate using the host cell's metabolic machinery.

Viruses can infect animals, plants, and even other microorganisms.

Since viruses lack metabolic machinery of their own and are totally dependent on their host cell for replication, they cannot be grown in synthetic culture media.

Structure of virus

Viruses vary in their structure. A virus particle consists of DNA or RNA within a protective protein coat called a **capsid.** The shape of the capsid may vary from one type of virus to another. The capsid is made from the proteins that are encoded by viral genes within their genome.

The shape of the capsid serves as one basis for classification of viruses. The capsid of the virus shown in **Figure** below is icosahedral. Virally coded proteins will self-assemble to form a capsid. Some viruses have an envelope of phospholipids and proteins. The envelope is made from portions of the host’s cell membrane. It surrounds the capsid and helps protect the virus from the host’s immune system. The envelope may also have receptor molecules that can bind with host cells. They make it easier for the virus to infect the cells.

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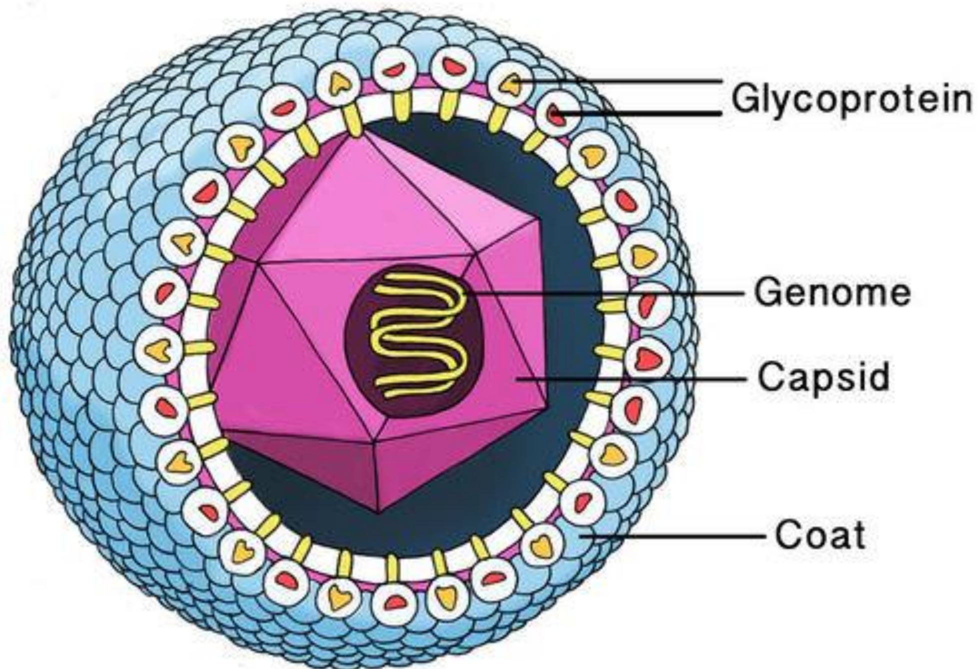
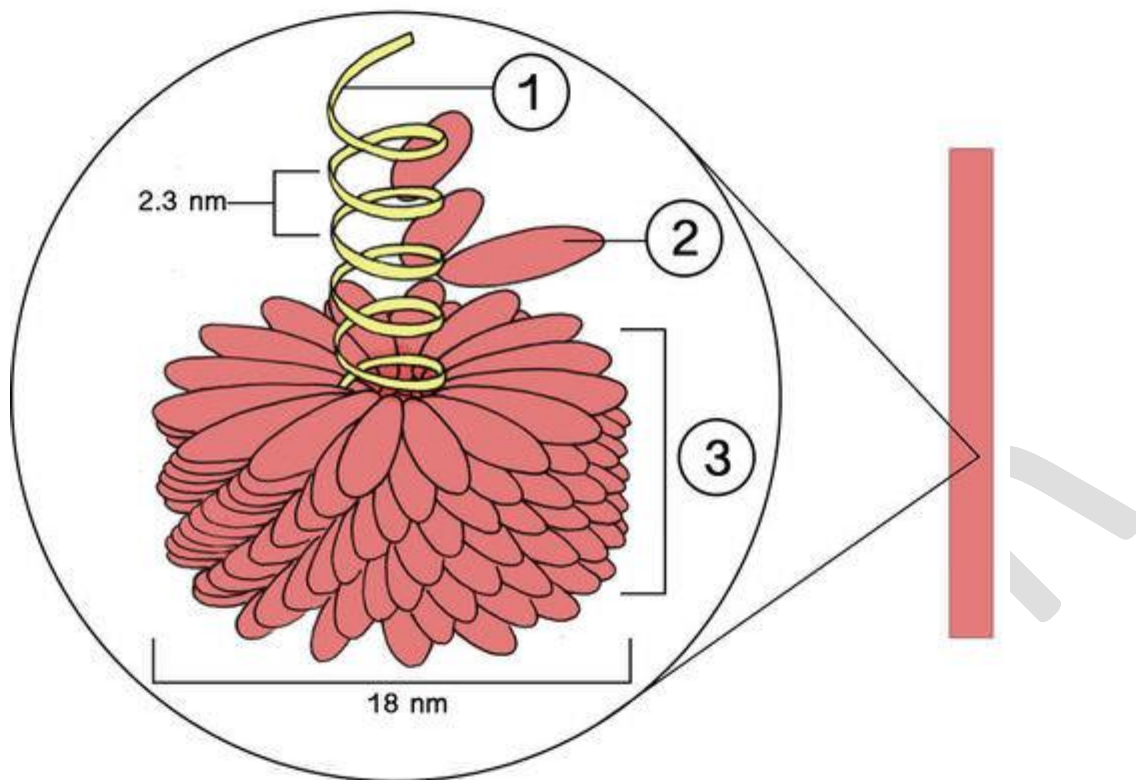


Diagram of a Cytomegalovirus. The capsid encloses the genetic material of the virus. The envelope which surrounds the capsid is typically made from portions of the host cell membranes (phospholipids and proteins). Not all viruses have a viral envelope.

Helical Viruses

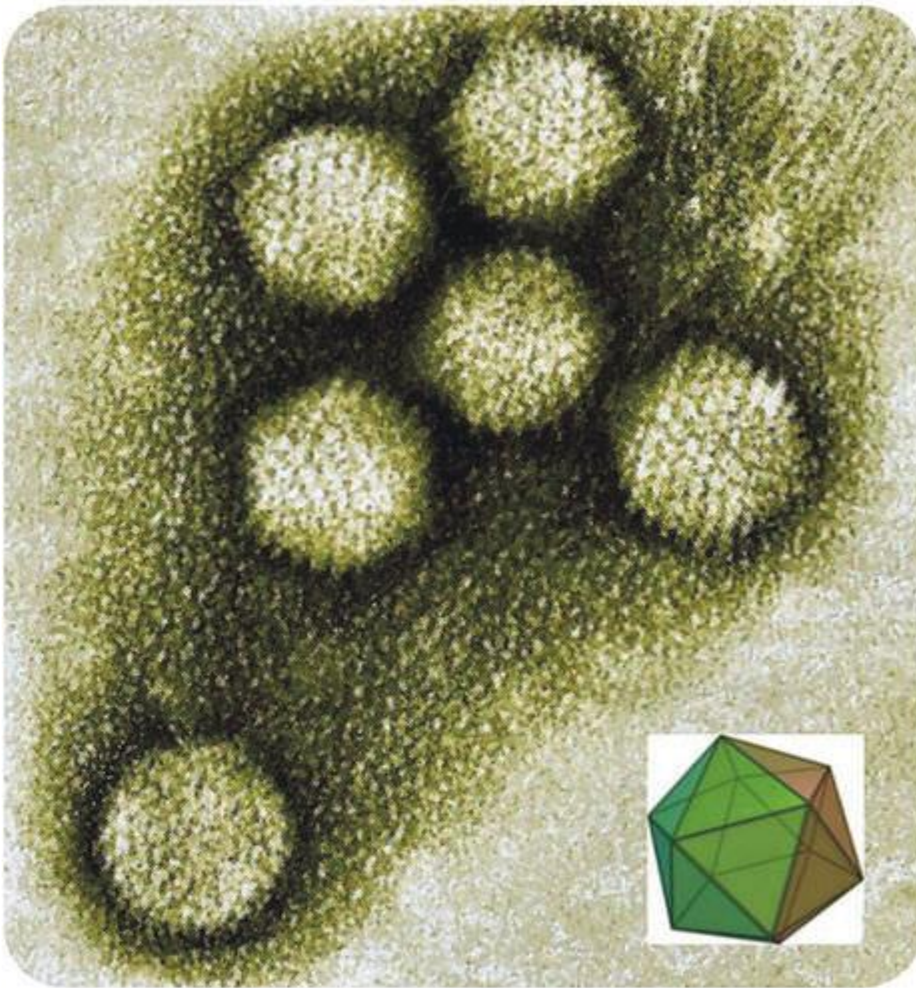
Helical capsids are made up of a single type of protein subunit stacked around a central axis to form a helical structure. The helix may have a hollow center, which makes it look like a hollow tube. This arrangement results in rod-shaped or filamentous virions. These virions can be anything from short and very rigid, to long and very flexible. The well-studied tobacco mosaic virus (TMV) is an example of a helical virus, as seen in the **Figure** below.



A helical virus, tobacco mosaic virus. Although their diameter may be very small, some helical viruses can be quite long, as shown here. 1. Nucleic acid; 2. Viral protein units, 3. Capsid. TMV causes tobacco mosaic disease in tobacco, cucumber, pepper, and tomato plants.

Icosahedral Viruses

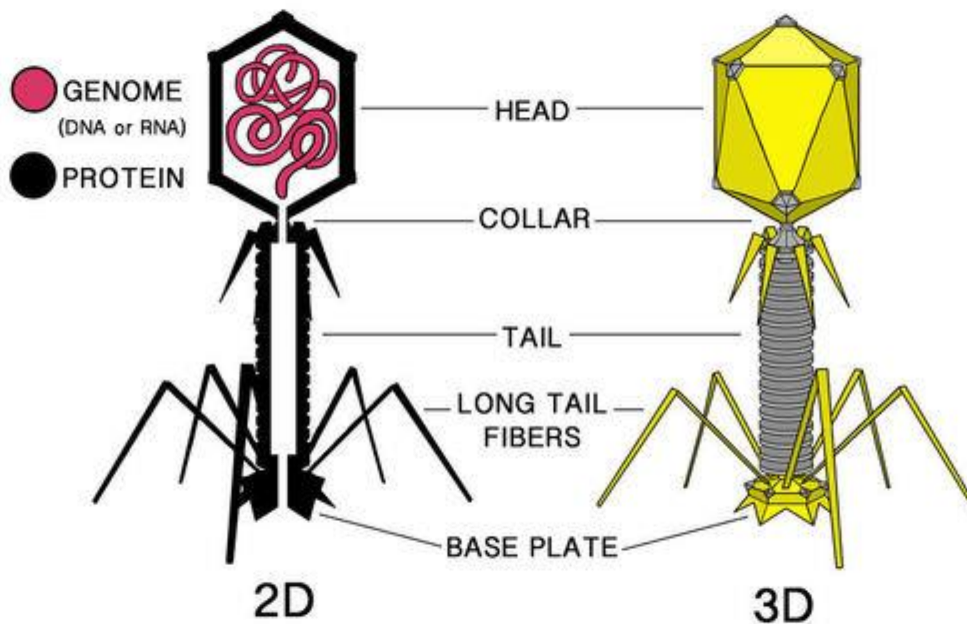
Icosahedral capsid symmetry gives viruses a spherical appearance at low magnification, but the protein subunits are actually arranged in a regular geometrical pattern, similar to a soccer ball; they are not truly spherical. An icosahedral shape is the most efficient way of creating a hardy structure from multiple copies of a single protein. This shape is used because it can be built from a single basic unit protein which is used over and over again. This saves space in the viral genome.



Adenovirus, an icosahedral virus. An icosahedron is a three-dimensional shape made up of 20 equilateral triangles. Viral structures are built of repeated identical protein subunits, making the icosahedron the easiest shape to assemble using these subunits.

Complex Viruses

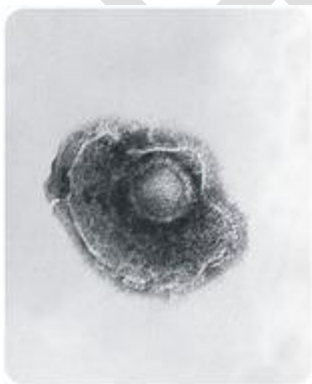
Complex viruses possess a capsid which is neither purely helical, nor purely icosahedral, and which may have extra structures such as protein tails or a complex outer wall. Viral protein subunits will self-assemble into a capsid, but the complex viruses DNA also codes for proteins which help in building the viral capsid. Many phage viruses are complex-shaped; they have an icosahedral head bound to a helical tail. The tail may have a base plate with protein tail fibers. Some complex viruses do not have tail fibers.



This "moon lander"-shaped complex virus infects *Escherichia coli* bacteria.

Enveloped Viruses

Some viruses are able to surround (envelop) themselves in a portion of the cell membrane of their host. The virus can use either the outer membrane of the host cell, or an internal membrane such as the nuclear membrane or endoplasmic reticulum. In this way the virus gains an outer lipid bilayer known as a **viral envelope**. This membrane is studded with proteins coded for by both the viral genome and the host genome. However, the lipid membrane itself and any carbohydrates present come entirely from the host cell. The influenza virus, HIV, and the varicella zoster virus (**Figure** below) are enveloped viruses.



An enveloped virus. Varicella zoster virus causes chicken pox and shingles.

The viral envelope can give a virus some advantages over other capsid-only viruses. For example, they have better protection from the host's immune system, enzymes and certain chemicals. The proteins in the envelope can include glycoproteins, which act as receptor molecules. These receptor molecules allow host cells to recognize and bind the virions, which may result in easier uptake of the virion into the cell. Most enveloped viruses depend on their envelopes to infect cells. However, because the envelope contains lipids, it makes the virus more susceptible to inactivation by environmental agents, such as detergents that disrupt lipids.

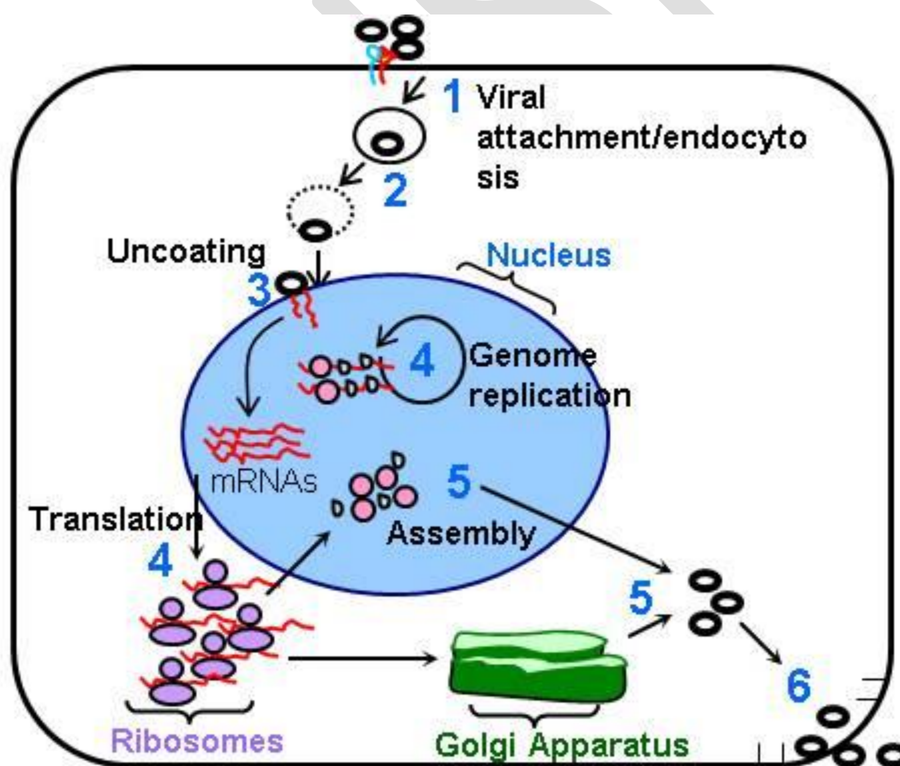
Virus replication

As viruses are obligate intracellular pathogens they cannot replicate without the machinery and metabolism of a host cell. Although the replicative life cycle of viruses differs greatly between species and category of virus, there are six basic stages that are essential for viral replication.

1. Attachment: Viral proteins on the capsid or phospholipid envelope interact with specific receptors on the host cellular surface. This specificity determines the host range (**tropism**) of a virus.

2. Penetration: The process of attachment to a specific receptor can induce conformational changes in viral capsid proteins, or the lipid envelope, that results in the fusion of viral and cellular membranes. Some DNA viruses can also enter the host cell through receptor-mediated endocytosis.

3. Uncoating: The viral capsid is removed and degraded by viral enzymes or host enzymes releasing the viral genomic nucleic acid.



4. Replication: After the viral genome has been uncoated, transcription or translation of the viral genome is initiated. It is this stage of viral replication that differs greatly between DNA and RNA viruses and viruses with opposite nucleic acid polarity. This process culminates in the *de novo* synthesis of viral proteins and genome.

5. Assembly: After *de novo* synthesis of viral genome and proteins, which can be post-transrciptionally modified, viral proteins are packaged with newly replicated viral genome into new virions that are ready for release from the host cell. This process can also be referred to as maturation.

6. Virion release: There are two methods of viral release: **lysis** or **budding**. Lysis results in the death of an infected host cell, these types of viruses are referred to as **cytolytic**. An example is *variola major* also known as smallpox. Enveloped viruses, such as influenza A virus, are typically released from the host cell by budding. It is this process that results in the acquisition of the viral phospholipid envelope. These types of virus do not usually kill the infected cell and are termed **cytopathic viruses**.

After virion release some viral proteins remain within the host's cell membrane, which acts as potential targets for circulating antibodies. Residual viral proteins that remain within the cytoplasm of the host cell can be processed and presented at the cell surface on MHC class-I molecules, where they are recognised by T cells.

Techniques of Virus Cultivation

Viruses are obligate intracellular parasites so they depend on host for their survival. They cannot be grown in non-living culture media or on agar plates alone, they must require living cells to support their replication.

The primary purpose of virus cultivation is:

To isolate and identify viruses in clinical samples.

To do research on viral structure, replication, genetics and effects on host cell.

To prepare viruses for vaccine production.

Cultivation of viruses can be discussed under following headings:

Animal Inoculation

Inoculation into embryonated egg

Cell Culture

1. Animal Inoculation

Viruses which are not cultivated in embryonated egg and tissue culture are cultivated in laboratory animals such as mice, guinea pig, hamster, rabbits and primates are used.

The selected animals should be healthy and free from any communicable diseases.

Suckling mice(less than 48 hours old) are most commonly used.

Suckling mice are susceptible to togavirus and coxsackie virues, which are inoculated by intracerebral and intranasal route.

Viruses can also be inoculated by intraperitoneal and subcutaneous route.

After inoculation, virus multiply in host and develops disease. The animals are observed for symptoms of disease and death.

Then the virus is isolated and purified from the tissue of these animals.

Live inoculation was first used on human volunteers for the study of yellow fever virus.

Advantages of Animal Inoculation

Diagnosis, Pathogenesis and clinical symptoms are determined.

Production of antibodies can be identified.

Primary isolation of certain viruses.

Mice provide a reliable model for studying viral replication.

Used for the study of immune responses, epidemiology and oncogenesis.

Disadvantages of Animal Inoculation

Expensive and difficulties in maintenance of animals.

Difficulty in choosing of animals for particular virus

Some human viruses cannot be grown in animals,or can be grown but do not cause disease.

Mice do not provide models for vaccine development.

It will lead to generation of escape mutants

Issues related to animal welfare systems.

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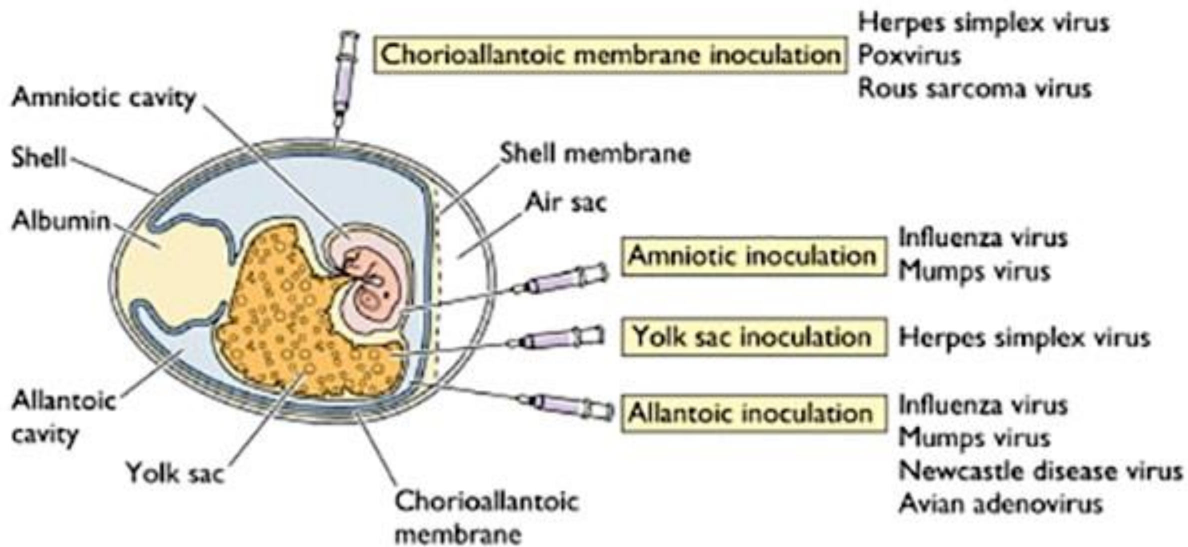
2. Inoculation into embryonated egg



Good pasture in 1931 first used the embryonated hen's egg for the cultivation of virus.

The process of cultivation of viruses in embryonated eggs depends on the type of egg which is used.

Viruses are inoculated into chick embryo of 7-12 days old.

For inoculation, eggs are first prepared for cultivation, the shell surface is first disinfected with iodine and penetrated with a small sterile drill.

After inoculation, the opening is sealed with gelatin or paraffin and incubated at 36°c for 2-3 days.

After incubation, the egg is broken and virus is isolated from tissue of egg.

Viral growth and multiplication in the egg embryo is indicated by the death of the embryo, by embryo cell damage, or by the formation of typical pocks or lesions on the egg membranes

Viruses can be cultivated in various parts of egg like chorioallantoic membrane, allantoic cavity, amniotic sac and yolk sac.

Chorioallantoic Membrane (CAM):

Inoculation is mainly for growing poxvirus.

After incubation and incubation, visible lesions called pocks are observed, which is grey white area in transparent CAM.

Herpes simplex virus is also grown.

Single virus gives single pocks

This method is suitable for plaque studies.

Allantoic cavity:

Inoculation is mainly done for production of vaccine of influenza virus, yellow fever, rabies.

Most of avian viruses can be isolated using this method.

Amniotic sac:

Inoculation is mainly done for primary isolation of influenza virus and the mumps virus.

Growth and replication of virus in egg embryo can be detected by haemagglutination assay.

Yolk sac inoculation:

It is also a simplest method for growth and multiplication of virus.

It is inoculated for cultivation of some viruses and some bacteria (Chlamydia, Rickettsiae)

Immune interference mechanism can be detected in most of avian viruses.

Advantages of Inoculation into embryonated egg

Widely used method for the isolation of virus and growth.

Ideal substrate for the viral growth and replication.

Isolation and cultivation of many avian and few mammalian viruses.

Cost effective and maintenance is much easier.

Less labor is needed.

The embryonated eggs are readily available.

Sterile and wide range of tissues and fluids

They are free from contaminating bacteria and many latent viruses.

Specific and non specific factors of defense are not involved in embryonated eggs.

Widely used method to grow virus for some vaccine production.

Disadvantages of Inoculation into embryonated egg

The site of inoculation for varies with different virus. That is, each virus have different sites for their growth and replication.

3. Cell Culture (Tissue Culture)

There are three types of tissue culture; organ culture, explant culture and cell culture.

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Organ cultures are mainly done for highly specialized parasites of certain organs e.g. tracheal ring culture is done for isolation of coronavirus.

Explant culture is rarely done.

Cell culture is mostly used for identification and cultivation of viruses.

Cell culture is the process by which cells are grown under controlled conditions.

Cells are grown in vitro on glass or a treated plastic surface in a suitable growth medium.

At first growth medium, usually balanced salt solution containing 13 amino acids, sugar, proteins, salts, calf serum, buffer, antibiotics and phenol red are taken and the host tissue or cell is inoculated.

On incubation the cell divide and spread out on the glass surface to form a confluent monolayer.

Types of cell culture

1. Primary cell culture:

These are normal cells derived from animal or human cells.

They are able to grow only for limited time and cannot be maintained in serial culture.

They are used for the primary isolation of viruses and production of vaccine.

Examples: Monkey kidney cell culture, Human amnion cell culture

2. Diploid cell culture (Semi-continuous cell lines):

They are diploid and contain the same number of chromosomes as the parent cells.

They can be sub-cultured up to 50 times by serial transfer following senescence and the cell strain is lost.

They are used for the isolation of some fastidious viruses and production of viral vaccines.

Examples: Human embryonic lung strain, Rhesus embryo cell strain

3. Heteroploid cultures (Continuous cell lines):

They are derived from cancer cells.

They can be serially cultured indefinitely so named as continuous cell lines

They can be maintained either by serial subculture or by storing in deep freeze at -70°c.

Due to derivation from cancer cells they are not useful for vaccine production.

Examples: HeLa (Human Carcinoma of cervix cell line), HEP-2 (Humman Epithelioma of larynx cell line), Vero (Vervet monkey) kidney cell lines, BHK-21 (Baby Hamster Kidney cell line).

Advantages of cell culture

Relative ease, broad spectrum, cheaper and sensitivity

Disadvantage of cell culture

The process requires trained technicians with experience in working on a full time basis.

State health laboratories and hospital laboratories do not isolate and identify viruses in clinical work.

Tissue or serum for analysis is sent to central laboratories to identify virus.

Cultivation of plant viruses and bacteriophages

Cultivation of plant viruses

There are some methods of Cultivation of plant viruses such as plant tissue cultures, cultures of separated cells, or cultures of protoplasts, etc. viruses can be grown in whole plants.

Leaves are mechanically inoculated by rubbing with a mixture of viruses and an abrasive. When the cell wall is broken by the abrasive, the viruses directly contact the plasma membrane and infect the exposed host cells. A localized necrotic lesion often develops due to the rapid death of cells in the infected area. Some plant viruses can be transmitted only if a diseased part is grafted onto a healthy plant.

Cultivation of bacteriophages

Bacteriophages are cultivated in either broth or agar cultures of young, actively growing bacterial cells.

Disinfection

Antisepsis: A process involving the destruction or inhibition of mico-organisms in living tissue thereby limiting or preventing the harmful effects of **infection.**

Antiseptic: Typically an antiseptic is a chemical agent that is applied to living tissue to kill microbes. Note that not all disinfectants are antiseptics because an antiseptic additionally must not be so harsh that it damages living tissue. Antiseptics are less toxic than disinfectants used on inanimate objects. Due to the lower toxicity, antiseptics can be less active in the destruction of normal and any pathogenic flora present.

Autoclave: An autoclave is a high pressure device used to allow the application of moist heat above the normal-atmosphere boiling point of water.

Biocidal: Active substances and preparations which serve to repel, render harmless or destroy chemically or biologically harmful organisms.

Biocide: Substance or chemical that kills biological organisms.

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Decontamination: The killing of organisms or removal of contamination after use, with no quantitative implication, generally referring to procedures for making items safe before disposal.

Disinfectant: A germicide that inactivates virtually all recognized pathogenic microorganisms but not necessarily all microbial forms. They may not be effective against bacterial spores.

Disinfection: A procedure of treatment that eliminates many or all pathogenic microorganisms with the exception of bacterial spores.

Germicide: An agent that destroys microorganisms, particularly pathogenic microorganisms.

Pathogenic: A microbe or other organism that causes disease.

Sanitization: The process of reducing microbial contamination to an acceptable "safe" level. The process of cleaning objects without necessarily going through sterilization.

Steam Sterilization: Autoclave, the process of sterilization by the use of heated steam under pressure to kill vegetative microorganisms and directly exposed spores. Common temperature and pressure for being effective is 121°C (250°F) at 15 psi (pounds per square inch) over pressure for 15 minutes. Special cases may require a variation of the steam temperature and pressure used.

Sterilization: The complete elimination or destruction of all forms of life by a chemical or physical means. This is an absolute not a relative term.

Many disinfectants are used alone or in combinations (e.g., hydrogen peroxide and peracetic acid) in the health-care setting. These include alcohols, chlorine and chlorine compounds, formaldehyde, glutaraldehyde, *ortho*-phthalaldehyde, hydrogen peroxide, iodophors, peracetic acid, phenolics, and quaternary ammonium compounds. Commercial formulations based on these chemicals are considered unique products and must be registered with EPA or cleared by FDA. In most instances, a given product is designed for a specific purpose and is to be used in a certain manner. Therefore, users should read labels carefully to ensure the correct product is selected for the intended use and applied efficiently.

Disinfectants are not interchangeable, and incorrect concentrations and inappropriate disinfectants can result in excessive costs. Because occupational diseases among cleaning personnel have been associated with use of several disinfectants (e.g., formaldehyde, glutaraldehyde, and chlorine), precautions (e.g., gloves and proper ventilation) should be used to minimize exposure. Asthma and reactive airway disease can occur in sensitized persons exposed to any airborne chemical, including germicides. Clinically important asthma can occur at levels below ceiling levels regulated by OSHA or recommended by NIOSH. The preferred method of control is elimination of the chemical (through engineering controls or substitution) or relocation of the worker.

The following overview of the performance characteristics of each provides users with sufficient information to select an appropriate disinfectant for any item and use it in the most efficient way.

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Microbial Resistance to Chemical Disinfectants:		
More Resistant	Type of Microbe	Examples
.	Bacterial Spores	*Bacillus subtilis*
.	Mycobacteria	*Mycobacterium tuberculosis* *Mycobacterium bovis*
.	Hydrophilic Viruses (non-liquid, non-enveloped)	*Coxsackievirus* *Rhinovirus*
.	Fungi	*Cryptococcus sp.* *Candida sp.*
.	Vegitative Bacteria	*Streptococcus pneumoniae* *Staphylococcus aureus*
Less Resistant	Lipophilic Viruses (lipid containing, enveloped)	Herpes Simplex Cytomegalovirus

Disinfectant Groups

a. Aldehydes: (Formaldehyde, Paraformaldehyde, Glutaraldehyde)

Formaldehyde – and its polymerized solid paraformaldehyde have broad-spectrum biocidal activity and are both effective for surface and space decontamination. As a liquid (5% concentration), formaldehyde is an effective liquid decontaminant. Its biocidal action is through alkylation of carboxyl, hydroxyl and sulfhydryl groups on proteins and the ring nitrogen atoms of purine bases. Formaldehyde's drawbacks are reduction in efficacy at refrigeration temperature, its pungent, irritating odor, and several safety concerns. Formaldehyde is presently considered to be a carcinogen or a cancer-suspect agent according to several regulatory agencies. The OSHA 8-hour time-weighted exposure limit is 0.75 ppm.

Paraformaldehyde – is a solid polymer of formaldehyde. Paraformaldehyde generates formaldehyde gas when it is depolymerized by heating to 232 to 246°C (450 to 475°F); the depolymerized material reacts with the moisture in the air to form formaldehyde gas. This process is used for the decontamination of large spaced and laminar-flow biological safety cabinets when maintenance work or filter changes require access to the sealed portion of the cabinet. A neutralization step, heating ammonium carbonate, is required prior to ventilation of the space. Formaldehyde gas can react violently

or explosively (7.0 – 73% v/v in air), when exposed to incompatibles, therefore, only individuals that have specific training and have been approved by the Dept. of Environmental Health & Safety are permitted to use this gas.

Glutaraldehyde – is a colorless liquid and has the sharp, pungent odor typical of all aldehydes, with an odor threshold of 0.04 parts per million (ppm). It is capable of sterilizing equipment, though to effect sterilization often requires many hours of exposure. Two percent solutions of glutaraldehyde exhibit very good activity against vegetative bacteria, spores and viruses. It is ten times more effective than formaldehyde and less toxic. However, it must be limited and controlled because of its toxic properties and hazards. It is important to avoid skin contact with glutaraldehyde as it has been documented to cause skin sensitization. Glutaraldehyde is also an inhalation hazard. The NIOSH ceiling threshold limit value is 0.2 ppm.

Cidex, a commercially prepared glutaraldehyde disinfectant is used routinely for cold surface sterilization of clinical instruments. Glutaraldehyde disinfectants should always be used in accordance with the manufacturer's directions.

b. Halogen-Based Biocides: (Chlorine Compounds and Iodophores)

1. Chlorine Compounds

Chlorine compounds are good disinfectants on clean surfaces, but are quickly inactivated by organic matter and thus reducing the biocidal activity. They have a broad spectrum of antimicrobial activity and are inexpensive and fast acting. Hypochlorites, the most widely used of the chlorine disinfectants, are available in liquid (e.g., Sodium hypochlorite), household bleach and solid (e.g., calcium hypochlorite, sodium dichloroisocyanurate) forms. Household bleach has an available chlorine content of 5.25%, or 52,500 ppm. Because of its oxidizing power, it loses potency quickly and **should be made fresh** and used within the same day it is prepared. The free available chlorine levels of hypochlorite solutions in both opened and closed polyethylene containers are reduced to 40% to 50% of the original concentration over a period of one month at room temperature.

There are two potential occupational exposure hazards when using hypochlorite solutions. The first is the production of the carcinogen bis-chloromethyl ether when hypochlorite solutions come in contact with formaldehyde. The second is the rapid production of chlorine gas when hypochlorite solutions are mixed with an acid. Care must also be exercised in using chlorine – based disinfectants which can corrode or damage metal, rubber, and other susceptible surfaces. Bleached articles should never be autoclaved without reducing the bleach with sodium thiosulfate or sodium bisulfate.

Chloramine T which is prepared from sodium hypochlorite and p-toluenesulfonamide is a more stable, odorless, less corrosive form of chlorine but has decreased biocidal activity in comparison to bleach.

2. Iodophors

Iodophors are used both as antiseptics and disinfectants. An iodophor is a combination of iodine and a solubilizing agent or carrier; the resulting complex provides a sustained-release reservoir of iodine and

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releases small amounts of free iodine in aqueous solution. Antiseptic iodophors are not suitable for use as hard-surface disinfectants because they contain significantly less free iodine than do those formulated as disinfectants.

Wescodyne, *Betadyne*, Povidone-Iodine and other iodophors are commercially available Iodine-based disinfectants, which give good control when the manufacturer's instructions for formulation and application are followed. **Both bleach and iodophors should be made up in cold water in order to prevent breakdown of the disinfectant.**

c. Quaternary Ammonium Compounds:

Quaternary ammonium compounds are generally odorless, colorless, nonirritating, and deodorizing. They also have some detergent action, and they are good disinfectants. However, some quaternary ammonium compounds activity is reduced in the presence of some soaps or soap residues, detergents, acids and heavy organic matter loads. They are generally ineffective against viruses, spores and *Mycobacterium tuberculosis*. Basically these compounds are not suitable for any type of terminal disinfection.

The mode of action of these compounds is through inactivation of energy producing enzymes, denaturation of essential cell proteins, and disruption of the cell membrane. Many of these compounds are better used in water baths, incubators, and other applications where halide or phenolic residues are not desired.

d. Phenolics: (O-phenophenoate-base Compounds)

Phenolics are phenol (carbolic acid) derivatives. These biocides act through membrane damage and are effective against enveloped viruses, rickettsiae, fungi and vegetative bacteria. They also retain more activity in the presence of organic material than other disinfectants. Cresols, hexachlorophene, alkyl- and chloro derivatives and diphenyls are more active than phenol itself. Available commercial products are Lysol, Pine-Sol, *Amphyl, O-syl, Tergisyl, Vesphene, L- Phase* and *Expose*.

e. Acids/Alkalis:

Strong mineral acids and alkalis have disinfectant properties proportional to the extent of their dissociation in solution. Some hydroxides are more effective than would be predicted from their values. In general acids are better disinfectants than alkalis. Mode of action is attributed to an increase of H^+ and OH^- species in solutions which interfere with certain microbial functions, however the total effect is not only dependent on pH alone. Weak organic acids are more potent than inorganic acids despite low dissociation rates in solution. Action is attributed to the disruption of 2° and 3° conformation of enzymes and structural proteins.

f. Heavy Metals:

Soluble salts of mercury, silver lactate, mercuric chloride and mercurous chloride are efficient bactericidal agents. Silver nitrate and mercuric chloride are commonly used as 1:1000 aqueous solutions. Action is through attack on protein sulfhydryl groups and disruption of enzyme functions. Organic matter can reverse the disinfectant properties of mercurials.

g. Alcohols:

Alcohols work through the disruption of cellular membranes, solubilization of lipids, and denaturation of proteins by acting directly on S-H functional groups. Ethyl and isopropyl alcohols are the two most widely used alcohols for their biocidal activity. These alcohols are effective against lipid-containing viruses and a broad spectrum of bacterial species, but ineffective against spore-forming bacteria. They evaporate rapidly, which makes extended contact times difficult to achieve unless the items are immersed.

The optimum bactericidal concentration for ethanol and isopropanol is in the range of 60% to 90% by volume. Their cidal activity drops sharply when diluted below 50% concentration. Absolute alcohol is also not very effective. They are used to clean instruments and wipe down interior of Biological Safety Cabinets and bottles, etc. to be put into Biological Safety Cabinets. Alcohols are generally regarded as being non-corrosive.

Factors affecting disinfection action

Factors affecting disinfection are

1. Concentration of disinfectant
2. Temperature
3. Time of contact
4. pH of environment
5. Surface tension
6. Formulation of disinfectant
7. Chemical structure of disinfectant
8. Types and number of micro-organisms present
9. Interfering substances in the environment
10. Potentiation, synergism, and antagonism of disinfactants

1. Concentration of disinfectants

The lethal effect of bacterial population is increased by increasing the concentration of disinfectant. However, the effectiveness is generally related to the concentration exponentially, not linearly. There is optimum concentration of phenol at about 1%. Beyond this concentration, the disinfecting effectiveness becomes less.

The dilution coefficient can be calculated from the following equation:

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n = logt2 -logt1 /logC2 -logC1

Where n=concentration exponent or dilution coefficient for disinfectant, t1= the death time with disinfectant concentration C1 t2= the death time with disinfectant concentration C2

2. 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 a temperature coefficient. The temperature coefficient per degree rise in temperature is denoted by θ where as per 100C rise in temperature is expressed by θ 10or Q10 values.

Thus θ 10 or Q10= $timerequiredtokillatT\ 0C/timerequiredtokillat\ (T{+}100C)$.

The value for Q10for phenol is 4,which means that over the100C range used to determine the Q10the activity will be increased by factor 4.

3. Time of contact

efficient time of contact must be allowed for the disinfectant to exert its action.

4. pH of the environment

A change of pH during the disinfection process can affect the rate of growth inoculum. A pH of 6-8 is optimal for the growth of many bacteria and the rate of growth declines on either side of the range. Phenolic and acidic antimicrobial agents usually have greatest activity in acidic conditions. Acridine dyes and quaternary ammonium compounds are usually more active in alkaline then in acidic solutions. Amphoteric antimicrobials (Tego compounds) have optimum activities at widely differing pH values.

5. Chemical structure of disinfectant

Chemical structures of compounds affects the disinfectant activity. Substitution of an alkyl chain upto 6 carbons in length in para position to phenolic –OH group increases activity but greater than 6 carbons in length decreases water solubility and disinfectant activity. Generally, halogenation increases the antibacterial activity of phenol but nitration increases antibacterial activity and systematic toxicity also.

6. Types and number of micro-organisms present

The efficiency of disinfection greatly depends on the nature and the number of contaminating microorganisms and especially on the presence and absence of bacterial spores. It can be seen that most vegetative bacteria are rapidly killed by most chemical disinfectants. Bacterial spores are difficult to destroy but some disinfectants e.g aldehyde are sporicidal.

7. Interfering substances in the environment

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Material such as blood, body fluids, pus, milk, food residues or colloidal proteins may reduce the effectiveness of disinfectant if present in small amounts. the presence of oil and fat markedly reduces the disinfecting ability of phenolics.

8. Potentiation, synergism and antagonism of disinfactants

Potentiation of a disinfectant leads to enhanced antimicrobial activity.

Synergistics effects are often shown by two antimicrobial agents which is giving an increased activity. Antagonism effects are often shown by two antimicrobial agents which is giving an decreased activity.

Evaluation of Anti-microbial agents and Disinfectants Techniques and methods used for evaluation of Disinfectants

1. Tube dilution and agar plate method.

2. Cup plate method or Cylinder plate method.

3. Ditch- Plate method.

4. Gradient plate technique.

5. Phenol coefficient method (Rideal-Walker test)

1. Tube Dilution And Agar Plate Method

The chemical agent is incorporated into nutrient broth or agar medium and inoculated with test micro-organisms. These tubes are incubated at 300C to 350C for 2 to 3 days and then the results in the form of turbidity or colonies are observed. The results are recorded and the activity of the given disinfectant is compared. 1. Tube Dilution And Agar Plate Method

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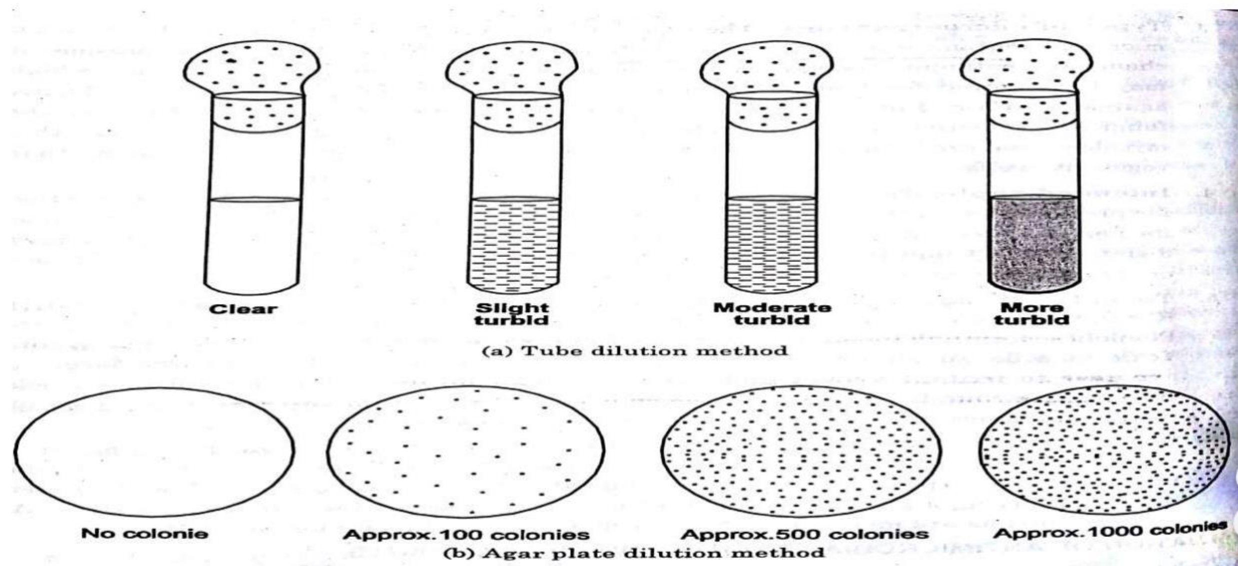


2. Cup Plate or Cylinder plate Method

The nutrient agar is melted, cooled suitably, poured into petri dish. Spread 0.2 ml of known concentration of inoculum on the surface of the solidified agar (Spread Plate Technique). Cups or cavities are made by using a sterile borer. Now 0.2 ml of drug is poured into the cups of agar plate and then incubated at 370C for 24 hr. If the drug has any anti-bacterial effect it will show the zone of inhibition

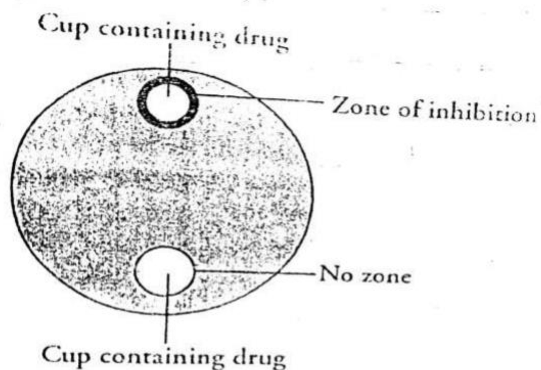
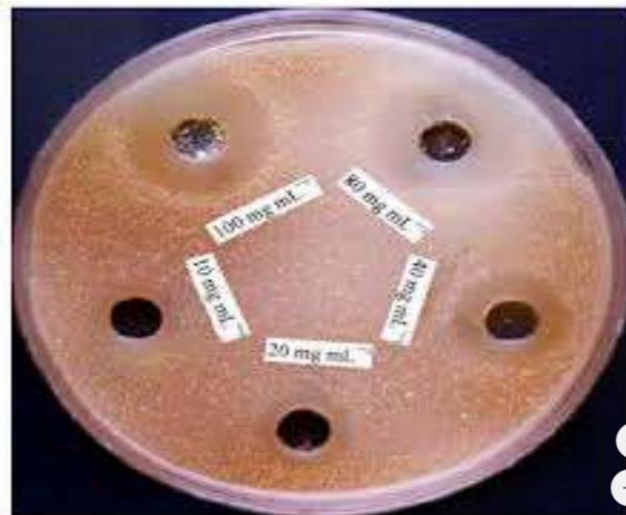
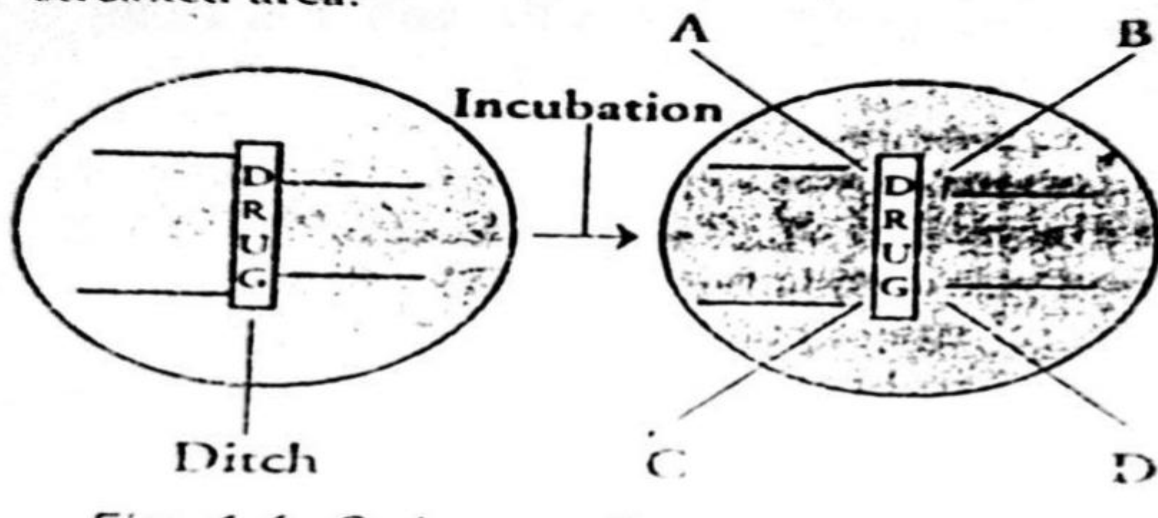


Fig. 4.4 Schematic representation of cup plate technique



3. Ditch-Plate Method

The nutrient agar is melted, cooled suitably, poured into petri dish. 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 the potency of drug against various microorganisms by the means of inhibition of growth on streaked area.



4. Gradient Plate Technique •

This technique is used to isolate the resistant mutants. The petri dish is kept in slanting position and; a sufficient amount of melted nutrient agar is poured and solidified in slanting position. Another layer of agar is poured over it, which contains antibiotic solution and solidified it. After solidification, 0.2ml of bacterial culture was speeded over the solid surface and incubated it at 370C for 24 to 48 hr. The microorganisms will grow, where the concentration of the drug is below the critical level. The antibiotics get diluted on the lower layer and the gradient of concentration will be produced. Thus, the resistant mutant can be isolated.

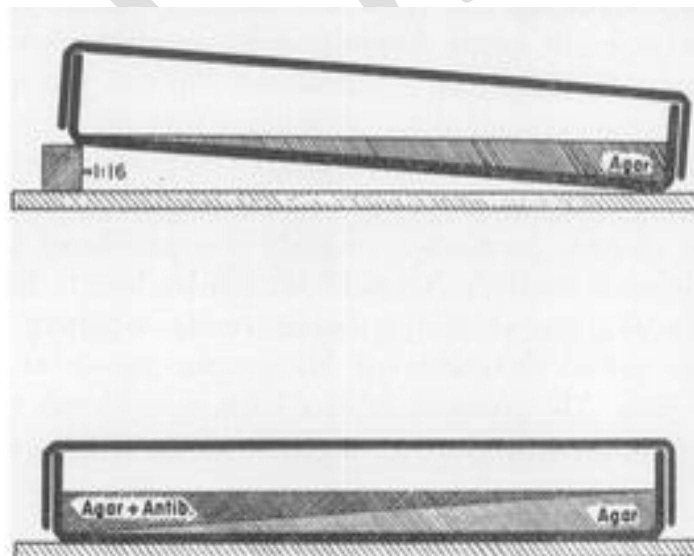


Figure 1. **Preparation of a gradient plate. Details of method described in text.**

5. Phenol Coefficient Method (Rideal-Walker test)

Phenol coefficient test is suitable for testing disinfectants miscible with water and which exert their antimicrobial action in manner similar to that of phenol. Test Organism: Salmonella typhi

Standard disinfectant: Phenol Different dilutions of the test disinfectants and phenol are prepared and 5ml of each dilution is inoculated with 0.5 ml broth culture of the organisms for 24 hr. All tubes (disinfectants + organisms and phenol + organisms)are placed in 17.5 0C water bath. Subcultures of each reaction mixture are taken and transferred to 5ml sterile broth after 2.5, 5, 7.5 and 10 min. The broth tubes are incubated at 370C for 48 to 72 hr and are examined for presence or absence of growth. If a phenol coefficient or Rideal-Walker coefficient of a given test disinfectant is 1, it means that disinfectant has same effectiveness as phenol. If a phenol coefficient or Rideal-Walker coefficient of a given test disinfectant is less than 1, it means that disinfectant is less effective than phenol. If a phenol coefficient or Rideal-Walker coefficient of a given test disinfectant is more than 1, it means that disinfectant is more effective than phenol. If the phenol coefficient of the test disinfectant is 20 it means that the disinfectant is 20 times more active than phenol.

Disinfectant	Dilution	Time interval for sub-culture (min.)			
		2.5	5	7.5	10
Test disinfectant	1 : 1000	+	–	–	–
	1 : 2000	+	+	–	–
	1 : 3000	+	+	+	–
	1 : 4000	+	+	+	+
Phenol	1 : 80	+	–	–	–
	1 : 100	+	+	–	–
	1 : 120	+	+	+	–
	1 : 140	+	+	+	+

(+ = growth; – = no growth)

$$\text{R.W. coefficient} = \frac{\text{Dilution of disinfectant killing in 7.5 but not in 5 min.}}{\text{Dilution of phenol killing in 7.5 but not in 5 min.}} = \frac{2000}{100} = 20$$

Sterility Testing

Sterility testing must be performed and be demonstrated throughout many stages of the production process of a biologic or viral.it offers sterility tests by **Direct Inoculation** and by **Membrane Filtration** to GMP regulations and in compliance with the European Pharmacopoeia (EP), Japanese Pharmacopoeia (JP) and the Unites States Pharmacopoeia (USP) standards.

There are two options for sterility testing that provides: **Membrane Filtration** and **Direct Inoculation**. Prior to testing, the sample matrix should be qualified in the assay to examine any inhibitory effects; this is achieved by spiking the test material with representative test organisms at low concentrations and acceptable growth should be observed. The following organisms are suitable as model organisms: Aerobic bacteria- Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa; Anaerobic bacterium- Clostridium sporogenes and fungi Candida albicans, Aspergillus brasiliensis.

In the sterility test, two different media are used: fluid thioglycollate and soya-bean casein digest medium. The sample is inoculated into the growth media and the test is incubated for 14 days. It is able to report the results of the assay with a Certificate of Analysis within 5 days of the final observations.

It should be noted that these sterility tests are not applicable for test articles that are not intended to be sterile; a Bioburden test should be used in this case.

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TABLE 2: SAMPLING SCHEDULE - MINIMUM NUMBER OF ITEMS TO BE TESTED FROM EACH BATCH[1]

Type of product	Number of items in the batch	Minimum number of items to be tested for each medium[2]
Injectable pharmaceuticals Injectable medical devices Ophthalmic and other non-injectable pharmaceuticals in single-dose containers Ophthalmic medical devices in single-dose containers	Not more than 100	10% of batch or 4 containers, whichever is the greater
	101-500	10 containers
	More than 500	2% of batch or 20 containers, whichever is the lesser
Ophthalmic and other non-injectable pharmaceuticals and medical devices not in single-dose containers	Not more than 200	5% of batch or 2 containers, whichever is the greater
	More than 200	10 containers
Bulk solid products	Not more than 4	Each container
	5-50	20% of batch or 4 containers, whichever is the greater
	More than 50	2% of batch or 10 containers, whichever is the greater
Pharmacy bulk packages of antibiotics	Less than 5g	20 containers
	Greater than or equal to 5g	6 containers
Solid medical devices	Not more than 100	10% of batch or 4 units, whichever is the greater
	101-500	10 units
	More than 500	2% of batch or 20 units, whichever is the lesser

NOTES

1. This Table incorporates Table 2.6.1-3 (BP and Ph Eur) and Table 3 (USP).
2. If the contents of one container are sufficient to inoculate the two media, this column gives the number of containers needed for both the media together.

The USP describes three general methods for sterility testing:

1) Membrane Filtration,

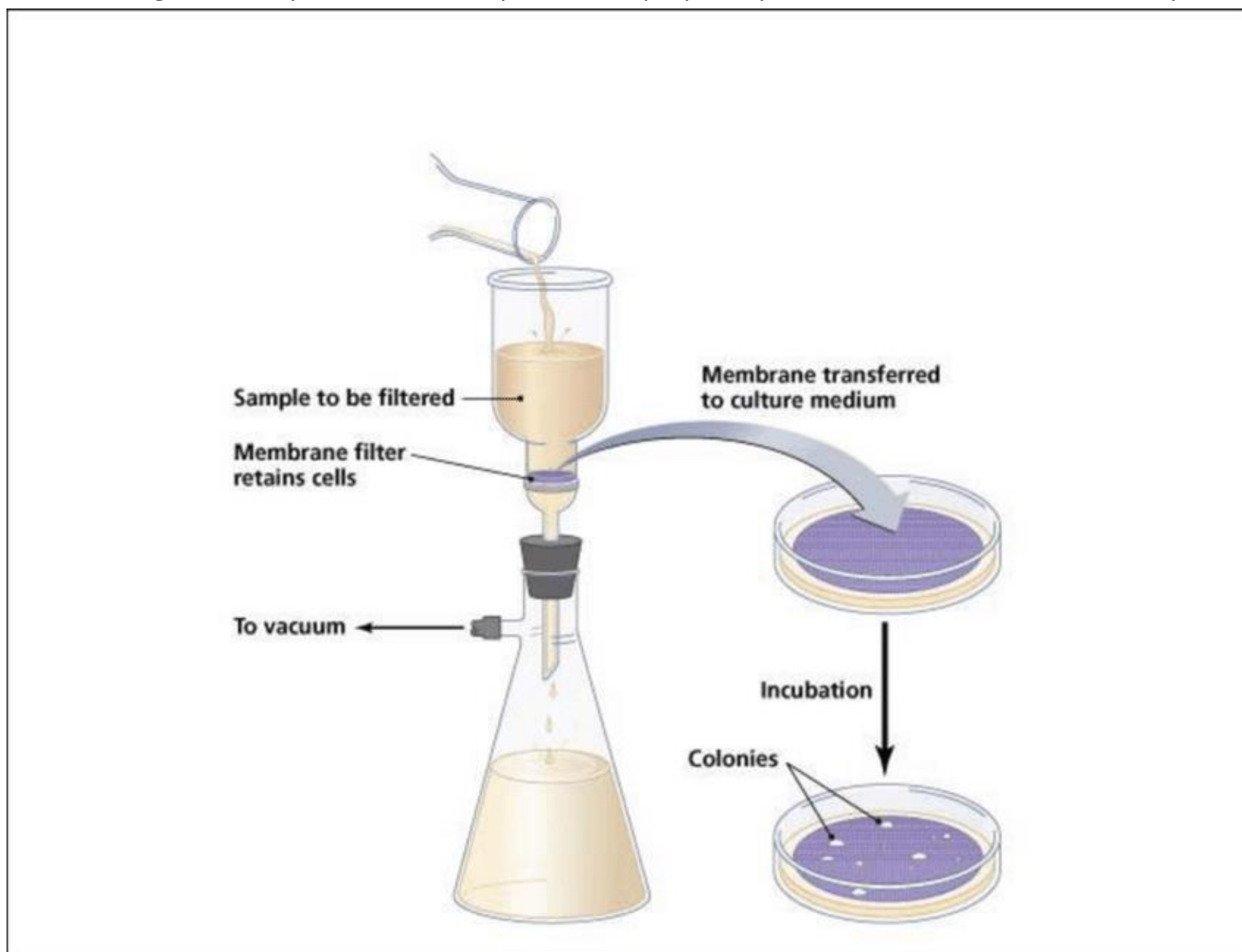
2) Direct Transfer (Product Immersion)

3) Product Flush.

Membrane Filtration Sterility Testing

The Membrane Filtration Sterility Test is the method of choice for pharmaceutical products. It is not the method of choice for medical devices; the FDA may question the rationale behind using the membrane filtration test over the direct transfer test for devices. An appropriate use of this test is for devices that contain a preservative and are bacteriostatic and/or fungistatic under the direct transfer method. With membrane filtration, the concept is that the microorganisms will collect onto the surface of a 0.45 micron pore size filter. This filter is segmented and transferred to appropriate media. The test media are fluid thioglycollate medium (FTM) and soybean casein digest medium (SCDM). FTM is selected based upon its ability to support the growth of anaerobic and aerobic microorganisms. SCDM is selected based upon its ability to support a wide range of aerobic bacteria and fungi (i.e. yeasts and molds). The incubation time is 14 days. Since there are many manipulations required for membrane filtration medical device sterility testing, the propensity for laboratory contamination is high. Therefore, in an open system, more sterility failures are expected when using this method. A closed system is recommended for drugs and small devices or combination products. Most pharmaceutical articles are

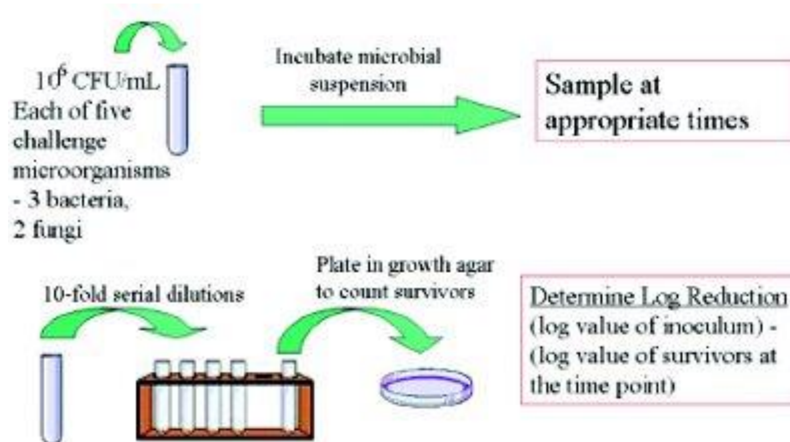
tested using a closed system. In closed systems, the propensity for extrinsic contamination is very low.



Direct Transfer Sterility Testing

Combination products: This method is the method of choice for medical devices because the device is in direct contact with test media throughout the incubation period. Viable microorganisms that may be in or on a product after faulty/inadequate sterilization have an ideal environment within which to grow and proliferate. This is especially true with damaged microorganisms where the damage is due to a sub-lethal sterilization process. All microorganisms have biological repair mechanisms that can take advantage of environmental conditions conducive to growth. The direct transfer method benefits these damaged microorganisms. The entire product should be immersed in test fluid. With large devices, patient contact areas should be immersed. Large catheters can be syringe filled with test media prior to immersion. Cutting catheter samples to allow for complete immersion is the method of choice.

The USP authors understand that appropriate modifications are required due to the size and shape of the test samples. The method requires that the product be transferred to separate containers of both FTM and SCDM. The product is aseptically cut, or transferred whole, into the media containers. The test article should be completely immersed in the test media. The USP limits the media volume to 2500 ml. After transferring, the samples are incubated for 14 days



Product Flush Sterility Testing

Combination products: The product flush sterility test is reserved for products that have hollow tubes such as transfusion and infusion assemblies where immersion is impractical and where the fluid pathway is labeled as sterile. This method is easy to perform and requires a modification of the FTM media for small lumen devices. The products are flushed with fluid D and the eluate is membrane filtered and placed into FTM and SCDM. This method is not generally used.

Distal Rinse Procedure

1 50mL PBS with 0.02% Tween 80 (PBST) (Cat. no. U334)

2 Aseptically transfer contents to sterile 120mL specimen cup (Cat. no. PC4064200S)

3 Dip brush in PBST. Sample elevator mechanism, recess, and channel. Brush under elevator mechanism and scrub the camera lens. Deposit brush in PBST.

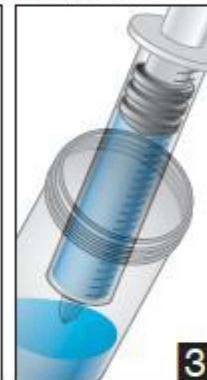
4 Tighten lid and seal with Parafilm. Hold sample at 4 °C until further processing.

Channel Rinse Procedure

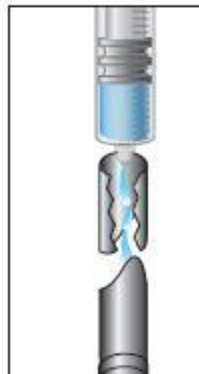
1 50mL sterile water (Cat. no. U336)

2 Aseptically transfer contents to sterile 120mL specimen cup (Cat. no. PC4064200S)

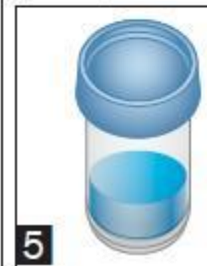
3 Withdraw the 50mL of water into a 60mL syringe.



4 Flush instrument channel and collect contents in sterile 120mL specimen cup.



5 Tighten lid and seal with Parafilm. Hold sample at 4°C until further processing.



Bulk Drug Products / Biologics and Pharmaceuticals

Bulk Pharmaceuticals (APIs) are tested for sterility per USP 71 prior to release to the manufacturing processes.

Bulk Biologics are tested according to 21 CFR 610.12 for sterility testing. This method requires one

media (FTM). The sample test sizes are listed in the document. Volumes are no less than 10 ml.10

Interpretation of Sterility Test Results

The technician must be trained in the method of detecting growth during the incubation period. Growth is determined by viewing the media, which is generally clear and transparent, against a light source. Turbid (cloudy) areas in the media are indicative of microbial growth. Once growth is detected, the suspect vessel is tested to confirm that the turbidity present is due to microorganisms and not due to disintegration of the sample; sometimes samples produce turbidity because of particulate shedding or chemical reactions with the media. Once a suspect container has been tested, it should be returned to the incubator for the remainder of the incubation period. Samples that render the media turbid are transferred on Day 14 of the test and incubated for four days. Growth positive samples require further processing such as identification and storage.

Sterility Test Failure Investigation

For every positive sterility test (OOS), the laboratory should perform an OOS investigation to determine the validity of the positive growth. This investigation encompasses the following items:

clean room environmental test (EER) data;

media sterilization records;

technician training records;

the relative difficulty of the test procedure;

control data (open and closed media controls);

technician sampling data (microbial counts on gloves and/or garments post testing).

HNSIPER