17MBU211 **VIROLOGY – PRACTICAL** **(3H – 2C)**

Instruction Hours / week: L: 0 T: 0 P: 3 **Marks:** Internal: **40** External: **60** Total: **100**
End Semester Exam: 6 Hours

SCOPE

Virology is a potential arena in the medical research companies, Pharmaceutical companies, governmental agencies, laboratory testing companies, or cancer treatment or research companies depending upon the specialization.

OBJECTIVES

- To study general aspects of viral morphology and classification.
- Cultivation of viruses and various methods of propogation.
- To discuss the application of various immunological and molecular diagnostic tools.

EXPERIMENTS

1. Study of the structure of important animal viruses (rhabdo, influenza, paramyxo hepatitis B and retroviruses) using electron micrographs – Demonstration.
2. Study of the structure of important plant viruses (caulimo, Gemini, tobacco ring spot, cucumber mosaic and alpha-alpha mosaic viruses) using electron micrographs – Demonstration.
3. Study of the structure of important bacterial viruses (φX174, T4, λ) using electron micrograph – Demonstration.
4. Isolation and enumeration of bacteriophages (PFU) from water/sewage sample using double agar layer technique
5. Studying isolation and propagation of animal viruses by chick embryo technique
6. Study of cytopathic effects of viruses using photographs
7. Perform local lesion technique for assaying plant viruses.

SUGGESTED READING

1. Dimmock, N.J., Easton, A.L., Leppard, K.N. (2007). Introduction to Modern Virology. 6th edition, Blackwell Publishing Ltd.
2. Carter, J., and Saunders, V. (2007). Virology: Principles and Applications. John Wiley and Sons.
3. Flint, S.J., Enquist, L.W., Krug, R.M., Racaniello, V.R., Skalka, A.M. (2004). Principles of Virology, Molecular biology, Pathogenesis and Control. 2nd edition. ASM pres.s Washington DC.
4. Levy, J.A., Conrat, H.F., Owens, R.A. (2000). Virology. 3rd edition. Prentice Hall publication, New Jersey.
5. Wagner, E.K., Hewlett, M.J. (2004). Basic Virology. 2nd edition. Blackwell Publishing.
6. Mathews. (2004). Plant Virology. Hull R. Academic Press, New York.
7. Nayudu, M.V. (2008). Plant Viruses. Tata McGraw Hill, India.
8. Bos, L. (1999) Plant viruses-A text book of plant virology by. Backhuys Publishers.
9. Versteeg, J. (1985). A Color Atlas of Virology. Wolfe Medical Publication.

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Coimbatore – 641 021.

LECTURE PLAN
DEPARTMENT OF MICROBIOLOGY

STAFF NAME: Dr. A. A. ARUNKUMAR
SUBJECT NAME: VIROLOGY PRACTICALS SUB.CODE:17MBU211
SEMESTER: II CLASS: I B.Sc. (MB)

S. No	Lecture Duration Period	Topics to be Covered	Support Material/Page Nos
		UNIT-I	
1	3	Study of the structure of important animal viruses (rhabdo, influenza, paramyxo hepatitis B and retroviruses) using electron micrographs – Demonstration.	
2	3	Study of the structure of important plant viruses (caulimo, Gemini, tobacco ring spot, cucumber mosaic and alpha-alpha mosaic viruses) using electron micrographs – Demonstration.	
3	3	Study of the structure of important bacterial viruses (φX174, T4, λ) using electron micrograph – Demonstration.	
4	3	Isolation and enumeration of bacteriophages (PFU) from water/sewage sample using double agar layer technique	
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7	3	Perform local lesion technique for assaying plant viruses.	

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CLASS: I BSc MB **COURSE NAME: VIROLOGY PRACTICALS**

COURSE CODE: 17MBU211

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OBJECTIVES

- To study general aspects of viral morphology and classification.
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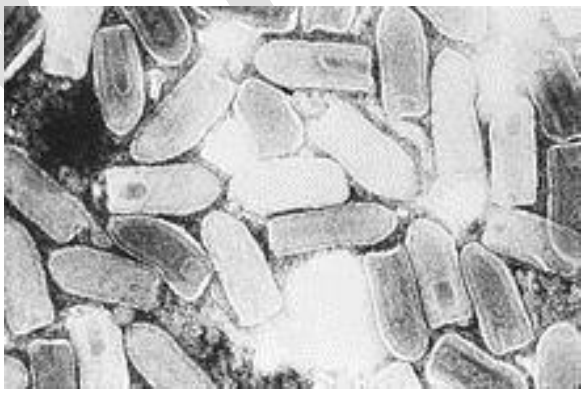
EXPERIMENT – 1

AIM

To Study the structure of important animal viruses (rhabdo, influenza, paramyxo hepatitis B and retroviruses) using electron micrographs – Demonstration.

Rhabdovirus, any of a group of viruses constituting the family Rhabdoviridae, responsible for rabies and vesicular stomatitis of cattle and horses. The virus particle is enveloped in a fatty membrane; is bullet-shaped, 70 by 180 nanometres (nm; 1 nm = 10^{-9} metre); and contains a single helical strand of ribonucleic acid (RNA)



Rhabdovirus

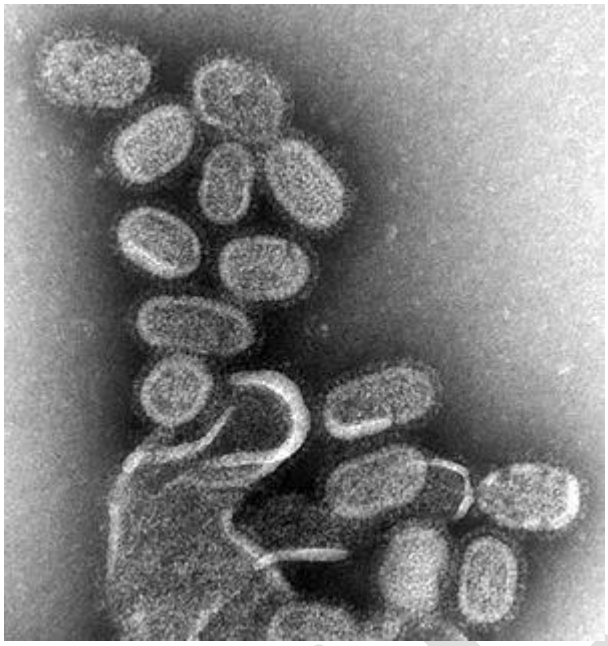
Influenza, commonly known as "**the flu**" or "**flu**", is an infectious disease caused by an influenza virus.[1] Symptoms can be mild to severe.[4] The most common symptoms include: a high fever, runny nose, sore throat, muscle pains, headache, coughing, and feeling tired.[1]These symptoms typically begin two days after exposure to the virus and most last less

than a week.[1] The cough, however, may last for more than two weeks.[1] In children, there may be nausea and vomiting, but these are not common in adults. Nausea and vomiting occur more commonly in the unrelated infection gastroenteritis, which is sometimes inaccurately referred to as "stomach flu" or the "24-hour flu". Complications of influenza may include viral pneumonia, secondary bacterial pneumonia, sinus infections, and worsening of previous health problems such as asthma or heart failure.

Three types of influenza viruses affect people, called Type A, Type B, and Type C.[2] Usually, the virus is spread through the air from coughs or sneezes. This is believed to occur mostly over relatively short distances. It can also be spread by touching surfaces contaminated by the virus and then touching the mouth or eyes. A person may be infectious to others both before and during the time they are showing symptoms. The infection may be confirmed by testing the throat, sputum, or nose for the virus. A number of rapid tests are available; however, people may still have the infection if the results are negative. A type of polymerase chain reaction that detects the virus's RNA is more accurate.

Frequent hand washing reduces the risk of viral spread. Wearing a surgical mask is also useful. Yearly vaccinations against influenza are recommended by the World Health Organization for those at high risk. The vaccine is usually effective against three or four types of influenza.[1] It is usually well tolerated. A vaccine made for one year may not be useful in the following year, since the virus evolves rapidly. Antiviral drugs such as the neuraminidase inhibitor oseltamivir, among others, have been used to treat influenza. Their benefits in those who are otherwise healthy do not appear to be greater than their risks. No benefit has been found in those with other health problems.

Influenza spreads around the world in a yearly outbreak, resulting in about three to five million cases of severe illness and about 250,000 to 500,000 deaths.[1] In the Northern and Southern parts of the world, outbreaks occur mainly in winter while in areas around the equator outbreaks may occur at any time of the year.[1] Death occurs mostly in the young, the old and those with other health problems.[1] Larger outbreaks known as pandemics are less frequent.[2]In the 20th century, three influenza pandemics occurred: Spanish influenza in 1918 (~50 million deaths), Asian influenza in 1957 (two million deaths), and Hong Kong influenza in 1968 (one million deaths).[9] The World Health Organizationdeclared an outbreak of a new type of influenza A/H1N1 to be a pandemic in June 2009.[10] Influenza may also affect other animals, including pigs, horses, and birds.[11]



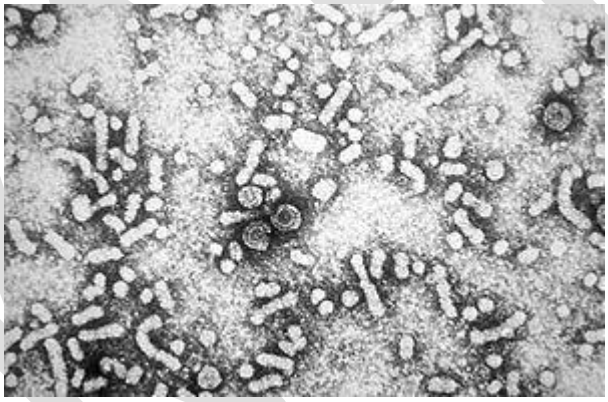
Influenza virus, magnified approximately 100,000 times

Hepatitis B is an infectious disease caused by the hepatitis B virus (HBV) that affects the liver.[1] It can cause both acute and chronic infections.[1] Many people have no symptoms during the initial infection.[1] Some develop a rapid onset of sickness with vomiting, yellowish skin, tiredness, dark urine and abdominal pain.[1] Often these symptoms last a few weeks and rarely does the initial infection result in death.[1][6] It may take 30 to 180 days for symptoms to begin.[1] In those who get infected around the time of birth 90% develop chronic hepatitis Bwhile less than 10% of those infected after the age of five do.[4] Most of those with chronic disease have no symptoms; however, cirrhosis and liver cancer may eventually develop.[2]These complications result in the death of 15 to 25% of those with chronic disease.[1]

The virus is transmitted by exposure to infectious blood or body fluids.[1] Infection around the time of birth or from contact with other people's blood during childhood is the most frequent method by which hepatitis B is acquired in areas where the disease is common.[1] In areas where the disease is rare, intravenous drug use and sexual intercourse are the most frequent routes of infection.[1] Other risk factors include working in healthcare, blood transfusions, dialysis, living with an infected person, travel in countries where the infection rate is high, and living in an institution.[1][4] Tattooing and acupuncture led to a significant number of cases in the 1980s; however, this has become less common with improved sterility.[7] The hepatitis Bviruses cannot be spread by holding hands, sharing eating utensils, kissing, hugging, coughing, sneezing, or breastfeeding.[4] The infection can be diagnosed 30 to 60 days after exposure.[1]The diagnosis is usually confirmed by testing the blood for parts of the virus and for antibodies against the virus.[1] It is one of five main hepatitis viruses: A, B, C, D, and E.[8]

The infection has been preventable by vaccination since 1982.[1][9] Vaccination is recommended by the World Health Organization in the first day of life if possible.[1] Two or three more doses are required at a later time for full effect.[1] This vaccine works about 95% of the time.[1] About 180 countries gave the vaccine as part of national programs as of 2006.[10] It is also recommended that all blood be tested for hepatitis B before transfusion and condoms be used to prevent infection.[1] During an initial infection, care is based on the symptoms that a person has.[1] In those who develop chronic disease, antiviral medication such as tenofovir or interferon may be useful; however, these drugs are expensive.[1] Liver transplantation is sometimes used for cirrhosis.[1]

About a third of the world population has been infected at one point in their lives, including 343 million who have chronic infections.[3][1][11] Another 129 million new infections occurred in 2013.[12] Over 750,000 people die of hepatitis B each year.[1] About 300,000 of these are due to liver cancer.[13] The disease is now only common in East Asia and sub-Saharan Africa where between 5 and 10% of adults are chronically infected.[1] Rates in Europe and North America are less than 1%.[1] It was originally known as "serum hepatitis".[14] Research is looking to create foods that contain HBV vaccine.[15] The disease may affect other great apes as well



Electron micrograph of hepatitis B virus

A **retrovirus** is a single-stranded positive-sense RNA virus with a DNA intermediate and, as an obligate parasite, targets a host cell. Once inside the host cell cytoplasm, the virus uses its own reverse transcriptase enzyme to produce DNA from its RNA genome, the reverse of the usual pattern, thus *retro*(backwards). The new DNA is then incorporated into the host cell genome by an integrase enzyme, at which point the retroviral DNA is referred to as a provirus. The host cell then treats the viral DNA as part of its own genome, translating and transcribing the viral genes along with the cell's own genes, producing the proteins required to assemble new copies of the virus. It is difficult to detect the virus until it has infected the host. At that point, the infection will persist indefinitely.

In most viruses, DNA is transcribed into RNA, and then RNA is translated into protein. However, retroviruses function differently, as their RNA is reverse-transcribed into DNA, which is integrated into the host cell's genome (when it becomes a provirus), and then undergoes the usual transcription and translational processes to express the genes carried by the virus. The information contained in a retroviral gene is thus used to generate the corresponding protein via the sequence: RNA → DNA → RNA → polypeptide. This extends the fundamental process identified by Francis Crick (one gene-one peptide) in which the sequence is DNA → RNA → peptide (proteins are made of one or more polypeptide chains; for example, haemoglobin is a four-chain peptide).

Retroviruses are valuable research tools in molecular biology, and they have been used successfully in gene delivery systems.[1]

Structure

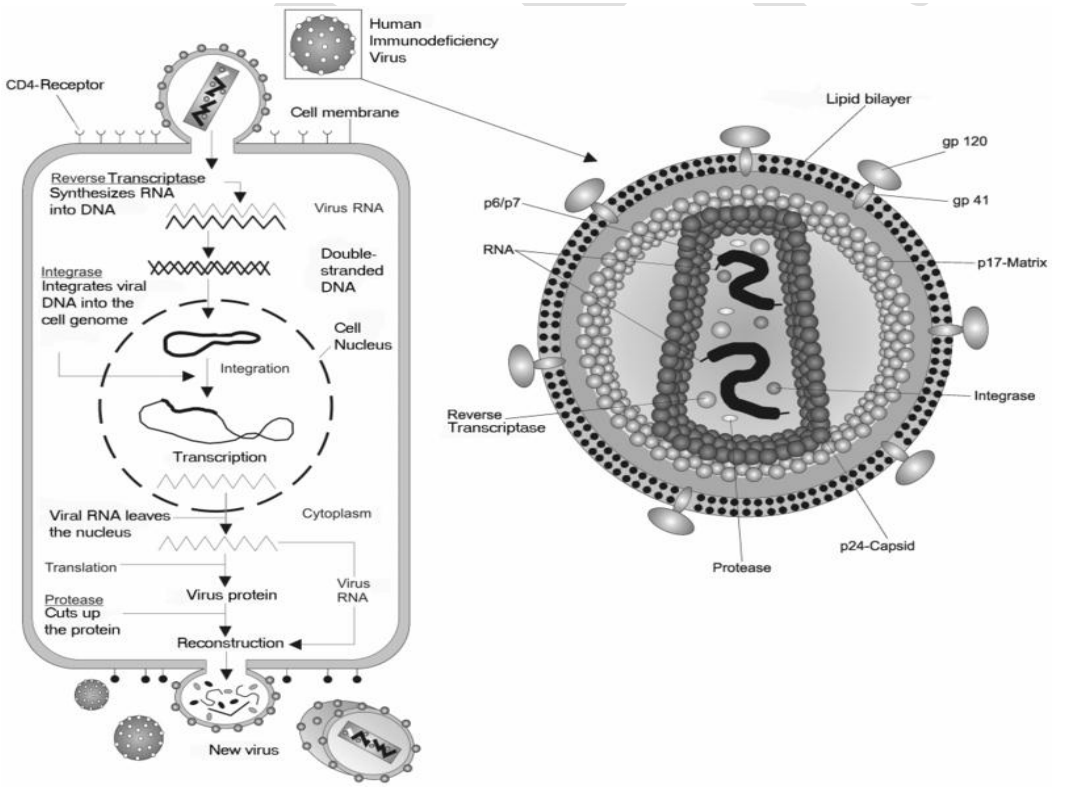
Virions of retroviruses consist of enveloped particles about 100 nm in diameter. The virions also contain two identical single-stranded RNAmolecules 7–10 kilobases in length. Although virions of different retroviruses do not have the same morphology or biology, all the virion components are very similar.[2]

The main virion components are:

- Envelope: composed of lipids (obtained from the host plasma membrane during the budding process) as well as glycoprotein encoded by the env gene. The retroviral envelope serves three distinct functions: protection from the extracellular environment via the lipid bilayer, enabling the retrovirus to enter/exit host cells through endosomal membrane trafficking, and the ability to directly enter cells by fusing with their membranes.
- RNA: consists of a dimer RNA. It has a cap at the 5' end and a poly(A) tail at the 3' end. The RNA genome also has terminal noncoding regions, which are important in replication, and internal regions that encode virion proteins for gene expression. The 5' end includes four regions, which are R, U5, PBS, and L. The R region is a short repeated sequence at each end of the genome used during the reverse transcription to ensure correct end-to-end transfer in the growing chain. U5, on the other hand, is a short unique sequence between R and PBS. PBS (primer binding site) consists of 18 bases complementary to 3' end of tRNA primer. L region is an untranslated leader region that gives the signal for packaging of the genome RNA. The 3' end includes 3 regions, which are PPT (polypurine tract), U3, and R. The PPT is a primer for plus-strand DNA synthesis during reverse transcription. U3 is a

sequence between PPT and R, which serves as a signal that the provirus can use in transcription. R is the terminal repeated sequence at 3' end.

- Proteins: consisting of gag proteins, protease (PR), pol proteins, and env proteins.
  - Group-specific antigen (gag) proteins are major components of the viral capsid, which are about 2000–4000 copies per virion.
  - Protease is expressed differently in different viruses. It functions in proteolytic cleavages during virion maturation to make mature gag and pol proteins.
  - Pol proteins are responsible for synthesis of viral DNA and integration into host DNA after infection.
  - Env proteins play a role in association and entry of virions into the host cell.[3] Possessing a functional copy of an env gene is what makes retroviruses distinct from retroelements.[4] The ability of the retrovirus to bind to its target host cell using specific cell-surface receptors is given by the surface component (SU) of the Env protein, while the ability of the retrovirus to enter the cell via membrane fusion is imparted by the membrane-anchored trans-membrane component (TM). Thus it is the Env protein that enables the retrovirus to be infectious.



HIV retrovirus schematic of cell infection, virus production and virus structure

Result:

The structure of important animal viruses (rhabdo, influenza, paramyxo hepatitis B and retroviruses) using electron micrographs has been studied.

Experiment -2

Aim

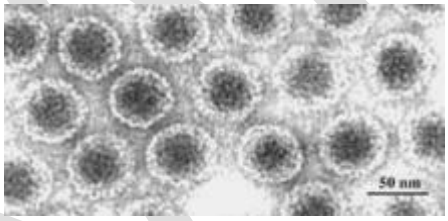
Study of the structure of important plant viruses (caulimo, Gemini, tobacco ring spot, cucumber mosaic and alpha-alpha mosaic viruses) using electron micrographs.

Caulimovirus is a genus of viruses, in the family Caulimoviridae. Plants serve as natural hosts. There are currently ten species in this genus including the type species Cauliflower mosaic virus. Diseases associated with this genus include: vein-clearing or banding mosaic.[1][2]

Structure

Viruses in Caulimovirus are non-enveloped, with icosahedral geometries, and T=7, T=7 symmetry. The diameter is around 50 nm. Genomes are circular and non-segmented. The genome codes for 6 to 7 proteins

Cauliflower mosaic virus (**CaMV**) is a member of the genus *Caulimovirus*, one of the six genera in the *Caulimoviridae* family, which are pararetroviruses that infect plants.[1] Pararetroviruses replicate through reverse transcription just like retroviruses, but the viral particles contain DNA instead of RNA



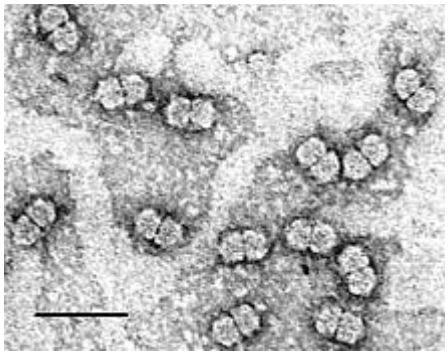
Electron micrograph of CaMV virions

Geminiviridae is a family of plant viruses. There are currently over 360 species in this family, divided among 9 genera.[1][2] Diseases associated with this family include: bright yellow mosaic, yellow mosaic, yellow mottle, leaf curling, stunting, streaks, reduced yields.[2][3] They have single-stranded circular DNAgenomes encoding genes that diverge in both directions from a virion strand origin of replication (i.e. geminivirus genomes are ambisense). According to the Baltimore classification they are considered class II viruses. It is the largest known family of single stranded DNA viruses.

Mastrevirus and curtovirus transmission is via various leafhopper species (e.g. maize streak virus and other African streak viruses are transmitted by *Cicadulina mbila*), the only known topocuvirus species, *Tomato pseudo-curly top virus*, is transmitted by

the treehopper *Micrutalis malleifera*, and begomoviruses are transmitted by the whitefly species, *Bemisia tabaci*.

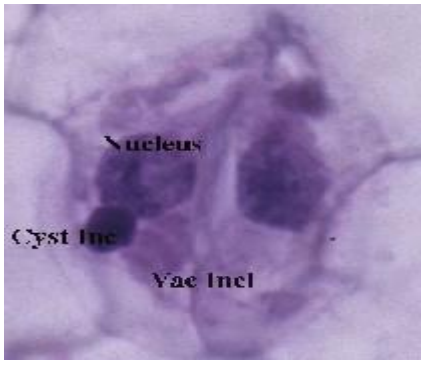
These viruses are responsible for a significant amount of crop damage worldwide. Epidemics of geminivirus diseases have arisen due to a number of factors, including the recombination of different geminiviruses coinfecting a plant, which enables novel, possibly virulent viruses to be developed. Other contributing factors include the transport of infected plant material to new locations, expansion of agriculture into new growing areas, and the expansion and migration of vectors that can spread the virus from one plant to another.

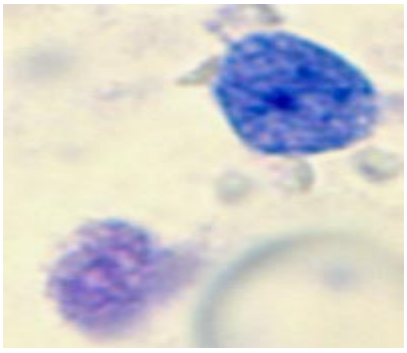


Purified Maize streak virus (MSV) particles stained with uranyl acetate. Size bar indicates 50 nm

Tobacco ringspot virus **(TRSV)** is a plant pathogenic virus in the plant virus family *Secoviridae*. It is the type species of the genus *Nepovirus*. Nepoviruses are transmitted between plants by nematodes, varroa mitesand honeybees.[1] TRSV is also easily transmitted by sap inoculation and transmission in seeds has been reported.[2] In recent cases it has also been shown to appear in bees.

TRSV was observed for the first time in tobacco fields in Virginia and described in 1927.[3] It is an isometric particle[4] with a bipartite RNA genome. The virus has a wide host range[5] that includes field grown crops, ornamentals and weeds. Its name comes from its most common symptom being chlorotic ringspots on the leaves of infected plants.[6] In some areas this virus has caused growers to stop growing affected crops.





Cucumber mosaic virus (**CMV**) is a plant pathogenic virus[1] in the family *Bromoviridae*.[2] It is the type member of the plant virus[3] genus, *Cucumovirus*.[4] This virus has a worldwide distribution and a very wide host range.[5] In fact it has the reputation of having the widest host range of any known plant virus.[6] It can be transmitted from plant to plant both mechanically by sap and by aphids in a stylet-borne fashion. It can also be transmitted in seeds and by the parasitic weeds, *Cuscuta sp.* (dodder).

Alfalfa mosaic virus (AMV), also known as *Lucerne mosaic virus* or *Potato calico virus*, is a worldwide distributed phytopathogen that can lead to necrosis and yellow mosaics on a large variety of plant species, including commercially important crops. It is the only *Alfamovirus* of the *Bromoviridae* family. In 1931 Weimer J.L. was the first to report AMV in alfalfa (*Medicago sativa*). Transmission of the virus occurs mainly by some aphids (plant lice), by seeds or by pollen to the seed.

The virion has a capsid (coat protein) but no envelope. The icosahedral symmetry of the capsid is round to elongated. The range for the length of the virion particle is about 30–57 nm. AMV is a multipartite virus and is composed of 4 particles (3 bacilliform and 1 spheroidal) with a diameter of 18 nm.[3] [4] The genetic material of AMV consists of 3 linear single strands RNAs (RNA 1, RNA 2 and RNA 3) and a subgenomic RNA (RNA 4) which is obtained by transcription of the negative- sense strand of RNA 3. RNA 1 and 2 encode proteins needed for replication. RNA 3 is required for the synthesis of the protein responsible for cell-to-cell movement. RNA 4 encodes the capsid. Beside encapsidation and its role in movement the viral coat protein also plays a role in the initiation of RNA replication. This property is called genome activation and means that the genomic nucleic acid is not infectious without the capsid. Specific association of the coat protein with the RNA 3'- terminal sequences or with the subgenomic mRNA is required for the infection. Bacilliform particles contain separately encapsidated RNAs 1, 2 and 3. Spheroidal particles each have two copies of RNA 4. The nucleotide sequence of the complete genome has been determined and the length of the genome is 8274 nucleotides (or 9155 including the subgenomic RNA). RNA 1, 2, 3 and 4 are respectively 3644 (3.65kb), 2593 (2.6kb), 2037 (2.2kb) and 881 (0.88kb) nucleotides long.

Symptoms of AMV on potato leaves

Result:

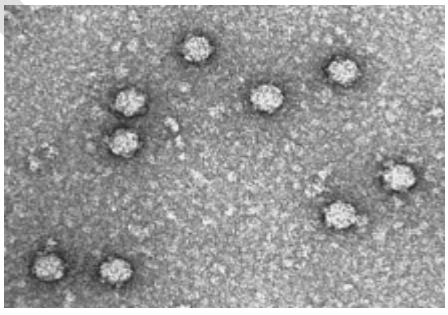
The structure of important plant viruses (caulimo, Gemini, tobacco ring spot, cucumber mosaic and alpha-alpha mosaic viruses) using electron micrographs has been studied.

EXPERIMENT – 3

Aim

To study of the structure of important bacterial viruses (φX174, T4, λ) using electron micrographs.

The **phi X 174** (or **ΦX174**) bacteriophage is a single-stranded DNA (ssDNA) virus and the first DNA-based genome to be sequenced. This work was completed by Fred Sanger and his team in 1977.[2] In 1962, Walter Fiers and Robert Sinsheimer had already demonstrated the physical, covalently closed circularity of ΦX174 DNA.[3] Nobel prize winner Arthur Kornberg used ΦX174 as a model to first prove that DNA synthesized in a test tube by purified enzymes could produce all the features of a natural virus, ushering in the age of synthetic biology.[4][5] In 2003, it was reported by Craig Venter's group that the genome of ΦX174 was the first to be completely assembled *in vitro* from synthesized oligonucleotides.[6] The ΦX174 virus particle has also been successfully assembled *in vitro*.[7] Recently, it was shown how its highly overlapping genome can be fully decompressed and still remain functional.

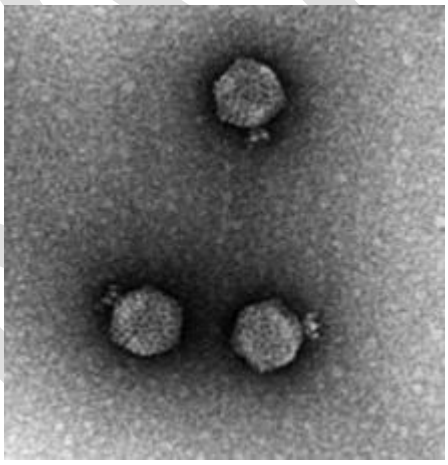


Electron micrograph of phage ΦX174

A **bacteriophage** (/ˈbækˈtɪərioʊˌfeɪdʒ/), also known informally as a ***phage*** (/feɪdʒ/), is a virus that infects and replicates within a bacterium. The term was derived from "bacteria"

and the Greek: φαγεῖν (*phagein*), "to devour". Bacteriophages are composed of proteins that encapsulate a DNA or RNA genome, and may have relatively simple or elaborate structures. Their genomes may encode as few as four genes, and as many as hundreds of genes. Phages replicate within the bacterium following the injection of their genome into its cytoplasm. Bacteriophages are among the most common and diverse entities in the biosphere.[1]Bacteriophages are ubiquitous viruses, found wherever bacteria exist. It is estimated there are more than 10^{31} bacteriophages on the planet, more than every other organism on Earth, including bacteria, combined.[2]

Phages are widely distributed in locations populated by bacterial hosts, such as soil or the intestines of animals. One of the densest natural sources for phages and other viruses is sea water, where up to 9×10^{8}virions per milliliter have been found in microbial mats at the surface,[3] and up to 70% of marine bacteria may be infected by phages.[4] They have been used for over 90 years as an alternative to antibiotics in the former Soviet Union and Central Europe, as well as in France.[5] They are seen as a possible therapy against multi-drug-resistant strains of many bacteria (see phage therapy).[6] Nevertheless, phages of Inoviridae have been shown to complicate biofilms involved in pneumonia and cystic fibrosis, and shelter the bacteria from drugs meant to eradicate disease and promote persistent infection



Bacteriophage P22, a member of the *Podoviridae* by morphology due to its short, non-contractile tail.

Enterobacteria phage λ (**lambda phage**, **coliphage λ**) is a bacterial virus, or bacteriophage, that infects the bacterial species *Escherichia coli* (*E. coli*). It was discovered by Esther Lederberg in 1950 when she noticed that streaks of mixtures of two *E. coli* strains, one of which treated with ultraviolet light, was "nibbled and plaqued".[1][2] The wild type of this virus has a temperate lifecycle that allows it to either reside within the genome of its host

through lysogeny or enter into a lytic phase (during which it kills and lyses the cell to produce offspring); mutant strains are unable to lysogenize cells- instead they grow and enter the lytic cycle after superinfecting an already lysogenized cell.[3]

The phage particle consists of a head (also known as a capsid), a tail, and tail fibers (see image of virus below). The head contains the phage's double-strand linear DNA genome. During infection, the phage particle recognizes and binds to its host, *E. coli*, causing DNA in the head of the phage to be ejected through the tail into the cytoplasm of the bacterial cell. Usually, a "lytic cycle" ensues, where the lambda DNA is replicated and new phage particles are produced within the cell. This is followed by cell lysis, releasing the cell contents, including virions that have been assembled, into the environment. However, under certain conditions, the phage DNA may integrate itself into the host cell chromosome in the lysogenicpathway. In this state, the λ DNA is called a prophage and stays resident within the host's genome without apparent harm to the host. The host is termed a lysogen when a prophage is present. This prophage may enter the lytic cycle when the lysogen enters a stressed condition.

Lysis plaques of lambda phage on *E. coli* bacteria

Result:

The structure of important bacterial viruses (φX174, T4, λ) using electron micrographs has been studied.

EXPERIMENT – 4

AIM

To perform Isolation and enumeration of bacteriophages (PFU) from water/sewage sample using double agar layer technique

Preparations

Cultures:

24- hour nutrient broth cultures of Escheria coli B and T2 coli phage.

Media:

Tryptone agar plates, Tryptone soft agar tubes (2ml/tube) and tryptone broth tubes (9ml/tube)

Equipments:

Bunsen burner

- Water bath
- Thermometer
- 1-ml sterile pipettes
- Sterile Pasteur pipettes
- Mechanical pipetting devices
- Test tube rack
- Glassware marking pencil

Media Preparation:

a) Tryptone agar:-

Add 10g Tryptone, 0.01-0.03M Calcium chloride (reagent), 5g Sodium chloride and 11g agar in 1L of water. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121°C for 15 minutes.

b) Tryptone Broth:-

Prepared as above without the addition of agar in the medium.

c) Tryptone soft agar:-

Add 10g Tryptone, 5ml Potassium chloride and 9g Agar in 1L of water. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121°C for 15 minutes.

Procedure:

Since viruses can grow to incredibly high concentrations, we need to dilute them in order to count them effectively. Perform dilution of the bacteriophage culture .

Label all dilution tubes and media as follows. Each tube represents a ten-fold dilution of the virus

a) Five tryptone soft agar tubes : 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}

b) Five tryptone hard agar plates : 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}

c) Ten tryptone broth tubes : 10^{-1} through 10^{-10}

Serial Dilution

Put on gloves, fill 9 ml of tryptone broth to ten culture tubes labeled as 10^{-1} through 10^{-10}. These tubes will be used for viral serial dilutions.

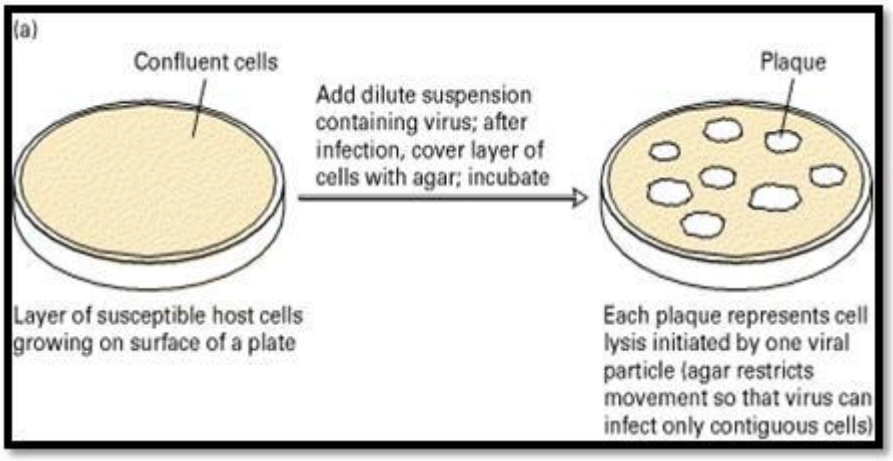
- Take 1 ml of the phage culture stock that you want to titer and transfer it to the tube titled 10^{-1} with a pipette. Mix the tube well. This is your first ten-fold dilution.(ie; a 1 in 10 dilution)
- Take 1 ml of the mixed culture from your tube labeled 10^{-1} and transfer it with a new pipette to the next tube, labeled 10^{-2} . Mix this tube as well.
- Continue this pattern to create a serial dilution series. You will end up with 9 tubes of 9 ml and 1 tube of 10 ml. The viral loads in your tubes will be diluted anywhere from 10 times (your first tube) or 100 times (your second tube) to ten billion times (your final tube).

Preparing Plates

Take five tubes of tryptone soft agar and five Petri plates labeled as 10^{-5} through 10^{-9}

- Place the five labeled soft tryptone agar tubes into a water bath. Water should be of a depth just slightly above that of the agar in the tubes. Bring the water bath to 100˚C to melt the agar. Cool and maintain the melted agar at 45˚C. This will ensure that your agar does not solidify in the tubes before you have a chance to pour it into the petri dishes.
- Aseptically transfer two drops of Escheria coli B culture with a Pasteur pipette to the agar and mix it gently. These are the bacteria that will be killed, allowing you to count the number of virus particles in a particular solution.
- Add 0.1 ml of each serial dilution to its corresponding soft agar tube while the tubes are still in the hot water bath. For example, 0.1 ml of your 10^{-5} serial dilution should go into the soft agar tube labeled 10^{-5}.
- Using separate Pasteur pipettes and sterile pipette tips, repeat the previous step for the tryptone broth phage dilution tubes labeled 10^{-6}through 10^{-9}.

- Mix the tubes well and then pour each tube into the Petri plate with the corresponding label. This will create a thin layer of agar that has been inoculated with bacteria and viruses in each plate. Incubate all plate cultures in an inverted position for 24 hours at 37 ˚C



Counting and Calculating Titers

The viral titer is a quantitative measurement of the biological activity of your virus and is expressed as plaque forming units (pfu) per ml.
To calculate the viral titer,

- Take your plates out of the incubator and examine them. You should see cloudy areas throughout the plate where bacteria have grown, except for small clear spots called plaques. These plaques are patches of dead bacteria, and each plaque represents one virus.
- Find a plate that has between 30 and 300 plaques and count the exact number of plaques on that plate.
- Then use the following formula to determine the titer (pfu/ml) of your viral stock.

$$\frac{PFU}{ml} = \frac{Number\ of\ plaques}{dXV}$$

Where, d = dilution

v = volume of diluted virus added to the plate

Sample calculation:

- An average of 50 plaques formed in the 1:10,000 dilution wells
- Volume of diluted virus added: 0.2 ml

$$\frac{50}{0.0001 \times 0.2} = 2.5 \times 10^6 \text{pfu/ml}$$

Experiment to Isolate Coliphage of a Virus!

Principle:

The bacteriophage or phage (virus) that infects the bacteria, Escherichia coli is called coliphage (coli: E. coli-, phage: bacteriophage).

It can be obtained from a variety of natural sources, such as soil, fecal matter and raw sewage. Its isolation from these sources is not very easy, as it is present in low concentration.

Therefore, first its number is increased by an enrichment step, in which a rich culture of the host bacteria (i.e. E. coli) is added to the coliphage-containing sample and incubated. The coliphage infects E. coli and increases in number profusely. This coliphage-rich culture is centrifuged to settle down the particulate matter.

The sediment is discarded and the supernatant is filtered through microbial filter to retain bacteria and other particles, while allowing the coliphage to pass through. The filtrate, rich in coliphage, is used to seed a suspension of E. coli, which is then allowed to grow as a confluent lawn on an agar plate.

The coliphage grows within the E. coli cells and lyse them. Lysis of the bacteria cells results in the formation of clear zones on the confluent lawn of bacteria. These clear zones are called plaques. Each clear zone is assumed to be formed by a single coliphage.

Materials Required:

Pipettes, conical flasks, petri dishes, test tubes, cotton plugs, bunsen burner, dispose jar, centrifuge, membrane filtration apparatus, laminar flow chamber, autoclave, incubator, bacteriophage broth, tryptone soft agar, tryptone hard agar, nutrient broth culture of the bacteria Escherichia coli (e.g., E. coli B), sample (e.g., raw sewage).

Procedure:

1. Five pipettes (in a stainless steel pipette case) and five petri dishes are sterilized in a hot air oven at 180° C for 3 hours. Alternatively, they can be covered with craft paper, tied with thread or rubber band and sterilized in an autoclave along with the media (Figure 8.5).

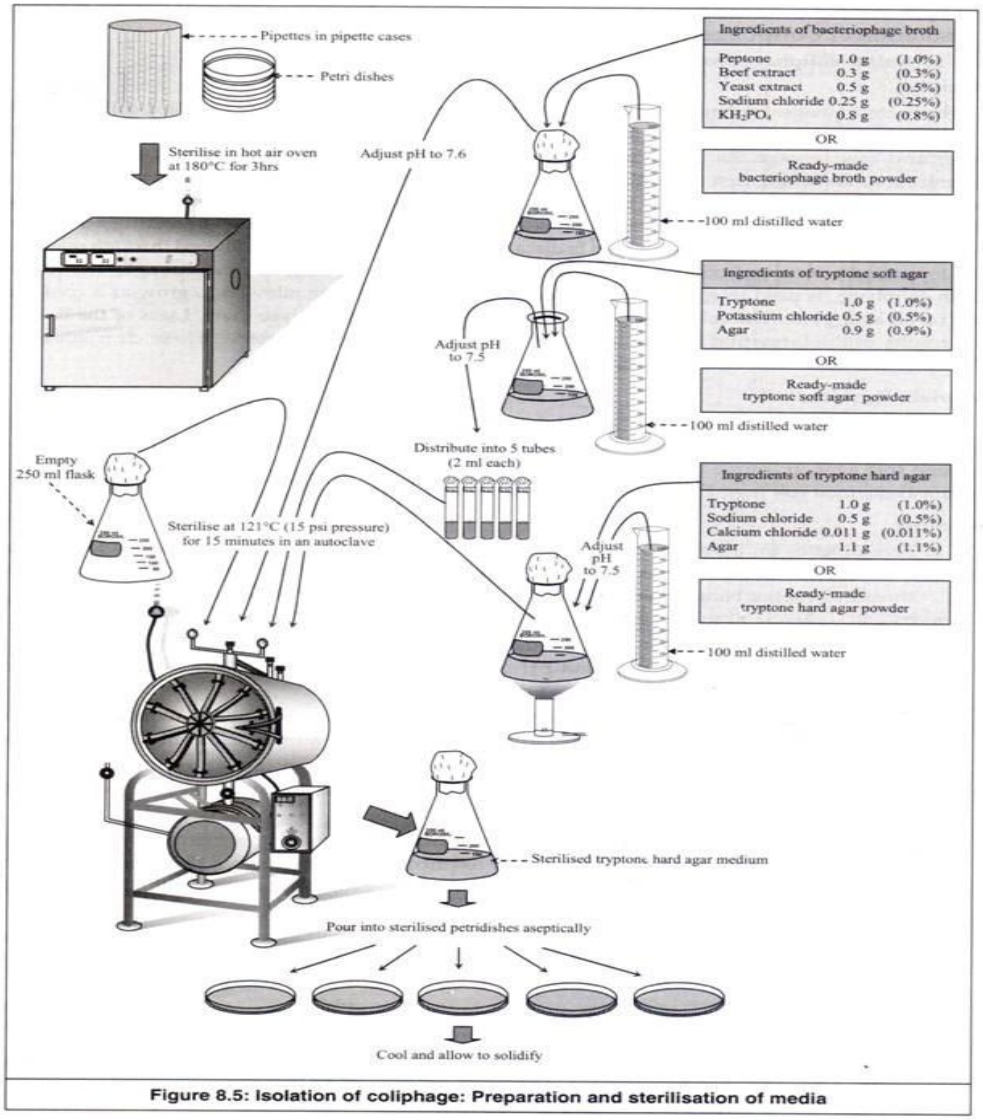


Figure 8.5: Isolation of coliphage: Preparation and sterilisation of media

2. The ingredients of bacteriophage broth medium or its ready- made powder, required for 100 ml of the medium is weighed, dissolved in 100 ml of distilled water in a 250 ml conical flask and pH adjusted to 7.6. The flask is cotton-plugged, covered with craft paper and tied with thread or rubber band.

3. The ingredients of tryptone soft agar medium or its ready-made powder, required for 100 ml of the medium is weighed, dissolved in 100 ml of distilled water in a 250 ml conical flask and pH adjusted to 7.5.

4. This liquid medium is distributed into 5 test tubes (2 ml each), cotton-plugged, covered with craft paper and tied with thread or rubber band.

5. The ingredients of tryptone hard agar medium or its ready-made powder required for 100 ml of the medium is weighed and dissolved in 100 ml of distilled water in a 250 ml conical

flask by heating after pH adjustment to 7.5. The flask is cotton-plugged, covered with craft paper and tied with thread or rubber band.

6. The flask containing bacteriophage broth, the 5 tryptone soft agar tubes, the flask containing tryptone hard agar medium and an empty cotton-plugged 250 ml conical flask are sterilized at 121 °C (15 psi pressure) for 15 minutes in an autoclave.

7. The sterilized tryptone hard agar medium in the conical flask is poured into the 5 sterilized petri dishes aseptically and allowed to cool, so as to get 5 tryptone hard agar plates.

8. Into the cotton-plugged sterilized empty 250 ml conical flask, 5 ml of sterilized bacteriophage broth, 5 ml of E. coli B broth culture and 45 ml of raw sewage are transferred aseptically, preferably in a laminar flow chamber (Figure 8.6).

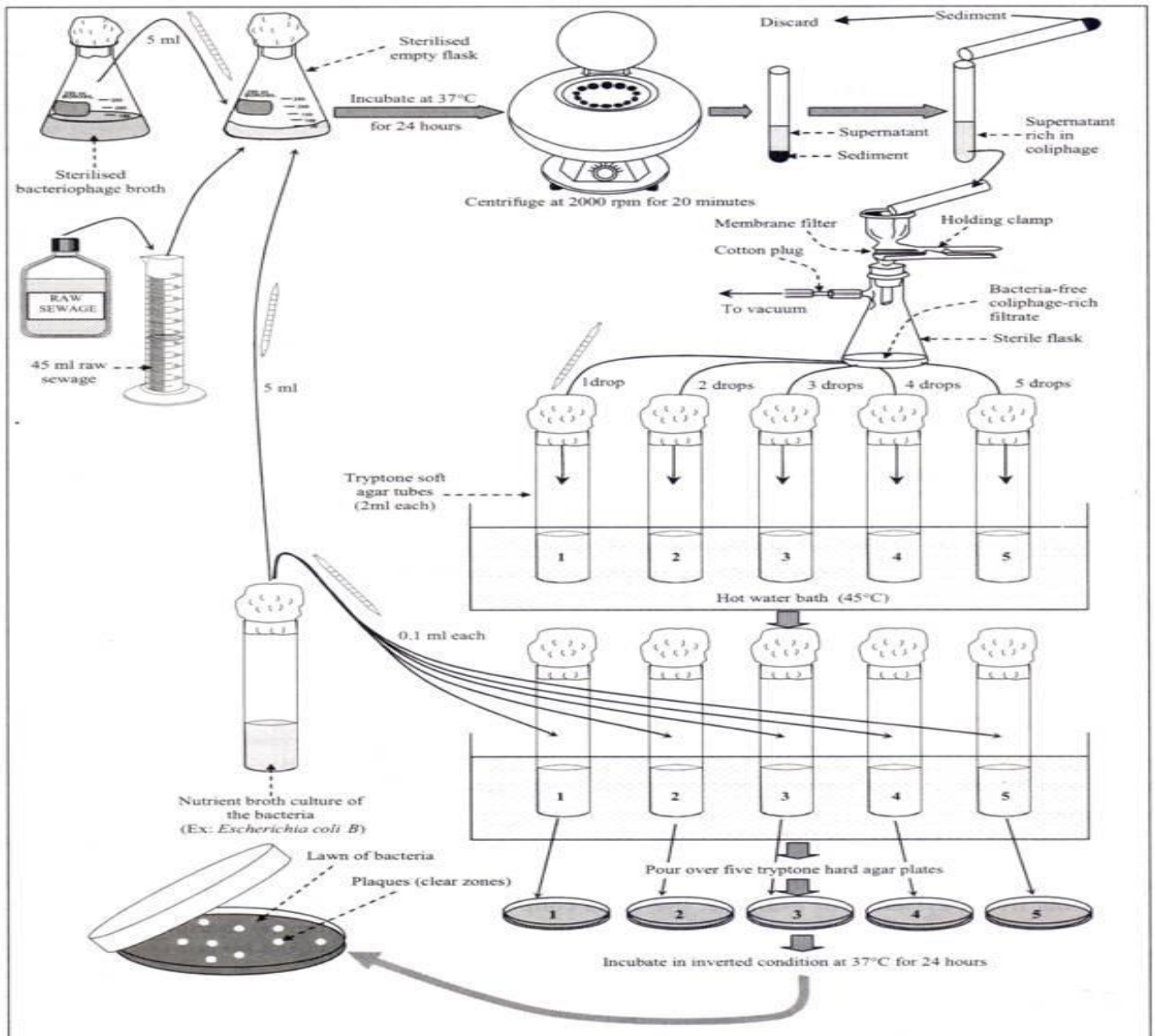


Figure 8.6: Isolation of coliphage: Inoculation, incubation and enumeration

9. The flask is incubated at 37°C for 24 hours, so as to allow the coliphage in the sewage to proliferate within the host bacteria cells (cells of E. coli B).

10. The contents of the flask is poured into 100 ml centrifuge tubes and centrifuged at 2500 rpm for 20 minutes in a centrifuge to settle down the particles. The sediment is discarded.

11. The supernatant, rich in coliphage, is filtered through a membrane filtration apparatus. The residue is discarded.

12. The five sterilized tryptone soft agar tubes are taken and heated on a water bath to 100°C, so as to melt the agar. The tubes are cooled and maintained at 45°C on a water bath.

13. Using a sterile pipette, 1, 2, 3, 4 and 5 drops of the bacteria-free filtrate, rich in coliphage is transferred aseptically to the five tryptone soft agar tubes.

14. To each tryptone soft agar tube, 0.1 ml of a nutrient broth culture of Escherichia coli B is transferred aseptically.

15. The contents of the five tryptone soft agar tubes having the virus, coliphage and the bacteria, E. coli B are rapidly mixed by rotating the tubes between the palms of hands.

16. The contents are poured over the five tryptone hard agar plates (labeled 1 to 5 drops), thereby forming a double layer plate culture preparation. The plates are swirled gently and allowed to harden.

17. The inoculated plates are incubated in an inverted position at 37°C for 24 hours in an incubator.

Results:

1. All the plates are observed for plaque forming units (PFUs) that develop as clear zones on the lawn of bacteria. Plaque formation is indicative of presence of coliphage in the culture.

2. Each plaque represents a rich growth of isolated coliphage.

3. The number of plaques is counted in all the plates and tabulated as follows (Table 8.1).

Table : Observations of the number of coliphage:

Number of Drops	Number of Plaques
1 drop	
2 drops	
3 drops	
4 drops	
5 drops	

EXPERIMENT – 5

AIM

Studying isolation and propagation of animal viruses by chick embryo technique.

CELL CULTURE TECHNIQUES

Subculture of a cell line

1. *Aedes albopictus* C6/36 (mosquito gut epithelium) cell line
2. Discard supernatant fluid from a confluent monolayer of C6/36 (Aedes albopictus) bottle cultures.
3. Add 3 to 5 ml trypsin (Pre-warmed at 37 C) to each culture bottle.
4. Leave the bottle at room temperature for 3-5 mins.
5. Discard the trypsin.
6. Add 5ml of growth medium to the bottle and gently detach the cells from the surface with the help of a pipette.
7. Add the required amount of medium (split ratio for this cell line is 1: 5) and distribute the diluted cell suspension to fresh flasks/bottles.
8. Incubate at room temperature for 3-7 days.

Primary cell culture technique

Chick embryo fibroblastic culture :

The development of routine cell culture methods has reduced the importance of eggs but they are still valuable for the isolation of many important viruses and for the production of vaccines. Fertile eggs must be obtained ideally from a specific pathogen free flock, should be clean, preferably unwashed and pale shelled to simplify candling. After laying they have to be incubated for 10 days at 37° C With 40-70% humidity and

good aeration and turned twice daily. After 6 days they are candled, infertile and dead eggs are discarded. On the day of culture those with satisfactory development of chorioallantioic blood vessels and showing embryonic movement are marked with pencil to indicate the limits of the air sac.

Materials required

1. Embryonated eggs preferably 10-12 days old.
2. Phosphate buffer saline (pH 7.2) .
3. 0.25% trypsin in PBS.
4. Growth medium (MEM, supplemented with 10% bovine serum)
5. Forceps, scissors, egg cup, petri dishes, filtration unit, silicone/teflon
6. Coated magnet, solution bottles and culture flasks.

Procedure:

1. Candle and select 10-11 day old eggs.
2. Place the egg in an egg cup, air sac upwards and wipe clean with spirit.
3. Break the shell with the sharp end of a sterile forceps, and lift the membrane. With a bent forceps pick up the embryo and place it in a petri dish containing PBS.
4. Dissect the embryo in the dish, remove and discard the head, limbs and viscera. Pick up the fibroblastic tissue and transfer into a wide neck bottle containing PBS.
5. Mince the tissue finely with scissors, and wash minced tissue several times in PBS to remove blood cells and debris.
6. Transfer tissue to transfusion bottle containing sterile silicone covered magnet and 50ml of 0.25 % trypsin solution and stopper securely.
7. Trypsinize on magnetic stirrer unit 37° C for 30 mins : avoid frothing of contents.
8. The tissue will disintegrate, forming a turbid suspension of cells. Filter the suspension through sterile gauze and centrifuge filtrate for 10min at 1000 rev/min.
9. Discard supernatant and resuspend cells in 100 ml growth medium.
10. Centrifuge once again and resuspend the cells in fresh medium.
11. Dilute 0.9ml suspension with 0.1ml trypan blue solution and count cells in haemocytometer.
12. Adjust concentration to 1 10 cells / ml growth medium and dispense into tubes or bottles.
13. Incubate at 37° C until monolayer is formed (2-3 days).
14. When cells have formed a monolayer, remove growth medium, inoculate virus, add maintenance medium (MEM, Eagles base with 1-2% bovine serum) and incubate.

Media preparation for cell culture

Dehydrated Tissue Culture Media

1. Take 900ml of triple distilled water.
2. Add the contents of one unit vial of dehydrated media to the water at room temperature with stirring until dissolved.
3. Rinse the vial with a small amount of triple distilled water to remove traces of powder and add to the above solution.
4. Add 2.2 grams of sodium bicarbonate or HEPES Buffer.
5. Adjust the pH if required between 7.1 to 7.4 using 1N HC1 or 1N NaOH or by bubbling carbon dioxide. Note that pH tends to rise during filtration and hence adjust it 0.2 to 0.3 units below the final desired pH.
6. Make up final volume to 1000ml with triple distilled water.

Sterilization of tissue culture Media

1. Sterilize the media by filtering through sterile membrane filter(sterilized by autoclaving at 15 lbs for 15 min & 121° C) of 0.22 micron or less porosity using positive pressure to minimise loss of carbon dioxide.

Antibiotics

2. The following antibiotics can be aseptically added to litre of media :
3. Amphotericin 2.5 mg
4. Gentamycin (50mg/ml. Solution) 1.0 ml
5. Benzyl Penicillin 10000 units
6. Streptomycin 100 mg

Sterility check

Add 0.5 - 1.0 ml of filtered media to a tube containing sterile thioglycolate broth and incubate at 37° C for 48 hours. If the broth is clear after 48 hours the media is sterile.

TVG (Trypsin Versene Glucose)

1. TVG consists of the following components in 1X PBS:
2. Trypsin 0.1%
3. Versene 0.2%
4. Glucose 0.05%

Stock solutions for TVG

1). 10x PBS

NaCl	80.00 gms
KCl	2.00 gms
Na2H P04	14.42 gms
KH2 PO4	2.00 gms
Dist.H2O	upto 1 litre

To prepare 1X PBS - add 100 ml of 1X PBS to 900 ml of D/W.

2) 2% TRYPSIN

Trypsin	2.00 gms
D/W	upto 100ml

Stir the above solution on a magnetic stirrer for 4h or O/N at 4°C. Sterilize by filtering through sterile membrane filter of 0.22u pore size. A sterility check can be done before using the solution.

3) 0.2 % VERSENE

EDTA	200 mg
D/W	100 ml

Sterilize by autoclaving at 15 lbs & 121°C for 15 minutes.

1) 10 % GLUCOSE

Glucose	10.00 gms
D/W	upto 100 ml

Sterilize by autoclaving at 15 lbs & 121° C for 15 minutes.

2) 1 % PHENOL RED
Phenol Red 1.00 gm
D/W upto 100ml

Preparation of working solution of TVG

Prepare 840ml of 1X PBS and to this add 1.0 ml of 1% phenol red (indicator). Sterilize by autoclaving at 15lbs and 121°C for 15 minutes. Cool this sterile solution and then add the following sterilized solutions to it :

Trypsin 2.0% 50 ml
Versene 0.2% 100 ml
Glucose 10.0% 5 ml
Do a sterility test as mentioned above before using this TVG.

WASHING OF GLASSWARE FOR TISSUE CULTURE WORK

1) Autoclave all glassware before washing, at 120°C, 15psi for 20min.
2) The outside surface of glassware can be cleaned with Vim and inside with only Teepol and scrubbed with a clean brush , which is only meant for brushing glassware. Wash in tap water and leave in 5% Teepol solution overnight (can be boiled)
3) Next morning wash in tap water at least 20 times and leave in 10% HC1 overnight.
4) Next day wash in tap water and rinse in 2 changes of demineralised distilled water at least 10 times in each, leave in third bucket of demineralised distilled water overnight.
5) Take out next day, dry it in hot air oven, pack and sterilize.

Packing and sterilization

Pipettes: Wrap the pipette with brown paper and sterilize in a hot air oven at 160°C for two hours.

Petridishes : Wrap the Petri dishes in brown paper and tie with Twine and sterilize by hot air oven at 160°C for two hours.

Culture & media storage bottles, measuring cylinders and beakers : Cover mouth with aluminum foil, over which brown paper is tied with twine at the neck and sterilize in a hot oven at 160°C. Plastic measuring cylinders and beakers are autoclaved at 120°C, psi for 30min.

Plastic centrifuge tubes, Eppendorf tubes, screw cap vials & tubes, : Arrange neatly the following items in a plastic or glass beaker and cover the mouth with aluminum foil and over that wrap brown paper tied with twine at the neck. Sterilize by autoclaving at 120°C, 15 psi for 30min.

Coverslips: Coverslips are put into a Petri dish , which is covered with brown paper and tied with twine. Sterilize in a hot air oven at 160°C for two hours.

Filter Apparatus: Wrap filter apparatus first with aluminum foil and then with brown paper, tie it with twine tightly. Sterilize by autoclaving at 120° C, 15 psi for 30min.

Solutions: Plug the mouth of the container with cotton, wrap it with brown

paper and tie it with twine. Sterilize by autoclaving at 121° C , 15 psi for 20 min.

EXPERIMENT – 6

AIM

Demonstration Of Cytopathic Effect (Cpe)

1) HSV - Demonstration of cytopathic effect (CPE) in Vero cell line.
2) Grow Vero cells to confluence in Nunc flasks / 24 well dishes.
3) Discard the growth medium.
4) Infect the cell line with HSV-1 suspension at an MOI (multiplicity of infection) of 1.
5) Adsorb the virus for 30 minutes at 37° C .
6) Add the maintenance medium - 5ml for Nunc flask/10-15ml for Milk dilution bottle/100ml for a Roux bottle.
7) Incubate the bottles at 37°C in CO incubator.
8) Observe daily for CPE both in the morning and in the evening.
9) HSV produces CPE by 24-48 h. Two forms of CPE are observed. The most common begins with cytoplasmic granulation after which the cells become enlarged or ballooned. These macrocytes then become rounded, take on a refractile appearance, and undergo lytic degeneration. The second type of CPE is formation of multinucleated giant cells.

Measles virus : Demonstration of CPE in Vero cell line

1) Grow Vero cells to form a monolayer in Nunc flasks/ Milk dilution bottles/Roux bottle.
2) Discard the growth medium.
3) Infect the cell line with measles virus suspension at an MOI(multiplicity of infection) of 1.
4) Adsorb the virus for 30 minutes at 37° C.
5) Add the maintenance medium - 5ml for Nunc flask/10-15ml for Milk dilution bottle/100ml for Roux bottle.
6) Incubate bottles at 37°C in CO incubator.
7) Observe daily for CPE both in the morning and in the evening.
8) Measles virus produces CPE after 4-5 days. Two types of CPE are described. One type seen after infection with wild virus and dilute inoccula is giant cell transformation. Multinucleated giant cells containing 10-100 nuclei form as a result of cell fusion induced by virus. The second type of CPE, spindle cell formation, is associated with measles virus , which has been passed repeatedly in tissue culture, or with repeated passage of undiluted inoccula.

Japanese Encephalitis virus : Demonstration of CPE in porcine stable kidney cell line (PS)

1) Grow PS cells to form a monolayer in Nunc flasks/Milk dilution bottles/Roux bottle.
2) Discard the growth medium.
3) Infect the cell line with measles virus suspension at an MOI (multiplicity of infection) of 1.
4) Adsorb the virus for 30 minutes at 37° C.
5) Add the maintenance medium - 5ml for Nunc flask/10-15ml for Milk dilution bottle/100ml for a Roux bottle.
6) Incubate the bottles at 37° C in CO incubator.
7) Observe daily for CPE both in the morning and in the evening.
8) JEV produces CPE after 18-20 hrs. CPE is seen in the form of rounding of cells followed by cell lysis.