**LAB WORK 13.**

**Subject: Isolation of bacteriophage from pathologic material and objects of the environment.**

**Session Purpose:** Introduction to qualitative and quantitative methods of detection of bacteriophages.

**Objectives**:

1. Isolate bacteriophage from the samples of water and soil with varied metods.
2. Make qualitative analyzing of bacteriophage in liquid and solid nutrient mediums.
3. Make phage-typing of *Staphylococcus aureus.*
3. Performexercises 1-4.

It is known that bacteriophages have parasitic properties that contribute the existence and reproduction only in homologous cultures of microorganisms. On this basis, the presence of the bacteriophage can be regarded as an indirect measure of infection of the test material by relevant microbe.

In practice of Microbiology bacteriophage can be detected when the isolation of microbe culture doesn't give a positive result, for example due to an excessively small amount of microbe in the material or contamination by foreign microflora.

Currently developed various methods for the qualitative detection of phage in the material, which are based on seeding the test material on solid and liquid nutrient media with bacterial strain which ishomologous to the required phage. Microbial cultures, sensitive to bacteriophage, are called the test cultures.

An important condition to the effectiveness of treatment with the phage medication is to identify the sensibility to phage by pathogen.

**Lab Exercise 1.Isolation of bacteriophagefrom the test material.**

**Methodical instructions:**Tested liquid is filtered from germs. The solid material (soil samples, food, feces) is grained in a mortar and emulsified, then filtered through paper, and through a bacterial filter. The presence of phage in obtained filtrate is identified on solid and liquid mediums with the use of test-cultures.

**1a. Isolation of bacteriophage with enrichment method "without seeding."**

This method is based on the principle of creating the most favorable conditions for reproduction.

**Procedure:**

1. The test material is inoculated into a liquid medium, which is optimal for the growth of microorganisms.
2. Cultures are incubated in thermostat with the optimum temperature for 18-20 hours.
3. After this period cultures are filtered through paper, and then with bacterial filter.
4. The filtrates are tested for the presence of bacteriophage on the solid and liquid nutrient media.

**1b. Isolation of bacteriophage with enrichment method with "seeding".**

The essence of this method is the enrichment of the material tested cells **of** the microorganism to which we search the bacteriophage.

**Procedure:**

1.Liquid material investigated directly, and solid material is grained in mortar and seeded in meat-peptone broth or into other liquid medium.
2. Simultaneously with the test material 3 0.5-1 ml 6 hour broth culture or flush with the agar culture bacteria which are homologous to the isolating phage.
3. For the control,bacteria culture is inoculated into a sterile nutrient medium.

4. Seeds are put into a thermostat at 37 C for 18 - 24 hours.
5. After incubation of the test and control tubes,we should take several milliliters of the liquid and filtered through a bacterial filter.
6. The filtrates are tested for the presence of bacteriophage which are homologous to the bacteria, applied for enrichment.

## Lab Exercise 2.Detection of bacteriophage on solid nutrient medium with the Ott's method.

**Methodical instructions:** Beef-extract 1.5%agar is poured into Petri dishes (higher concentration of agar inhibits the growth of negative colonies of bacteriophage).We can put several samples on the one dish. We should divide the bottom of the dish in sectors, and insert in each sector drops of filtrate.

Interpretation of results: evidence of a bacteriophage is the lack of growth in culture filtrate falling drops (active bacteriophage) or the appearance of this small plot of bald spots - colonies of bacteriophage (phage weak activity).Account or the results: presence of bacteriophage is shown by the absence of culture growth where the drops of filtrate(active bacteriophage) were inserted or appearing in this places little sterile spots-colonies of bacteriophage(bacteriophage of weak activity).

**Procedure:**

1. Dishes with agar are inoculated with 3 -6 –hour broth culture or washout with daily agar culture of bacteria , which are homologous to the phage.
2. For deriving continuous growth 2-3 drops of culture is pounded with a pallet over its entire area and accurately distribute the broth culture (approximately 2ml) on the plate surface , the excess is removed by pipette.
3. Then dried for 30-40 minutes at $37^0$C, and cover it with sterile filter paper.
4. On the dried surface of the seeding we must put drops of the test-filtrate.
5. When the liquid is absorbed in the medium , the cup turned upside down and placed in a thermostat at $37^0$C for 18-24 hrs.

## Lab Exercise 3. Titration of bacteriophage on solid nutrient media with the method of agar layers by Gracia.

**Methodical instructions:**After detection of bacteriophage in test-material ,it is necessary to identify it's quantitative content or find phage's titre.

To express the titer of bacteriophage we can use two indicators: the number of active phage particles contained in 1 ml of the test material, or the value of the highest dilution at which exerts its bacteriophage lytic action. The resulting value is expressed with the negative 10 logarithm, where the rate indicates the dilution of phage.

There are several methods of bacteriophage titration,but the most popular is the titration method of phagein liquid nutrient media,offered by Appelman and method of agar layers by Gracia. It is based on inserting various dilutions of titrated bacteriophage in corresponded bacteria culture and seeding of mixture on solid nutrient media with the purpose of deriving negative colonies of bacteiophage.

During the titration with the method of agar layers,seeding should be done no less than two dishes of phage with same dilution.Phage titration is detected by the counting of quantity of negative colonies on parallel dishes and multiplying arifmeticverage on dilution index. For example,seeding of 1ml of phage in $10^5$ dilution we can observe 128 negative colonies on the one dish,and 146 on the secondtitration phage is equal to $1,37*10^{7.}$

**Procedure:**

1. Nutrient Medias are prepared the previous day.25-30 ml of 1.5% of beef-extract agar is poured into Petri dishes. Before the pouring we should add 0,004 % alcohol solution of gentian violet: 0,1 of dye in each 100 ml of beef-extract agar.
2. Dished with medium is covered with sterile blotting paperand dry in thermostat for 30 min,close it with lid and stay for night.
3. Prepared medium must be dried,because even little moisturization can change quantitative index of particles of phage in testes liquid.
4. Beef-extracted 0,7 % agar,poured into tubes for 2,5 ml can be prepared a several days before the experiment.
5. On the day of making an experiment,we should prepare consequent dilutions in tubes.For the dilution of phage it is necessary to use sterile broth or isotonic solution of sodium chloride.
6. After that add 1 ml of dilutions of titrated phage into tubes with 0,7 % of beef-extracted agar,which is melted in water-bath and cooled till 44-46$^0$C.
8. Simultaneously with phage put on each tube 0,1 ml of similar and sensitive to phage culture.
9. Content of tubes is mixed by rolling tubes between the palm,and pour out with 2$^{nd}$ layer into the 1,5% agar dishes.
10. After the cooling of medium,dishes are incubated at 37$^0$C for 18-20hrs.