

A LABORATORY COURSE IN MICROBIOLOGY

International Foundation of Microbiology
729 South Western Avenue
Chicago, Illinois 60612

TO THE STUDENT

You have joined this program to learn, to understand, to acquire skills. To help you achieve these goals, we have compiled this work-study manual. It contains much useful information about germs and how to work with them. It also gives details of tests which are done in hospitals, in industrial laboratories such as food, pharmaceutical and cosmetics, and in sewage and water treatment plants. Your objective during this summer should be to become as skillful as possible in carrying out these tests.

Learning is a systematic process. It is not possible to learn by merely skimming at the surface. Learning is made easier by listening and observing, by understanding and

doing, and finally by discussing and re-understanding.

The manual is written with this view in mind. Not only the details of what you will be doing in the laboratory is given in the manual but you will also be working closely with a laboratory supervisor who will demonstrate and discuss the various techniques.

Things have a way of looking simple when they are explained. Do not let this fact deceive you into believing that you have learned what is being taught. You have not learned it until you can do it and explain it all by yourself. Resolve to become so self-sufficient.

We are hoping that your summer will be a rewarding experience. On the basis of what you will learn here, you should be able to earn the certificate which will be awarded to those individuals who earn a total of 70% or more points in various examinations. The skills and knowledge that you will gain will also be helpful in finding a job as well as in your future educational endeavors.

Orientation

Your instructor will discuss the history and importance of germs. Some of the facts that will be discussed are given in the chapter entitled "Germs - their history and importance." Each student should read this to become more familiar with this subject.

Two or more students will be assigned to one microscope. This is the microscope which you will use throughout the work-study program. You are responsible for keeping it clean and to handle it according to the directions to be given later. For the time being, learn the proper way to carry it.

Each of you will also receive one basket containing the following laboratory supplies:

- Innoculation loop
- Innoculating needle
- Wax marking pencil
- Lens paper
- wash bottle
- A beaker or a small wide mouth bottle
- Dropper bottles with reagents

Staining tray
Oil Immersion in a small dropper bottle
Match box
Racks for holding dropper bottles (two)
Test tube rack
Slide 1 box

The following additional equipment and supplies will be available in each class room:

First-aid kit
Incubator
Sponge and a jar of disinfectant
Separate jars for receiving used pipettes
Separate jars for receiving: (1) stains smears (2) cavity slides and (3) wet mounts

Burners 1 per students (two)
Tripod or ring stands
Paper clip, xylene, paper towels, hand soap, & scouring powder.
Waste baskets for receiving waste other than cultures and media.
Test tube baskets for receiving used media and culture tubes.
(use one basket for one size of tubes)
Containers for receiving used agar plates.
Absorbent cotton, non-absorbent cotton, applicator sticks, swabs.
Empty cans or cups for incubating tubes. These conserve space in the incubator.
Vaseline and cover slips.

Autoclaves will be available in the preparation room.

Your instructor will set up some demonstrations and will show you plates containing colonies of bacteria, fungi and viruses. You will also see a negative (nigrosine) stained smear of material from teeth and a slide of a mold, such as *Aspergillus niger*.

GENERAL RULES FOR THE LABORATORY

1. Each person is required to wear a clean laboratory coat while working in the laboratory. Do not place books, not immediately needed, or coats on the laboratory benches or seats.
2. Wipe the work area of your laboratory bench with disinfectant at the beginning and end of each laboratory session.
3. This procedure will reduce the incidence of accidental contamination of experimental cultures.
4. Wash your hands thoroughly with soap and water at the beginning and end of each laboratory session.
5. Do not lick labels or put pencils or other objects in your mouth.
6. Do not chew gum, eat, drink, or smoke in the laboratory.
7. Do not leave burners, while lit, unattended.
8. Always return all equipment and supplies to their proper places.
9. Discard papers and other uncontaminated materials in the waste baskets.
10. Do not discard tubes, plates, and other glassware containing live microorganisms in the waste baskets. Since these are to be sanitized or sterilized before disposal, place them as per special instructions given by your instructor.
11. Do not place tubes containing cultures, media, or reagents directly on the tables. They must always be kept in an upright position in the test tube racks.
12. Under no circumstances shall the plugs or closures provided on the tubes or flasks be allowed to get wet with the liquid within these containers.
13. Do not let cultures accumulate in the incubator. Promptly discard them, as per required procedure, at the end of each experiment.
14. Place all pipettes after use in the jars containing disinfectant.
15. All contaminated swabs and tongue depressors are similarly discarded in separate jars containing disinfectant.
16. Do not attempt to clean slides after use. Place them in the container provided for them.
17. Do not mix cavity or depression slides with the regular slides. After use, place them in a separate jar containing disinfectant.
18. Unless specifically required, never pipette reagents with your mouth, use dropper equipped with a rubber bulb or a safety bulb.
19. Boiling is not a simple technique. First check if the solution can be heated; it may be combustible. Some viscous solutions will get super-heated if not stirred and then boil over vigorously upon slightest agitation. It is easy to burn ones hands with such solutions. Agar is one such substances. It does not dissolve until boiled. Its solutions are viscous and must be stirred during preparation. Otherwise they will not only get super-heated but agar will also settle down in the container and burn and stick to the bottom. Exercise care.
20. If a tube of bacteria or a petri plate falls on the floor and breaks, do not attempt to sweep it out of the way. Let the instructor show you how to first disinfect the object and the area and remove it for disposal.
21. If some material accidentally gets into your mouth, DO NOT PANIC. Nothing that is used in the laboratory is in any way dangerous. Spit the material out and simply rinse your mouth thoroughly with plenty of tap water and inform your

laboratory instructor.

22. Wear eye protection whenever working with or near hazardous chemicals.
23. Steel reinforced shoes are recommended in the laboratory.

RECORD KEEPING

1. Always use bound note books.
2. Record all information in ink.
3. Start work by writing the date (day and time of the day) and the name of the project.
4. Plan the experiment, and write down its purposes. As the experiment progresses record the results.
5. Never erase any entry. Instead draw a line through it neatly. Make sure that you can still read what has been crossed out.
6. Be specific, clear, and precise while making your entries.
7. Your records must be complete and legible in every detail so that anyone can follow them.
8. Do not crowd your entries. Leave a margin and center your entries. Divide thoughts in paragraphs and separate paragraphs by leaving one line.
9. At the end of the program, hand in your record book. This will be graded with regards to the above points. Total grade for keeping a neat and orderly record book is 100 points.

Laboratory Session # 1 cont.

Laboratory session 1:

Before we proceed with the actual laboratory work, let us get familiar with a few of the laboratory tools. These are:

1. Test tubes and their closures: Your instructor will show you various sizes of test tubes and closures. Test tubes most commonly used are:

Plain tubes:

a. 6x50 ml tubes; b. 13x100 mm tubes; c. 16x150 mm tubes and d. 20x150 mm tubes.

Screw cap tubes:

1. 13x100 and b. 16x125 mm.

Closures most commonly used are:

a. screw caps; b. non-absorbent cotton for plugging tubes; c. test tube closures made of plastic, aluminum, or stainless steel.

Your instructor will show you how to plug test tubes with non-absorbent cotton. Then plug five tubes. Hand these in to your instructor.

2. Laboratory glassware: Study the drawings shown on the following pages and learn the names of various types of glassware. Plug one Erlenmeyer flask and hand it in to your instructor. Use non-absorbent cotton.
3. Petri plates: Your instructor will show you a Petri plate. These are available in three sizes: 10x60 mm, 15x100 mm, and 15x150 mm.
4. Pipettes: Your instructor will show you serological pipettes of various sizes. These are the most popular types. Note that they are graduated to the tip. They are used for dispensing small volumes of liquids. Learn to recognize their capacities and the divisions that they have. This information is printed near the top of the pipette. For instance: 10 in 1/10 ml means that this is 10 ml pipette and each ml, is divided into 1/10 ml.
Your instructor will show you how to plug pipettes. Then you plug two 1.0 ml and 2 10.0 ml pipettes. Place these separately in envelopes and hand these in to your instructor for sterilization. Your instructor will also show you how to remove a sterile pipette from its envelope and how to use it in an aseptic manner. Your instructor will also show you a pipette can and how to properly use it.

Miscellaneous laboratory tools: These are shown in the drawing on the following

Laboratory Session # 1 cont.

page. Your instructor will show you how to use them properly.

Sterility and aseptic techniques:

You have heard about sterility, sterile, sterilization, and you know that these terms mean complete absence of life. You also know that when a surgeon operates, he is required to use sterile instruments and materials.

LABORATORY GLASSWARE.



SQUIBB
FUNNEL



CYLINDER
FUNNEL



SIMPLE
FUNNEL



PEAR-SHAPED
FUNNEL



BUCHNER
FUNNEL



GRADUATED
CYLINDER



PLAIN
CYLINDER



BEAKER



VOLUMETRIC
FLASK

FLASKS



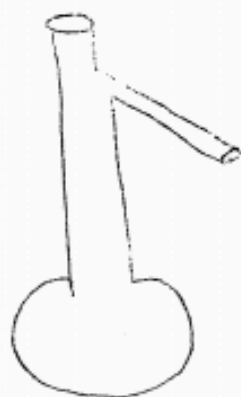
BOILING
FLAT-BOTTOM



BOILING
ROUND-BOTTOM



BOILING
SHORT NECK



DISTILLING

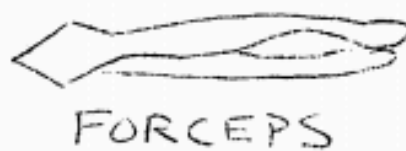
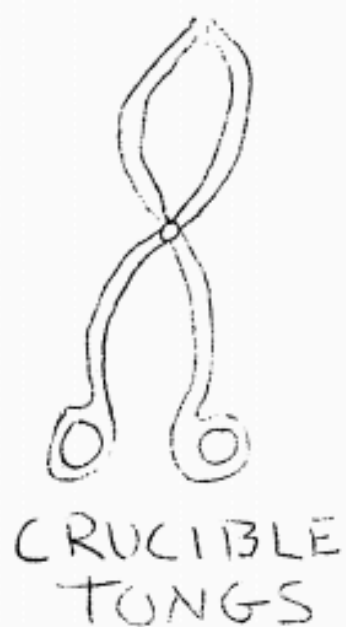
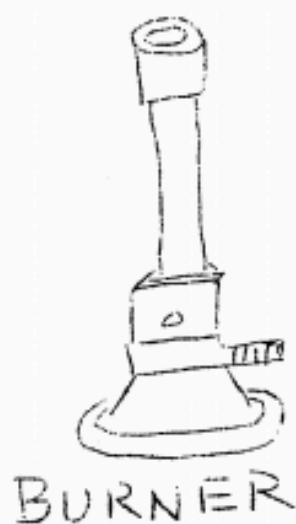
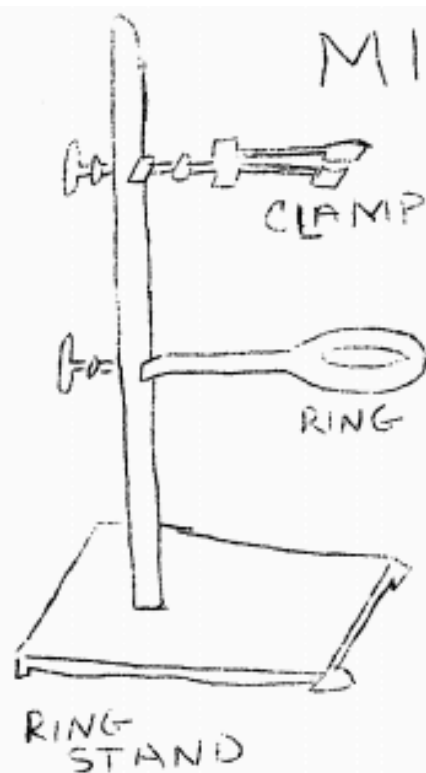


FILTRATION



ERLENMEYER

MISCELLANEOUS



Laboratory Session # 1 cont.

When a nurse makes an injection she is required to use a sterile syringe and needle, and of course, the medication with which the patient is injected must also be sterile. When a microbiologist works in a laboratory, he is required to use sterile media, sterile tubes, and glasswares etc.

For sterilization heat is mostly used in one form or another, for example, if we heat a metallic object red hot in an open flame it will become sterile. Also if we place glassware and other materials not destroyed by heat in an oven such as we have in the kitchen and heat these objects to 160° C for 1 hour or 180° C for 1/2 hour then these objects become sterile. Heat can also be used as moist heat such as when we boil an object in boiling water. This is not a good method, however, because it takes a long time to sterilize things this way. However, we can use moist heat as steam under pressure such as in a pressure cooker at home. Here as the steam accumulates inside the cooker it exerts pressure. Water therefore, fails to boil at 100° C and the temperature within the cooker begins to rise so much that at 15 pounds of pressure the temperature inside the cooker reaches 121° C. At this temperature objects, providing that they are not too bulky, become sterile within 15-20 minutes. This is the most useful method of sterilization and is used in hospitals and laboratories. Home pressure cookers, however, are too small for such purposes and large vessels called autoclaves are used instead.

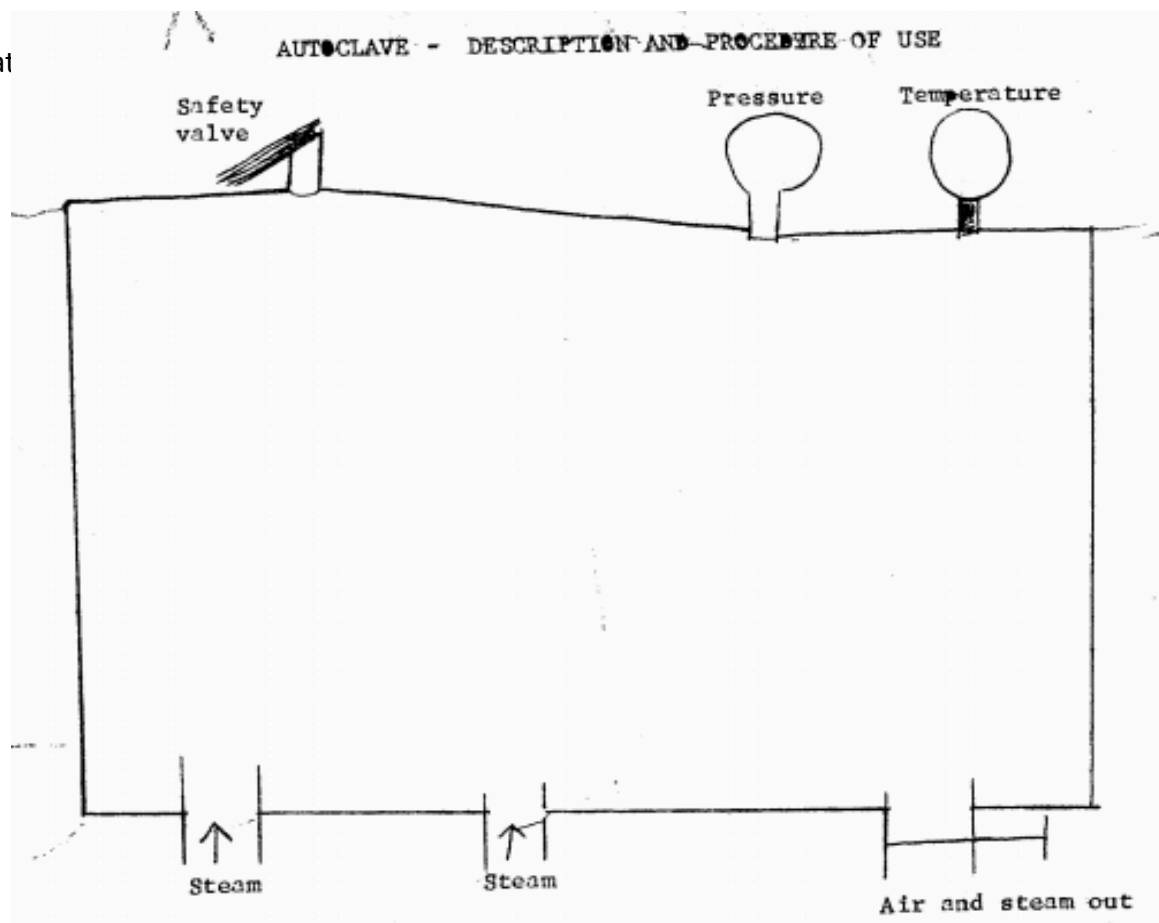
Your instructor will show how to use an autoclave using the diagram on the next page.

Chemicals are used for sterilizing those objects which cannot withstand heat. Liquids which do not withstand heat are sterilized by filtration. Viruses, however, pass through such filters. Liquids so sterilized should thus be regarded as bacteria free, but not sterile.

The requirements for sterilizing various objects are easy to fulfill. What is difficult, however, is to insure that they will remain sterile after they have been sterilized. This is a long problem because microorganisms of various kinds are present not only in the environment, such as air, but also on the persons. Being invisible, they get in without us ever knowing it. We must, therefore, take certain precautions. These are:

- 1) Place the objects to be sterilized in container.
- 2) Close the containers in a way that the microorganisms cannot get in.
- 3) Wrap the objects in paper or cloth. Again, in such a way that microorganisms cannot get in.

This sounds easy enough, but it is not so. For instance, microorganisms can eventually pass through certain kinds of paper, and if staples are used, then the holes left by the staple can aid in the entry of the organism. Moisture further complicates the problem of keeping microorganism out. It is also easy to contaminate objects while using them. One must, therefore, always be watchful and:



Procedure:

1. Place in the chamber material which is to be sterilized.
2. Close door.
3. Leave air and steam outlet open.
4. Allow steam to enter chamber.
5. When steam begins to come out at the air and steam outlet close this outlet. Now the chamber will begin to fill with steam and since there is no air left inside, uniform temperature will be obtained for the sterilization of the object.
6. Watch the pressure and the temperature gauges. As the pressure reaches 15-17 lbs. The temperature will also slowly rise to 248° F (121° C) which is the sterilizing temperature. At or slightly over 17 lbs. of temperature, the safety valve will open and will allow the excess steam to escape. It will close back at 15 lbs. of pressure.
7. When the sterilizing temperature 248° F (121° C) has been reached, note the time. Wait 15 minutes for the sterilization to be completed.
8. Now shut off the steam. Do not attempt to open the door of the sterilizer. Also do not open the air and steam outlet valve because if you did the steam will rush out but the temperature inside the chamber will remain at or near 248° F. At this temperature without the pressure the liquids will boil over.
9. As you shut off the steam, the chamber will cool, and the steam will condense back to water. Pressure and the temperature will, therefore, drop. When the pressure has dropped down to zero and the temperature to below 212° F (100° C) then open the air-steam outlet valve. Then standing aside carefully open the

Laboratory Session # 3 cont.

door and using gloves remove the objects from the chamber.

NOTE: The above is the standard procedure for sterilizing liquids. If dry goods such as glassware are to be sterilized, use the above procedure up to step 7. Then shut off the steam. Since there is no danger of boiling over in the case of sterilizing glassware you should partly open the air-steam outlet valve and let steam escape. This procedure prevents the steam from condensing over the glass-ware. These, therefore, come out fairly dry.

Laboratory Session # 1 cont.

- 1) Sterilize everything thoroughly
- 2) Wrap everything before sterilization, and do so keeping in mind the minute invisible microorganisms which are bound to get in if you fail to block their way, and
- 3) Handle objectives while using them with caution, otherwise they will get contaminated while being used.

In the exercise for today we will see how much contamination one can get by simply transferring liquid from one container to another.

Materials:

2 tubes of Thioglycollate broth per student

Sterile 1-3 ml, syringe with needle and 11.0 ml sterile pipette per student

1 tube sterile Trypticare soy broth (TS broth), 5 ml/ tube

Procedure:

Your instructor will demonstrate how to obtain the pipette from its container and how to use it to transfer both from its tube to the Thioglycollate broth tube.

After the demonstration, use the pipette and transfer 1 ml. quantities of the TS broth to each of the tubes of Thioglycollate broth. Then use the syringe and transfer 1/2 ml. quantities of the glucose solution to each of the remaining thioglycollate broth tubes.

Incubate all these tubes at 37° C. until the next laboratory session. Also calculate the TS broth tube. Then see if they have remained clear or have become turbid. Presence of turbidity means that the organisms had entered into the tubes when the TS broth was being added to them. These had thus gotten contaminated and are now showing growth. The tubes which did not get contaminated will remain clear. If the TS broth is now showing turbidity it has somehow gotten contaminated also. Only if you are careful will all the tubes remain clear. So be careful today and always.

Laboratory Session # 2 cont.

USE OF THE LIGHT MICROSCOPE

Laboratory work assignment:

1. Obtain a light microscope and carry it to your desk.
2. Your instructor will identify its parts and show you how to operate these. Simultaneously you locate the parts on the light microscope and learn to operate them. The various parts of the microscope and their functions are listed below. Your instructor will further elaborate on them.

Parts of the microscope	Function
1. Base	1. To support and align the optical systems.
2. Condenser	2. To: focus rays of light on the specimen.
3. Iris diaphragm	3. To increase or decrease the effective size of the condenser lens. Closing it also cuts down the light and reduces glare. For best results with oil immersion objective, it should be all the way open.
4. Illuminant	4. Source of light
5. Stage	5. For supporting the slide containing the specimen. Specimen side must always face up.
6. Mechanical stage	6. To move the slide back and forth for conveniently examining it. Some microscopes do not have this convenience. In such cases a set of clips holds the slide and the slide is moved manually.
7. Coarse adjustment	7. For focusing. Generally used to bring the object in view and then focused with the fine adjustment.
8. Fine adjustment	8. See 7 above
9. Revolving nose piece	9. Holds objective lenses. Since it can be revolved, it enables one to view the object with different lenses conveniently.
10. Objective lenses	10. Magnify the object
Scanning lens magnifies	4X
Lower power magnifies	10X
High dry magnifies	40-43X
Oil immersion magnifies	97-100X
11. Eye piece	11. Magnifies the image formed by the objective
12. Immersion oil	12. See figure on the following page. Oil immersion objective examines a very small part of the object. It also operates very close to the object. Without oil, rays of light coming through the object will sharply bend away and thus will not be collected by the objective lens. A drop of oil

Laboratory Session # 3 cont.

placed between the object and the lens prevents this bending of the light. Use oil only with the oil immersion lens.

Once you are familiar with the light microscope your instructor will demonstrate how to focus it on a stained smear using the low, high dry, and the oil immersion objectives. After the demonstration you examine the stained smear yourself under the low, high dry, and the oil immersion objectives. The procedure is as follows:

1. Obtain the stained slide of mixed bacteria. Locate the smear; it is generally in the center of the slide. Position the low power (10X) objective for viewing. Place the slide on the stage so that the smear is in the path of the light coming from the condenser and is facing towards the objective lens. Check that the iris diaphragm is open and the condenser is raised all the way up.
2. Focus the stained smear under the low power (10X). Note the appearance of the smear under this magnification (100X). You will most likely see a pattern of fine dots and dashes. Each one of the dots or dashes is a bacterium. See how tiny they are; you have magnified them 100 times already.
3. Without changing the focus rotate the nose piece and bring the 40X or 43X objective in line. Adjust focus with the fine adjustment if needed and note the appearance of the preparation under this. They are bigger and clearer, but still see how small they appear. You have magnified them 430 times.
4. Partially rotate the nose piece and bring the oil immersion (97X or 100X) objective half way toward the object. Place a drop of immersion oil on the slide, directly over the area being examined. Now carefully bring the oil immersion lens over the object. Adjust the focus with the fine adjustment if needed and note the appearance of the preparation under this magnification (970X or 1000X).

5. Move into an area of the smear which is not crowded. Note the shape of bacteria. Even after magnifying 970X to 1000X (under the oil immersion lens) they still appear as tiny dots or rods. Each dot or rod is a bacterium. The dot shaped spherical organism is called a coccus. The rod shaped organism is called a bacillus.

Next your instructor will demonstrate how to focus the smear directly under the oil immersion lens and how to remove the oil from the slide afterwards. Then you practice and master this technique. The procedure is:

With the oil immersion lens a sufficient distance away from the slide, rotate the fine adjustment toward the slide as far as it will go. While looking from the side carefully bring the oil immersion objective in contact with the oil until it almost touches the slide. Focus with the fine adjustment away from the specimen until you see the image. Adjust further for sharp focus.

Laboratory Session # 3 cont.

NOTE: Since the oil immersion lens when in focus is only 0.13 mm away from the object, care is necessary in its use so as to avoid damage to the oil immersion lens. This can be assured by always focusing upward away from the slide.

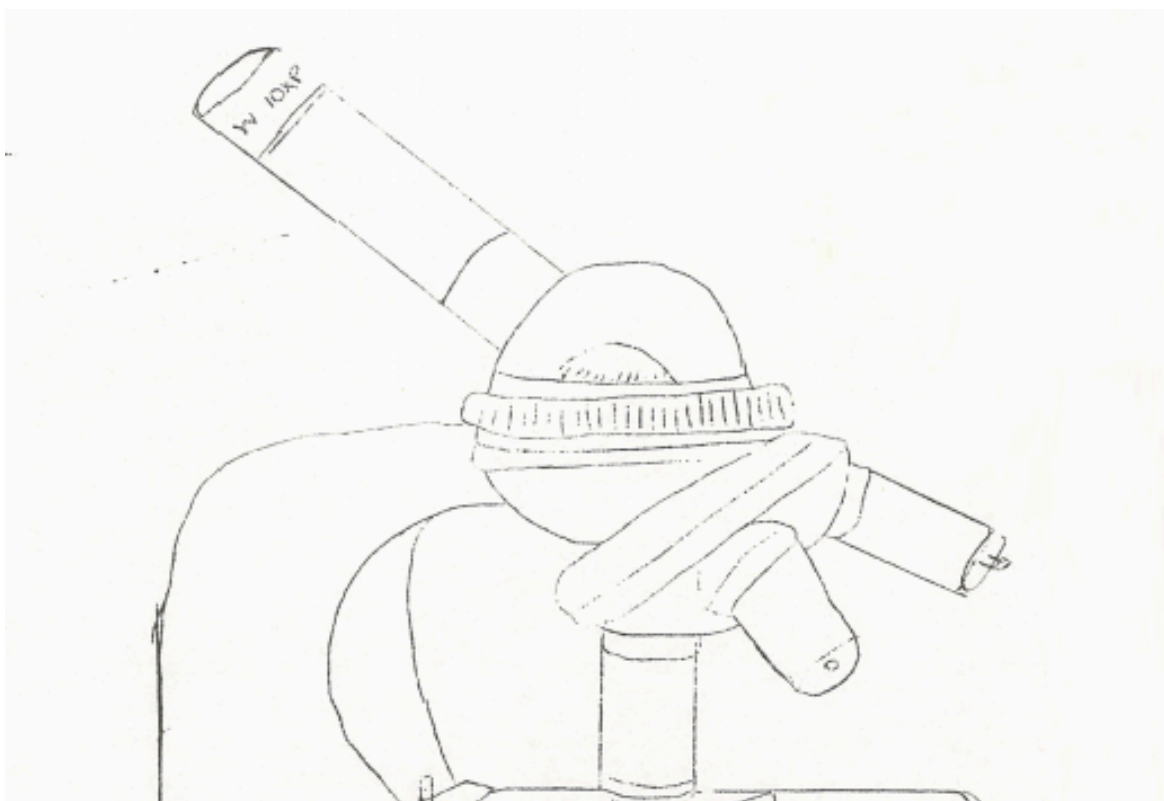
Remove the slide from the stage. Remove oil from the slide. To do so, fold a lens paper lengthwise as a narrow strip approximately equal to the width of the slide. Dip one end of this strip in xylene. Place the strip flat on the slide and pull it across a few times.

What could happen if you place the slide upside down (smear side away from the objective) on the stage and tried to focus it with the low, high dry, and the oil immersion objectives. Try and find out. Under which of the three objectives were you able to focus the smear placed upside down on the stage?

Identify each parts of the microscope by writing its name next to it. Do this as your instructor is explaining the microscope.

SPECIAL ASSIGNMENT FOR LAB SESSION# 2

Your instructor will allow you time to read the following pages and a written examination will follow.



GERMS: THEIR DISCOVERY AND IMPORTANCE

You have heard of germs. They are very small living things, so small that we cannot see them unless we use a microscope.

Man learned about germs in 1674. It was all on account of a curious person named Anton Van Leeuwenhoek (1632-1723). He used to work in a fabric store in Delft, Holland and used to use magnifying lenses to test the quality of fabrics by counting the number of threads that they contained per square inch. He was quite fascinated by the details of the weave that he could see with his lenses. As a hobby he started to make better and better lenses and then with these he examined not only the fabrics but also many other things such as saliva, rain water, and material scraped from his teeth. He was astounded at what he saw. These samples contained little objects which were moving about. He thought that these were some form of very tiny animals and called them "little animalcules."

Leeuwenhoek wrote about his little animalcules to the Royal Society of London and became quite famous but no one attached any significance to his little animalcules. After all they were so very small and looked so very insignificant what purpose can they serve! Now we know different but for almost 200 years after their discovery they were regarded as nothing more than objects of curiosity.

How did we come to realize their importance is a story in itself. It all began around 1860 when someone asked Louis Pasteur (1822-1895), who was a chemist, if he knew why sometimes the wine goes sour and what can be done to prevent it from souring. Pasteur examined both the good wine and the bad (sour) wine and found that good wine always contained one type of yeast (a germ) while the bad wine contained other types of germs. He figured out that it was the yeast which converted the sugar of the fruit juices to alcohol and vinegar. Some other types of germs spoiled the juice as they changed it to many other products. Pasteur then suggested that if one wants to produce good wine all the time, one should heat the juice to destroy the germs that it contained and then after cooling the juice add to it a bit of good wine which contained the yeast. This process of heating to destroy the harmful germs is now called pasteurization and is also used to destroy the harmful germs which get into milk during and after milking.

Laboratory Session # 3 cont.

What Pasteur discovered about wine making was very important but still it was hard to imagine that germs could have anything whatsoever to do with making people, animals and plants sick. A bit of tragedy, however, showed the way. In 1865, the silk industry of France was almost destroyed by a disease called Pebrine and Pasteur was asked by the Government of France to see if something could be done to save it. Pasteur demonstrated that the disease was caused by a Protozoan (a type of germ) which infects the larvae of the silk worm and if one carefully separated the healthy larvae from the sick ones by examining them under the microscope, the disease could be eliminated. This was done and the silk industry was saved. From then on, germs were no longer regarded as unimportant. Later, Pasteur along with Robert Koch (1834-1910) showed that Anthrax, a disease of animals and man was caused by a bacterium (another type of germ). The importance of germs was thus further established and the study of germs and their effects became a science. This was around 1880. This is the science of Microbiology and we owe its origin to Pasteur.

Pasteur, during his many years of research made numerous other discoveries. He showed, for instance, that chicken cholera was caused by a bacterium and he also discovered how to make vaccines and make people and animals immune to various diseases. This he discovered quite accidentally but under circumstances which were otherwise quite embarrassing. In order to show that chicken cholera was caused by the bacterium that he had isolated, he had arranged to inject a number of chickens with the organisms in front of the public so all could see. Unfortunately, he did not have a freshly isolated culture, so he used an old one. To his embarrassment the chickens injected with this culture did not die. He thought about it and figured that the failure was because he used the old culture. Experiments that he subsequently did in his laboratory proved him right. So he arranged for another public demonstration and this time he made certain to use a freshly isolated culture but this time he did not have enough new chickens so he included in the experiment some of the chickens which were previously injected with the old culture. To Pasteur's surprise, the culture killed the new chickens but the chickens which had previously received an injection of the old culture did not die.,,

Laboratory Session # 2 cont.

Pasteur argued that the old culture somehow not only had lost its ability to produce disease but it also made the animals immune. By putting these findings to experimental test, Pasteur was able to develop vaccines against chicken cholera, anthrax and rabies. Since rabies is a disease caused by a virus, Pasteur is also regarded as the father of the science of Immunology and Virology. Immunology deals with the mechanisms by which the body protects itself from disease and from foreign materials. Virology deals with the study of viruses.

Just about this time, another scientist entered the picture. His name was Robert Koch (1834-1910). He was a physician who concentrated on developing methods to isolate and study the various germs. Such methods were necessary to answer a very important question. The problem was that almost any sample which was studied in the laboratory, whether it came from healthy or sick persons contained germs and the question which had to be answered was whether all or some of the germs produced disease or whether they had to be present in a certain combination or ratio and only then they could produce disease.

Koch reasoned that to answer this question one will have to isolate each type of germ and then see if it alone or together with others produced disease. He and his students developed methods to isolate pure culture and also introduced methods to stain bacteria because without staining bacteria, it is just too difficult to see them under microscope as they are quite transparent. Equipped with these methods Koch turned his attention to the study of tuberculosis. It was a major disease in those days and took quite a toll. He isolated the organism which caused this disease and published instructions as to how one can protect oneself from catching tuberculosis. This is one of Koch's major contribution to microbiology.

Once the methods of isolation and cultivation of microorganisms became available, the science of microbiology developed very rapidly. Soon it became apparent that organisms even smaller than bacteria existed and that some of these also produced diseases. These are viruses. The credit of showing that they produced certain diseases goes to Iwanowski who in 1892 showed that the causative agent of mosaic disease of tobacco was a virus and to Loeffler and Frosch who in 1897 showed that the cause of the foot and mouth disease was also a virus. The science of virology was thus founded. It was thought that perhaps they cause many other disease but isolating and growing viruses was not an easy matter. Viruses are strict parasites and grow only within cells. The progress in this area of science was thus slow. A good

method of growing viruses was developed by Woodruff and Good Pasteur, in 1930. This inoculating the sample containing the viruses in embryos of chickens. Later a much better method became available. This is the tissue culture method. It involves growing viruses in cells which are grown in flasks or bottles. This method was developed by Enders, Waller and Robbins and for which they were awarded the Nobel Prize in 1954. It was the tissue culture method which enable the produced of Polio vaccine and thus bring a crippling disease under control.

Laboratory Session # 3 cont.

In 1909, Howard Ricketts discovered another group of germs. These are called Rickettsiae. They were found in the blood of persons suffering from Rocky Mountain Spotted Fever. These organisms are also strict parasites and grow within cells. Dr. Ricketts died working with these organisms. He was at the University of Chicago. Rickett Hall at the University of Chicago is named after him.

Laboratory Session # 2 cont.

Another type of microorganisms called PPLO (short for Pleuropneumonia-like organisms) were discovered by Kleiberger in 1935. These appear to have evolved from bacteria and are now considered to be responsible for causing certain respiratory disease.

The discoveries which established that many diseases were produced by germs brought about many changes in man's way of life. Vaccination against certain disease became a necessity. Milk which previously transmitted diseases like tuberculosis and strep throat began to be pasteurized and thus made safe. Water for drinking and bathing was likewise made safe by filtration and treatment with chlorine which killed germs. Laws were passed which required that food of all types must be free of those germs which produce diseases via mouth. Similarly all medicine which are to be injected must be sterile. Aseptic way of surgery and delivery were introduced. These procedures were made safe. All in all these changes made it possible for mankind to live longer and healthier lives. These changes also brought about unprecedented need for newer products, and services. They thus simultaneously resulted in economic growth within these countries which availed of the newer knowledge of microbiology. Food, pharmaceutical, cosmetics, and toilet goods industries grew into billion dollar enterprises and brought forth increased employment. In 1970, the processed food industry in the United State of America alone produced goods worth 65 billion dollars. It employed, 1,639,000 employees with a payroll of \$ 11,711,000,000. Pharmaceutical and toilet goods industries accounted for an additional \$ 6,792,000,000 and 8,183,000,000 worth products respectively. None of these industries could have achieved such a growth without microbiology knowledge. Similar economic growth can be brought about the world over by making the public aware of microbiological principles. The developing countries especially stand to benefit from such an approach.

So far we have considered only the disease producing activities of germs and what man has been able to accomplish by understanding how to control the disease producing organisms. Disease production, however, is not the main function of the germs. Disease in fact is an unfortunate side effect which results when two or more living organisms such as certain germs and an animal come at odds with each other and the germs by virtue of their life activities cannot help but win over the animal. This is an un-economical situation for both. When the animal dies as a result of the disease so do the germs which produced the disease. The disease producing germs thus slowly get eliminated. Now, over the years, only those types of germs have survived which rarely if ever produce disease. Presently, out of a total of 200,000 known species of germs only 200 or so produce disease and even most of these do so only under special circumstances. The rest of the organisms are very useful. Most of these live free in nature and perform many important functions. Of these, the two most important ones are:

1. Certain germs such as Rhizobium change nitrogen air into chemicals which are needed by plants as their food. Without this activity of germs the plants, as we

Laboratory Session # 3 cont.

known them will not be able to grow and since the animal depend on plants for their food, animals and so also we as humans will not be able to survive.

Laboratory Session # 2 cont.

2. Most germs decompose the dead bodies of plants and animals and thus recycle the wastes. Without this natural process of recycling the resources of our earth will be soon exhausted and life as we know it will come to an end.

The role of the microbiologists of the future thus is to preserve and not to destroy germs. Germs in fact are the key elements which maintain the balance of nature. Destroy them and you endanger life. Destroy them and you stop the recycling process. Upset nature's checks and balances and you create chaos. Man's future rests on understanding and protecting these balances. Your understanding of microbiology is, therefore, vital to man's future. Think of microbiology with this view point.

From the above one should not conclude that man should become passive and abandon all exploration of nature. The human race as a whole barring catastrophe and despite measures of population control, will go on increasing in number. This will necessitate learning from and exploring nature for newer sources of energy, food and raw materials. This process of growth, of search and of advancement, therefore, cannot and should not be stopped because without it we are sure to push our future generations into the Valley of Darkness from which it took millions of years for man to emerge. With it there is hope but it will demand newer skills-skills in disposing wastes, in preserving the wholesomeness of the environment and in finding new sources of food. Knowledge of microbiology is playing a key role in these areas now and will continue to do so in the future.

You can be a contributing part of that future by learning and understanding microbiology.

Laboratory Session # 3 cont.

Reading Assignment for Lab. Session # 3:

WHAT ARE MICROORGANISMS: PLANTS OR ANIMALS?

When Leeuwenhoek saw microorganism he called them "little animals." Was he right, or was he looking at "little Plants?" In order to settle this question all types of organisms including plants and animals were studied to see if the microorganisms resembled plants or animals. Surprisingly, microorganisms turned out to be both plant like and animal like and, therefore, could neither be regarded as plants nor animals.

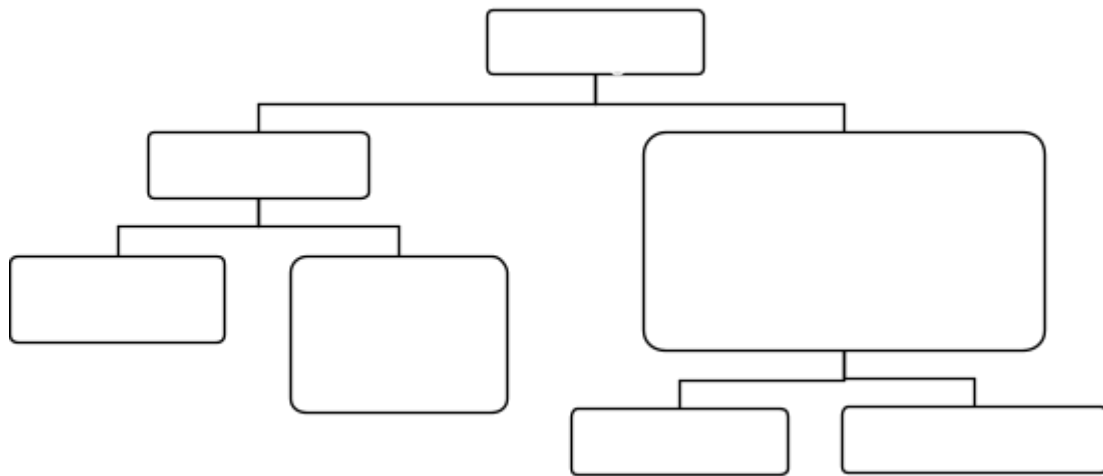
There started a controversy which lingered on for quite a while and which in a way persists until today. In 1886, however, E.H. Haeckel, a German zoologist suggested a solution to this controversy which is now more and more being accepted. He placed all microorganisms in a new kingdom and called it the kingdom PROTISTA which means the kingdom of primitive organisms. This kingdom thus includes not just the microorganisms, but all primitive organisms. Many organisms which were previously placed in the plant or the animal kingdom but which nevertheless primitive, were also placed in the kingdom Protista. Removing the primitive organisms from the plant and the animal kingdom also allowed Haeckel to redefine plant and animal kingdoms.

The decision as to which organisms should be called primitive was based on the number and type of cells that an organism was made of. Thus any organism which was made of one type of cells was regarded as a primitive organism. Such organisms could be unicellular (consisting of one cell) or multicellular (consisting of more than one cell) but all of its cells should be alike.

A non-primitive or an advanced organism would thus be the one which had more than one cell, and in which all cells were not alike. In such organism, one type of cells are grouped together to form tissues and a number of tissues are brought together to form an organ which performs one or more specialized functions. This is the case with animals and plants. They have specialized organs such as heart, liver, kidneys and roots and leaves which perform specialized functions. Such organisms are placed in the Animal or Plant kingdom.

If we summarize Haeckel's way of classifying living things we will have the following picture:

Laboratory Session # 3 cont.



Although only primitive organisms are placed in the kingdom protista, these organisms do show different degrees of primitive characteristics. For instance, cells of some organisms are not any different from the cells of plants or animals, i.e. they have a nucleus which is enclosed in a membrane. Such cells are called EUCARYOTIC and the organisms which have this type of cells are placed in the sub kingdom Higher Protista. Cells of some organisms on the other hand have a naked nucleus, i.e. it is

Laboratory Session # 3 cont.

not enclosed in a nucleus membrane. In fact, it appears to be pretty much a part of the cytoplasm. Such cells are called PROKARYOTIC and the organisms which have this type of cells are placed in the sub-kingdom Lower Protista.

PROTISTA KINGDOM	
Lower Protista	Higher Protista
Cells are simple; the nucleus is diffused in the cytoplasm; the presence of a membrane around the diffused nucleus has not been demonstrated and is considered absent. Groups of organisms included here are:	Cells are similar to the cells of plants or animals, i.e. they have a well developed nucleus which is enclosed in a membrane. Groups of organisms , included here are:
1. Actinomycetes	1. Algae (except blue-green algae)
2. Blue-green Algae	2. Protozoa
3. Bacteria	3. Fungi a) Molds b) Yeasts
4. Mycoplasma a) Pleuropneumonia-like organisms (PPLO)	4. Slime molds.
5. Rickettsia	
6. Viruses	

Which of the organisms included in the kingdom protista should be considered microorganisms? From the standpoint of definition, only organisms too small to be seen by naked eye should be regarded as microorganisms. But this definition is occasionally stretched and all organisms belonging to the kingdom protista are considered microorganisms. Some of the seaweed; for instance, are very large as they grow to many feet. Even these are regarded as microorganisms. From this standpoint microorganisms are defined as primitive organisms mostly too small to be visible to the naked eye.

Class discussion:

You have isolated an organism which you found growing on the bark of a tree. How would you know if it was a lower or higher protista? When can you say that you are dealing with a primitive organism?

Laboratory Session # 3 cont.

Check the tubes of Thioglycollate broth which you had incubated during the last laboratory session. Are they still clear? If so, then you did not contaminate them while transferring sterile glucose solution into them. If they are turbid, then contamination had occurred. The organisms which entered into the tubes have now grown in number to produce the cloudiness in the broth. You have to be a lot more careful to avoid the entry of the organisms.

A Survey of Major Groups of Microorganisms

We have established that any organism too small to be seen by the unaided human eye is a microorganism. The human eye is not able to see objects smaller than 100 micron (0.01 mm or 0.004 in.). From the standpoint of size, therefore, any organism smaller than this dimension will be regarded as a microorganism. We have also established that certain larger organisms such as certain fungi (mushrooms) and certain algae (seaweed) because of simplicity of their structure are also regarded as microorganisms.

Microorganisms are, therefore, defined as simple, unicellular or multicellular living organisms, generally too small to be seen by the naked eye.

The number and variety of microorganisms that one finds on this earth is truly enormous. They represent the single most numerous form of life. Both soil and the bodies of water are richly endowed with them. A single gram of soil, for instance, contains as many as twenty billion bacteria plus a host of protozoa, algae, and fungi and this at best is an underestimation. So far approximately 200,000 species of microorganisms have been recognized. This also represents only a partial picture because many organisms are yet to be isolated and studied. The organisms so far known are placed in the following 10 groups:

- Algae
- Protozoa
- Fungi (molds and yeasts)
- Slime molds
- Actinomycetes
- Blue-green algae
- Bacteria
- Mycoplasma (PPLO and L-forms)
- Rickettsia
- Viruses

In this exercise our aim will be to learn to recognize their major features. For each group of organisms as far as the availability of live or prepared material or of audio-visual aids will permit, we will observe the form as seen through a microscope and the size, shape, and color of the colony as seen by the naked eye.

Laboratory Session # 3 cont.

In order to further assist you in forming a mental picture of each of the major groups of microorganisms a brief description of each of the groups is given at the end of this exercise. Your instructor will further elaborate on this description and if possible will supplement it with films, slides, pictures, or drawings.

Materials:

Algae

Cultures and/or prepared slides and preserved specimens of algae such as spirogyra and volvox.

Protozoa

Cultures and/or prepared slides and preserved specimens of protozoa such as paramecium and Amoeba.

Fungi

Prepared slides and preserved specimens of molds such as Penicillium, Rhizopus, and Aspergillus and yeasts such as Saccharomyces Cerevisiae (baking yeast). Also plates showing colonies of these organisms.

Slime molds

Preserved specimens of slime molds or a culture of Physarum Polycephalum.

Actinomycetes

Cultures and/or prepared slides and preserved specimens of actinomycetes such as Streptomyces species.

Blue-green algae

Cultures and/or prepared slides and preserved specimens of blue-green algae such as oscillatoria and nostoc.

Bacteria

Prepared slides of Escherichia coli, Streptococcus Pyogenes or Staphylococcus aureus, Bacillus subtilis, Spirillum serpens, or Rhodospirillum Rubrum. Also plates of cultures showing isolated colonies of bacteria such as E. Coli and Bacillus subtilis.

Mycoplasma

Preserved specimens of PPLO or L-form colonies

Rickettsiae

Photomicrograph of Rickettsiae

Viruses

Tissue culture bottles or plates showing plaques due to viruses or bacterial cultures showing plaques due to bacteriophage

Laboratory work assignment:

The laboratory work will depend upon the availability of material. If at all possible examine members of all the 10 groups of microorganisms. In any event members of 3 groups, namely fungi, bacteria, and viruses must be examined.

Algae, Protozoa

You will be provided with pond water. Your instructor will demonstrate how to prepare a wet mount. You will then prepare a wet mount of the pond water and examine under the 10X objective. Note the Eucaryotic nature of the cell. Partially

Laboratory Session # 3 cont.

close the iris diaphragm and search for Protozoa and Algae. Consult chart for identifying certain forms, where feasible, note.

Slime Mold.

Your instructor will demonstrate how to prepare a wet mount. You will then prepare a wet mount of the pond water. Observe slime mold culture under the 10X objective and note the streaming protoplasm and reversal of the direction of streaming. Examine the plates showing colonies of molds. Note that they are cotton-like and fluffy in nature and are of various colors. The color are due to spores. Examine the prepared slides of fungi under the 10X objective and note that the fungus appears as a filamentous structure in which the filaments are intertwined. Each of the filaments is called a hypha (plural hyphae) and collectively the hyphae are called the mycelium. The entire body of the fungus, i. e. the mycelium and the spores and root-like structures if any (called Rhizoids) is called the thallus. Note if the filaments are septate or aseptate, i.e. if the filaments are divided into cells.

Next look at the spores and the spore bearing structures. In *Rhizopus* you will note a ball-like structure called the sporangium which contains the spores. This structure usually ruptures and you will see spores either free or some still attached to the sporangium. Spores are round refractile ball-like structures. The hyphae which bears the sporangium is called the sporangiospore.

In *Aspergillus* you will note that some of the spores are free but some are still attached to the spore bearing body. Here the spores are called conidia and they are borne on special structure which originate from a hyphae. This hyphae is called a conidiophore. To the conidiophore is attached a ball-like structure called the vesicle and to the vesicle you will see attached small finger-like projections called the sterigmata (use 43X objective to see these in detail). Note that some spores are still attached to the sterigmata.

In the case of *Pencillium*, you will also see conidia but you will note that there is no vesicle. Instead the sterigmats are originating from the hyphae (coniodphore) and bear the conidia.

Note that in each of the 3 molds it was mainly their spores and how they were attached to the hyphae which differed. Shape and arrangement of spores are thus most features for classifying and identifying molds.

Laboratory Session # 3 cont.

Next examine the colonies of yeast. Note that they are not fluffy or cotton-like but they are pastry and quite smooth. Eventually you will learn that they look very much like the colonies of bacteria. The only sure way of telling whether such a colony is a bacterial or yeast colony is to spread a portion of the colony on a slide, stain it and examine it under the microscope. Then you will note that a yeast colony contained large oval cells which had a well developed nucleus (hence Eucaryotic cells) while the bacterial cells are small and had no visible nucleus (hence Prokaryotic cells). Examine a prepared slide of yeast (*Saccharomyces cerevisiae*) under the 43X and the oil immersion objectives and note the shape of the yeast cells.

Actinomycetes:

Examine the nature of growth of the *Streptomyces* colonies. Note that they are hard and almost like cartilage or plastic objects. Also examine the prepared slide and note that the mycelium is present, but if it is of such a small dimension that you will need to examine it under the oil immersion lens.

Laboratory Session # 4 cont.

Bacteria

Examine the plate showing bacterial colonies. Note that some of these can be easily confused with the yeast colonies. Use oil immersion lens for examining the slides of bacteria. After focusing find an area of the specimen which is not crowded. Note if it is a bacillus (elongated) or spherical, or spiral shaped.

NOTE: Some bacteria are so small that their shape may be difficult to determine. These are generally described as cocco-bacillary.

Also note the arrangement of cells. Bacterial cells are arranged as follows:

All bacilli are arranged singly or in chains. The arrangement of bacilli in chains is called the streptobacillus arrangement.

Cocci are arranged in one of the following ways:

- In pairs called the diplococcal arrangement
- In chains called the streptococcal arrangement
- In grape-like clusters called the staphylococcal arrangement
- In groups of four cells each called the tetrad arrangement
- In packets of eight cells each called the sarcina arrangement

Spirals are arranged singly.

Blue-green algae:

Examine under the low, high dry, and oil immersion objectives and note the Procaryotic nature of the cell. There is no defined nucleus. The photosynthetic pigments are contained as granules throughout the cytoplasm. The cytoplasm is also watery.

Mycoplasma:

PPLO or L-forms:

Your instructor will show you a plate or a specimen on which colonies of Mycoplasma (PPLO, or L-form) are growing. The colonies of these organisms are so tiny that you will have to see them under the 10X objective. Your instructor will set this up as a demonstration. Examine the demonstration and note that the colonies look like tiny fried eggs. For this reason the PPLO colonies are described as fried egg type colonies.

Rickettsiae:

Note the small cocco-bacillary shape of these organisms. They are approximately 0.2 micron in size.

Viruses:

Observe the tissue culture or the bacteria culture which has been infected with the virus. Note that there are small clear areas where the cells of bacteria or the tissue have not grown, because these have been destroyed (lysed) by the viruses. The clear area is called a plaque and represents a colony of the virus. Although you do not see anything in this clear area, merely touching this area with a needle is sufficient to pick up a large number of viruses and transfer them to a fresh tissue or a bacterial

Laboratory Session # 3 cont.

culture. Viruses so transferred will infect cells, lyse them, and will produce more plaques.

Laboratory Session # 4 cont.

Description of Various Groups of Microorganisms

(A) Higher Protista

1. Algae: Any microorganisms which is made up of Eucaryotic cells, which has cell wall, and which performs photosynthesis with the production of oxygen is regarded as an algae. Both unicellular and multicellular forms are known. The group represents approximately 54,000 species. All contain chlorophyll or some other pigment and thus are capable of photosynthesis. Their size varies from microscopic to that of huge kelps and other seaweeds which are many feet in length. The natural habitat of algae is both fresh and salt water. They are also found in soil, on rocks, and on tree trunks. Some species are motile. Reproduction is either asexual, sexual or both.

Algae are useful organisms. Besides photosynthesis and replenishing oxygen they are also involved in soil fertility by fixing nitrogen, i.e. they convert atmospheric nitrogen to nitrogenous compounds used by plants as food. Algae also serve as food for man. Presently the large sea kelps and miscellaneous other sea algae are so used. In the future it may become necessary to use smaller algae such as chlorella as food for man. None is known to be pathogenic to man. Some are pathogenic to plants, though. Certain algae, however, are poisonous. These, when eaten by shellfish, make the latter extremely poisonous for man and animals,

The classification of algae is not entirely agreed upon, but they are generally placed in ten classes. The classification is mainly based on their mode of reproduction, the nature of photosynthetic pigments, and the type of products synthesized and accumulated in the cells.

Not all algae consist of Eucaryotic cells. Some algae placed in the class cyanophyceae consist of Procaryotic cells. These are called Blue-green algae and are regarded to be closer to bacteria than to algae. The reason that the Blue-green algae are not grouped with the photosynthetic bacteria is that their photosynthetic mechanism is closer to algae and to plants than to bacteria. Bacteria do not produce oxygen as a result of their photosynthetic mechanism while blue-green algae and plants do.

2. Protozoa: Any organism which is made up of a Eucaryotic cell in which the cells do not have a cell wall which also lacks chlorophyll and thus is not capable of photosynthesis is regarded as a Protozoan. These are thus single celled Eucaryotic organisms which do not contain chlorophyll and cell wall. Some 35,000 species of protozoa are known. Most of them are free-living organisms and abound in both salt and fresh water. Some species are found in soil. The top six inches of soil is particularly rich with protozoa. A few species are parasitic. i.e. they do not live freely, but parasitize both lower and higher animals and occasionally produce both acute and chronic disease. Many protozoa in the face of changing physiological conditions of their environment become encysted. The cysts are resistant to dessication and thus are important for the survival of the

Laboratory Session # 3 cont.

protozoa. Cysts also play an important role in the transmission of protozoal infections.

Since protozoa are non-photosynthetic, their food consists of organic matter dissolved or suspended in water. The main diet of protozoa, however, is smaller living organisms such as bacteria and algae.

From a medical standpoint the important protozoa are those which parasitize man and domesticated animals. We have limited knowledge of these protozoa because we have not been able to grow them on artificial media and study their physiology to any great extent. We also seem to be fighting a losing battle as far as treating the maladies caused by them is concerned. As a consequence parasitic diseases are increasing. Malaria alone takes a death toll of approximately two million annually, resulting in at least 200 million cases of the diseases.

The classification of protozoa is based on their mode of locomotion. Accordingly they are placed in the following classes:

Sarcodina: The representative member of this class is amoeba. Members of this class are regarded as naked globs of protoplasm which move by projecting pseudopods (false feet) in the direction of the motion. The parasitic organism belonging to this class is *Entamoeba histolytica* which causes amoebic dysentery. The organism forms cysts which pass in the faeces. Contamination of food with faecal matter through improper washing of hands and through flies results in the transmission of the cysts to other persons.

Mastigophora: Organisms placed in this class are also called flagellates because they possess one or more thread-like structures called flagella. All members are motile by means of these flagella. Some members of this class are pathogenic for man. *Giardia lamblia*, for instance, produces diarrhea mostly in children. The cysts of the organism pass mostly in feces and spread to other persons when the food gets contaminated with the fecal matter. *Trichomonas vaginalis* produces severe urogenital infection mostly in female. The organisms spread by personal contact as it may be found in the urethra of males. *Leishmania donovani* causes kala-azar. The organism spreads mainly by sand flies. *Trypanosoma gambiense* causes African sleeping sickness and is spread by tse-tse flies.

Infusora or ciliata: Members of this class show most elaborate internal structure of the cell. The unicellular organism is surrounded with numerous cilia which are responsible for the sweeping motion of the organism. The common representative of this class is the paramecium. The pathogenic member of this class is *Balantidium coli* which causes dysentery in man.

Sporozoa: All sporozoa are parasites. Members of this class have complex life cycles and pass through different hosts through various stages of their development. Unlike other protozoa the mature organisms are not motile. They also do not ingest food particles, but rather use soluble food material. The important member of this class is the plasmodium which causes malaria. It has a complex life cycle and is transmitted by the mosquito of the genus anopheles. The sexual cycle of the parasite takes place in the mosquito and the asexual cycle takes place in the red blood cells of man.

3. Fungi: Any organism which is made up of Eucaryotic cells in which the cells have a cell wall but do not contain chlorophyll and thus are not capable of photosynthesis is regarded as a fungus. Fungi are a diverse group which includes both unicellular and multicellular organisms. There are approximately 70,000 species of fungi. The group includes such contrasting organisms as molds, mushrooms, and yeasts. Fungi are most abundant in soil where they live mostly as saprophytes, i.e. they live on dead organic matter. They do not engulf solid particles, but rather use dissolved organic matter as food. Some species produce disease in plants while a few are parasitic to man and animals where they produce either superficial or deep (involving internal organs) fungal infections.

Molds are multicellular and consist of ten to twenty micron thick extremely long thread-like filaments called hyphae. These are either septate i.e. divided into cells non-septate (coenocytic). The non-septate hyphae have many nuclei.

Collectively the hyphae from the body of the fungus which is called the

Laboratory Session # 3 cont.

mycelium. Everyone is familiar with at least some molds as they occasionally grow as fluffy cotton-like growth on old bread and vegetables.

Mushrooms do not look like molds, but they are also fungi. The mycelium in their case is not made of loosely bound hyphae, but is a compact structure.

Yeasts are unicellular oval or round shaped fungi. Some yeasts are dimorphic, i.e. they are mold-like at room temperature and yeast-like at 37°C. The natural habitat of yeasts is the surface of flowers and vegetable rather than the soil. Some, however, live in the soil. Some yeasts are parasites and live within the cavities on the body surfaces of man and animals. Yeasts multiply both asexually and sexually. The asexual process is through the formation of buds (Blastospores). These buds enlarge to the size of the mother cell when the nucleus of the parent divides and passes onto the bud. The bud then separates.

Some yeasts multiply by binary fission much like bacteria. Some yeasts reproduce only through asexual mechanisms. These are placed in the class Fungi Imperfecti.

Some yeasts reproduce through sexual means also. This is through fusion of two cells. The resulting sac is called the ascus in which the spores are produced. Such yeasts are classified with the ascomycetes.

Laboratory Session # 4 cont.

Certain yeasts are also placed in the class Basidiomycetes as they reproduce through the formation of basidia from which the basidiospores are violently discharged just like the basidiospores of mushrooms.

Phycomycetes: A number of common molds such as the Rhizopus (the black bread mold) and mucor are placed in this class. The hyphae of these molds are not septate. They reproduce both sexually and asexually. Sexual reproduction is via the formation of zygospores. The asexual spores are called sporangiospores as they are formed in a closed structure called the sporangium. Most phycomycetes are saprophytic organisms. Some are pathogenic for plants.

Basidiomycetes: Mushrooms and other similar organisms are placed in this class. Some smaller forms called smuts and rusts and some yeasts are also included in this class. They are important plant pathogens, but none produce disease in man or animals although some mushrooms are poisonous. The mycelium is compact and fleshy. The hyphae, if present, are septate. They reproduce sexually and the spores are produced on club-like structures called basidia,

Ascomycetes: Common mode of reproduction is asexual and the spores so produced are called conidia. At times they produce sexual spores called ascospores because they develop in a sack-like structure called the ascus. Some members of this class are single-celled yeasts while the others are molds.

Fungi imperfecti or hyphomycetes: No sexual stage is known in these fungi, Reproduction is asexual and different members are recognized on the basis of shape and arrangement of their spores. The fungi pathogenic for man and animals belong to this class. Some yeasts which multiply asexually only are included in this class.

Slime molds (myxomycetes): These are very peculiar organisms and are described as naked multinucleate masses of protoplasm lacking chlorophyll. They are saprophytes living in damp, shady places on decaying wood and leaves. Their body which can be many centimeters in diameter has no particular form and is called a Plasmodium.

There are approximately 400 species of slime molds. As organisms they are intermediate between fungi and protozoa. Their locomotion and feeding mechanism are those of protozoa, i.e., they move by means of pseudopodia and engulf solid particles. Their reproduction is like that of some fungi, i.e., they produce sporangia in which are produced numerous uninucleate spores. The spore walls are made of cellulose and these spores are scattered by the wind when the sporangia rupture.

Laboratory Session # 3 cont.

Laboratory Session # 4 cont.

Special Assignment for lab. session # 4. Your instructor will allow you time to read the following pages and will give you a written learning examination at the end of the allotted time.

Description of Various Groups of Microorganisms (contd).

(A) Lower Protista

Actinomycetes.

Any organism consisting of Procaryotic cells in which the cell size is approximately 1 micron in width and which has a tendency to form elongated hyphae like filaments is regarded as a member of the Actinomycetes group. The structure of this group of organisms is, therefore, very much like that of molds, but the hyphae are only 1 micron rather than 10-20 microns thick. The hyphae like filaments are aseptate. These organisms also have a tendency to break up into smaller fragments which look very much like bacteria. Each of the fragments is capable of growing and giving rise to a colony.

The colonies of actinomycetes are hard almost like a cartilage. They are of various colors such as green, white, brown, blue, yellow and orange.

Most members of the actinomycetes group are found in soil and damp places. The musty odor found in cellars is due to these organisms. Their abundance in soil can be appreciated from the fact that 30% of the organisms isolated from soil are actinomycetes. They are very active, metabolically, and are important vehicles of recycling the dead and decaying organic matter. The group includes the following families.

Mycobacteriaceae: These organisms are much closer to bacteria. They tend to form filaments, but the predominant shape is small rod shaped individual cells. The family does not form spores. The important member of this family is Mycobacterium tuberculosis which causes TB (tuberculosis) in man and animals. :

Actinomycetaceae: Here the organism forms long filamentous hyphae. These bear spores which originate by the fragmentation of the hyphae. Two genera are recognized on the basis of their oxygen requirement for growth. The aerobic organisms (those requiring atmospheric oxygen for growth) are placed in the genus Nocardia. The anaerobic organisms (those growing without atmospheric oxygen) are placed in the genus Actinomycetes. Most actinomyces and Nocardia are saprophytic but some are pathogenic. Nocardia asteroides and Actinomyces israeli produce slow but fatal disease in man. These diseases, however, are not common. Actinomyces bovis produces lumpy jaw in cattle.

Streptomycetaceae: Here the spores are borne in the form of chains on the tips of the hyphae. Two genera are recognized on the basis of the number and the arrangement of spores. The genus streptomyces bears the spores in the form of a chain on the hyphae while the genus micromonospora bears single spores on the tips

Laboratory Session # 3 cont.

of the hyphae. Many members of *Streptomyces* produce important antibiotics. Two examples are Streptomycin and Aureomycin.

Laboratory Session # 4 cont.

Actinoplanaceae: Here the spores are found in sac-like structure called the sporangia.

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Blue -green algae:

Any Prokaryotic organism which is capable of photosynthesis and in which oxygen produced as a result of the photosynthesis is regarded as blue-green algae. They are very simple organisms and resemble bacteria in almost all but one respect, i.e. their mechanism of photosynthesis is like other algae and plants and results in the production of oxygen. There are about 1,500 species of blue-green algae which are found in fresh water and in soil. Both unicellular and multicellular forms are found but each cell is a complete organism. They multiply by binary fission which is how bacteria also divide. At times suggestions have been made to place this group of organism with the photosynthetic bacteria, but since the photosynthetic bacteria do not produce oxygen the group has remained isolated.

Bacteria:

Any Prokaryotic unicellular organisms which has a cell wall and which either does not undertake photosynthesis or if it does the end products are not carbon dioxide and water is regarded as a bacterium. There are approximately 3,000 known species of bacteria. Most of these are saprophytes and live on dead and decaying matter in soil and water. Some have come to be associated with higher forms of life and have thus acquired a parasitic existence.

The size of a bacterial cell is generally very small. Most are approximately 1 micron in size. From the standpoint of shape, different bacteria have any one of the following shapes:

Spherical shape, also called coccoid shape – coccus (singular)
cocci (plural)

Elongated, called bacillary shape. This shape could be:

- a. sausage shape also called bacillus or a rod bacillus singular and bacilli, plural)
- b. Cigar shape also called fusiforms.
- c. comma shape also called vibrio
- d. spiral shaped with one or more curves

Mycoplasma:

These are a curious group of organisms which seem to have evolved from bacteria via some unknown process in which the bacteria have lost all or portions of their cell wall. Theoretically such an organism would not survive because water will enter into it, causing it to expand and burst. But through some evolutionary mechanism they have come to survive and perpetuate. They are parasitic organisms and are found on the mucous membrane of man and animals and occasionally also produce disease.

In the laboratory these organisms can be grown provided the medium contains 5% sodium chloride (salt) or 20% sucrose (sugar), and serum. Under artificial laboratory conditions the salt or the sugar prevents the water from entering into the cell. The organism thus survives and grows. On agar plates these organisms

Laboratory Session # 3 cont.

produce very small colonies which have to be viewed under the 10X objective of a microscope. Under the microscope these colonies will appear like fried eggs, i. e they will have a central portion which looks like the yolk (sunny-side up) and a lacy periphery which looks like the white of the egg. For this reason the colonies of mycoplasma are called fried egg shaped colonies.

L-forms:

These organisms are like the PPLO except that they are produced in the laboratory by treating bacteria with antibiotics or with other similar substances. In the presence of penicillin, for instance, bacterial cells which are sensitive to penicillin do not make their cell walls. The bacterial cells thus cannot maintain their shape. They round up and become what is called a protoplast. Some of these protoplasts survive and grow into colonies provided that the growth medium contained 5% salt or 20% sugar and 10% animal or human serum.

The colonies of L-forms are also fried egg type colonies.

L-forms are resistant to antibiotics such as penicillin. They thus survive in the presence of penicillin. Upon removal of penicillin or upon its destruction by the enzyme Penicillinase, the cells in the L-form colony begin to make the cell wall and the colony soon reverts to a regular bacterial colony. Is not this an interesting situation? Some persons use this observation to explain why certain patients when treated with penicillin get well but later come down with the same infection again. It is argued that in the presence of penicillin, some of the organisms survive as L-forms. These, when the treatment is discontinued, revert back to the original bacteria and produce disease again. An interesting hypothesis. Perhaps some of you will some day prove or disprove it

Rickettsiae

These are very small organisms which look very much like bacteria only smaller. They can barely be seen with the light microscope. They are strict parasites and grow only inside living cells. From the standpoint of their shape, they are described as cocco-bacillary. They are transmitted by insects such as ticks and produce such disease as Rocky Mountain Spotted Fever and typhus fever. The group of organisms is named Rickettsiae in honor of Dr. Ricketts of the University of Chicago who died working with these organisms. Ricketts Hall in the University of Chicago is also named in his honor.

Laboratory Session # 3 cont.

Laboratory Session # 4 cont.

Viruses:

Viruses are the most remarkable of all microorganisms. By themselves, they are unable to grow or reproduce. So much so that some persons consider viruses as lifeless chemicals. But this is true only as long as viruses are outside the cells. Once they get inside the cells they literally take over the machinery of the cell. From that time on the cell works to duplicate and produce more viruses. Eventually, when about 200 or so viral particles have been produced, the cell bursts (lysis) and the viruses are released. Viruses thus are strict parasites and cannot reproduce by themselves.

As microorganisms, viruses are quite simple. They are made of two main chemicals. One is the genetic material which is ribonucleic acid (RNA) in some viruses and deoxyribonucleic acid (DNA) in other viruses. The other is the protein coat which surrounds and protects the genetic material. The most important part of the virus is its genetic material.

You have already learned that viruses grow by parasitizing cells. They are thus called intracellular parasites. Viruses, however, do not infect and grow in every type of cell. They are quite specific, i.e. a particular virus will grow only within certain type of cell. Thus some viruses grow only within certain types of cells of animals. Such viruses are called animal viruses. Certain other viruses grow only within certain cells of plants. Such viruses are called plant viruses. Still, certain other viruses grow only within certain types of bacterial cells. Such viruses are called bacterial viruses or bacteriophages or phages for short.

When a virus infects its specific host cell, it is the genetic material which enters the cell. The protein coat remains outside the cell. It has thus merely acted as means of protecting and injecting the genetic material into the specific host.

Some viruses have head portion and a tail portion. The genetic material is in the head. Tail mainly acts as a channel which attaches to the cell. Through this channel, then the genetic material passes to the cell.

Once inside the cell, the genetic material has two alternatives:

1. It can direct the cell to alter its machinery and produce more viruses. This is called the infectious cycle, or
2. It becomes part of the genetic material of the cell and passes from generation to generation. Such a phage when it has attached to the genetic material of the host is called a prophage. Occasionally, however, this prophage becomes loose and directs the cell to produce more viruses. The cell thus goes through the infectious cycle (also called lytic cycle).

Laboratory Session # 3 cont.

Laboratory Session # 4 cont.

Just think. If all the machinery of the cell depends upon its genetic material, then with a change in the genetic material (as when a virus attaches to it), the machinery of the cell will also change. Such a cell may now grow too fast and consume all of the food from the rest of the cell. Such cell could thus be regarded as a cancer cell. The theory which proposes that the cancer is caused by viruses uses this reasoning as its basis. It sounds reasonable, but is it true! The answer will come from research. You can get ready for it by learning the principles presented in this course.

Laboratory Session # 4 cont.

Names of Microorganisms

All organisms are named by the system of Binomial Nomenclature, i.e. they all have two names. The first name represents the name of genus and the first letter of this name is always capitalized. The second name represents the name of the species. This is always written in lower case letters. Since the names are of latin derivation, the letters when used in print are italicized. When written by hand or when typed the names are separately underlined.

Some of the cultures used in this manual are listed below. Learn to pronounce and spell their names correctly:

Escherichia coli
Aerobacter aerogenes
Sarcina lutea
Staphylococcus aureus
Staphylococcus epidermidis
Bacillus cereus
Bacillus subtilis
Bacillus megaterium or Bacillus megatherium
Pseudomonas aeruginosa
Alcaligenes faecalis
Serratia marcescens
Penicillium notatum
Aspergillus niger
Streptomyces griseus
Saccharomyces cerevisiae
Proteus vulgaris
Neisseria catarrhalis
Pneumococcus pneumoniae
Streptococcus lactis
Streptococcus pyogenes

Laboratory Session # 3 & 4

Assignment# 1

Student identification number _____

Make drawings of all the organisms made available to you during this exercise.

The drawings for the bacteria and actinomycetes should be made by observing the slide under the 100X objective lens.

For fungi draw what you see under the 10X and 40X objectives

For yeast draw what you see under the 40 x objectives.

Keep drawing as close to the size that you observe under the respective magnifications.

Make certain that your drawings are under the proper category and that the names of the organisms are correctly spelled.

Where applicable, label the various parts of the organisms.

In the case of bacteria, label the drawings with the shape and arrangement of cells.

This assignment is worth 50 points.

Drawings of

Organism observed	Procaryotic Protista	Eucaryotic Protista
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Laboratory Session # 3 & 4

Assignment# 1
Drawings of

Organism observed	Procaryotic Protista	Eucaryotic Protista
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Cultivation of Microorganisms

In order to grow microorganisms we must provide them the type of food material that they need and we must also grow them at a temperature which they need for their best growth. Some organisms also require light to grow and this must be provided. Some organisms also require that they be incubated in air, others die out if they are incubated in air. Such organisms are grown in the absence of air...

From the standpoint of food, all organisms fall into 3 major categories:

1. Those which grow on dead and decaying organic material - Protozoa fungi, slime molds, actinomycetes, bacteria, and mycoplasma belong here.
2. Those which grow on simple chemicals and do not need dead organic material - algae, and certain bacteria belong here
3. Those which grow only if living cells are available to them. Here they grow inside the cells. Viruses and rickettsiae belong here.

From the standpoint of temperature, all organisms fall into 3 categories:

1. Thermophiles - these grow within 45°-65° C
2. Mesophiles - these grow within 25°-42°c
3. Psychrophiles - these grow within 0°-22° C

The average temperature for growing:

Thermophiles is 55° C

Mesophiles 37° C

Psychrophiles 4 °C

Some mesophiles grow best at 25° C (room temperature)

From the standpoint of needing light all organisms fall into 2 categories:

1. Photosynthetic
2. Non-photosynthetic (also called chemosynthetic)

Photosynthetic need light energy to grow. The chemosynthetic do not. They harvest energy trapped in chemicals and use it for growth.

Laboratory Session # 5 cont.

From the standpoint of oxygen requirement, all organisms fall into 4 categories:

1. Aerobic - need free oxygen of air
2. Microaerophilic - need less air than the aerobic organisms
3. Anaerobic - do not need air (oxygen). In fact, oxygen is poisonous for these.
4. Faculative - these can grow both in air and in the absence of air.

NOTE: Some organisms require that they be incubated in air containing 5-10% carbon dioxide.

Now, let us look at all the above information in a summary form.

Group of Organisms	Food requirement	Temperature	Photosynthesis	Air Harmful
Algae	Simple chemicals such as phosphate, carbon dioxide, and minerals dissolved in water	25° C	Yes	No
Protozoa	Organic matter. Engulf particles, feeds on bacteria etc.	25° C	No	No
Fungi	Dissolved organic matter – such as proteins, sugar carbohydrates	25° C	No	No
Slime molds	Like protozoa	25° C	No	No
Actinomycetes	Same as fungi	Some 25° C Some 37° C	No	For some yes
Blue-green Algae	Same as Algae	25° C	Yes	No
Bacteria	Some like Algae Some Like Fungi	Some 25° C Some 4° C Some 37° C	Most no, some yes	For some yes
Mycoplasma	Like Fungi	37° C	No	No
Rickettsiae	Need living cells	37° C	No	No
Viruses	Need living cells	37° C	No	No

Laboratory Session # 5 cont.

Now that we have general idea about the growth requirements of organisms, we will concentrate on growing bacteria. Since they will grow on organic matter dissolved in water their growth in the laboratory is brought about by placing (inoculating) the sample (soil, water, swabs, from, nose, throat, surfaces and food etc.) in extracts of meat and vegetables. These we call media. They are now available, commercially as powders and to prepare them all one needs to do is to dissolve the quantity (indicated on the bottle) in water and sterilize the solution. Such solutions are called broth or liquid media.

If we add 1.5% agar to the broth medium and boil it, we get a medium which will gel upon cooling. This is how the agar or solid medium is made. Solid medium is used in the form of agar slants, plates and deeps.

Sometimes we add only 0.3 to 0.5% agar to the broth medium. This gives us a semi-solid media. This is used as deeps and is useful for detecting if the bacteria are motile and also if they produce gases as they grow.

In order to grow the bacteria present in a sample, we can use a liquid (broth) medium or an agar plate medium.

If we use broth medium, we merely add a small amount of the sample into the sterile broth and then we incubate the tube. Here different bacteria present in the sample will have to grow together. They will thus consume for the food and space and those which find the condition suitable for their needs will grow. The resulting culture thus will have many types of bacteria and will not be a pure culture.

If we use an agar plate medium, then we inoculate the sample onto the plate by a method that we call the streak plating method. We place the sample on one side of the agar surface (see figure for streak plating) and streak the rest of the plate by following the method described in the figure. This procedure enables us to isolate different bacteria from each other. These are allowed to grow, will form isolated colonies. All isolated colonies is thus regarded as a pure culture of one type of bacteria. Streak plating is very important technique. You must become thoroughly proficient in it.

Pure cultures are also inoculated onto plated by the:

1. Radial streak or stab or spot method. This allows us to grow more than 1 culture (up to 10 on one plate).
2. Swab method - This involves completely smearing the plate with the culture. This is done when wish to find which antibiotic will kill or stop the growth (inhibit) the bacteria.

Laboratory Session # 3 cont.

After the samples are inoculated (plated) on the media, the medium is incubated at the temperature and under the atmosphere that the culture needs for growth. The method of incubating under anaerobic conditions (without air) and under 5-10% of carbon dioxide in air are:

- I. Take a jar with a screw cap lid. Mouth of jar should be wide enough for the Petri plate to go through. Smear the lip of the jar with vaseline.
- II. Place two Petri plates in the bottom on the jar.
- III. Place the inoculated plates upside down on these two Petri plates.
- IV. Place a small candle on the top plate. Make sure that the jar can still be closed and the candle has room above it to burn.
- V. Light the candle.
- VI. Close the lid tightly. When the candle goes out incubate the jar.

Incubation of cultures under Anaerobic conditions.

1. Take a jar with a screw cap lid. Mouth of jar should be wide enough for the Petri plate to go through. Smear the lip of the jar with vaseline.
2. Place 2 Petri plates in the bottom of the jar.
3. Place the inoculated plates upside down on these 2 Petri plates.
4. Take a tube of anaerobic indicator to the inside wall of the jar (see formula below).
5. Place Sodium Hydroxide 20% solution (50 ml) in the bottom of the jar.
6. Add 20g Pyrogalllic acid crystals.
7. Immediately seal the jar and incubate.

Anaerobic indicator:

This is used to ensure that there was no oxygen present in the jar during incubation, i.e. air was not leaking into the jar.

0.1 N Sodium hydroxide 6.0 ml dilute to 100 ml with distilled water.

0.5% aqueous methylene blue 3 ml dilute to 100 ml with distilled water

Glucose 6 gram dissolve in 100 ml distilled water (add a crystal or 2 of thymol to this solution).

Mix equal parts of the above three solutions in a test tube. Place the test tube in a breaker of boiling water and boil the water until the blue color of the solution disappears. Immediately place the tube in the jar where anaerobic incubation of the culture is to be done. Then proceed with obtaining anaerobic conditions in the jar. If the oxygen of the jar was completely consumed, then the indicator will stay colorless throughout the period of incubation. If the blue color returns it means the container was leaking and anaerobic conditions were not maintained.

Laboratory Session # 3 cont.

Laboratory Session # 5 cont.

Learn and practice inoculating various types of media. You will be provided with the following materials:

- 1 broth culture of *Escherichia coli*
- 1 slant culture of *Escherichia coli*
- 1 Trypticase soy broth tube
- 1 Kilgler's agar slant
- 5 Trypticase Soy agar plate
- 5 Trypticase Soy agar plates
- 1 Trypticase Soy agar deep
- Candle jar with candle - 1 per class
- Gas pack assembly - 1 per class
- Solution for making anaerobic indicator (see below) a set per class.

Your instructor will demonstrate transferring cultures aseptically to a slant and a broth tube. The procedure is as follows:

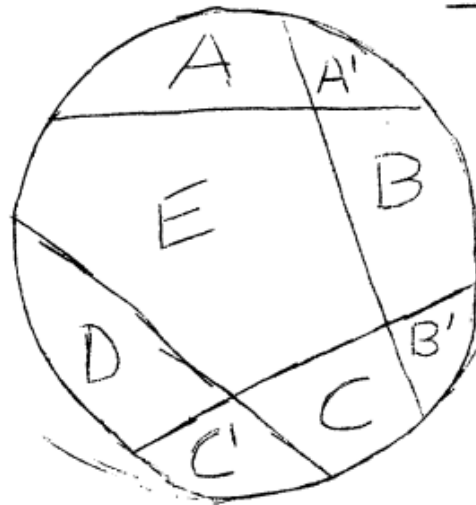
1. Hold the inoculating loop in your hand as you would hold a pencil while writing. Flame it red hot and then let it cool.
2. Hold the tube containing the culture to be transferred in your other hand. Bring the culture towards your hand in which you are holding the inoculating loop.
3. With the palm and the little finger of this hand remove the cap of the tube. This will require practice, but you must learn this because you will be required to handle tubes in this way.
4. Flame the lip of the tube.
5. Introduce the inoculating loop in the tube. Remove a very little amount of the culture by just dipping the loop in the broth culture. If the growth is on a slant culture, remove a little of the growth by just touching the growth.
6. Withdraw the loop.
7. Flame the lip of the tube.
8. Replace plug of closure on the tube.
9. Pick up the tube to be inoculated. Remove plug. Flame the lip of this tube also and introduce the loop containing the culture and stir it if you are inoculating a broth culture or rub it with a zig-zag motion if you are inoculating a slant.

After the demonstration, transfer the culture of *E.coli* into a broth tube and also to a slant. Incubate the cultures so inoculated at 37°C. Next your instructor will demonstrate the streak plate method of inoculating a petri plate. Following the demonstration you will streak trypticase soy agar plates from the broth culture of *Escherichia coli*. The streak plate method is detailed below and in figure 1.

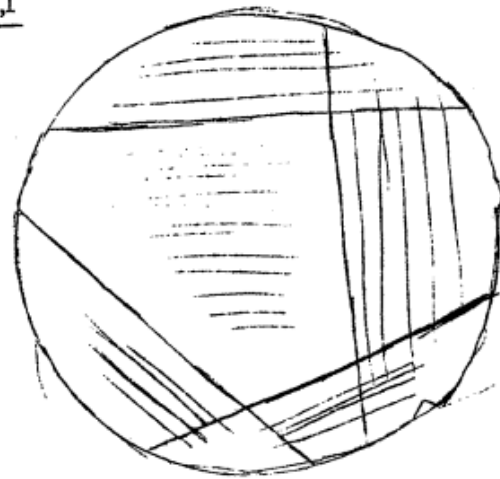
1. Place the petri plate to be inoculated in an upside down position on the table.
2. Sterilize the bacteriological loop and allow it to cool in your hand. Obtain a very small amount of the sample from the broth or the slant culture.
3. Spread the sample in the area of the petri plate marked A and A' in Figure 1. Do not dig into the agar. Return the plate to its lid.
4. Sterilize the bacteriological loop and allow it to cool.

Laboratory Session # 3 cont.

5. Run the loop simultaneously with continuous strokes in the areas marked A', B' and B'. Note that each stroke of the loop brings bacteria from area A' and spreads it on the areas B and B'. Return the plate to its lid on the table.
6. Sterilize the loop and allow it to cool.

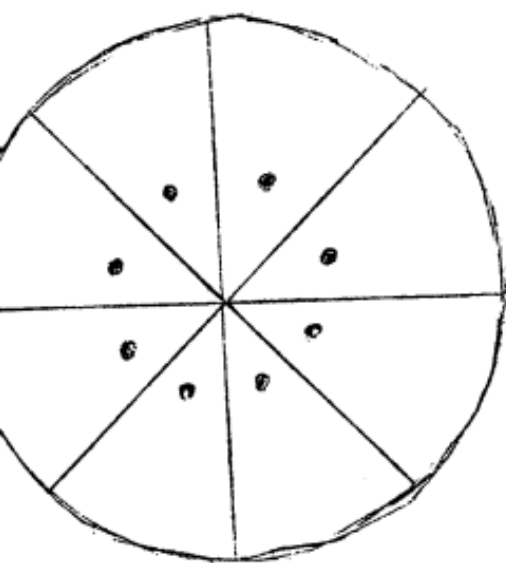
FIGURE 1

Guidline for streaking

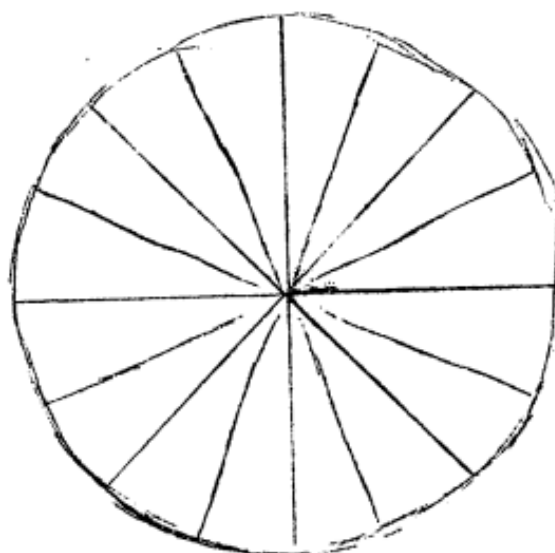
Streak marks on the surface
of a properly streaked plate.The streak plate method

The method merely calls for spreading the sample over a large area so that the organism in the sample will separate from each other. In order to accomplish this one needs to use the entire area of the plate effectively. This is done by first spreading the sample in the area marked A and A'. The loop is then sterilized and cooled and the material from the area A' is spread over to B and B'. The loop is again sterilized and cooled and the material from area B' is spread over to C and C'. Finally the material from C' (without sterilizing the loop) is spread over to D and the streaking is continued into area E. While in area E one does not ever let the loop enter into any of the other areas.

FIGURE 2

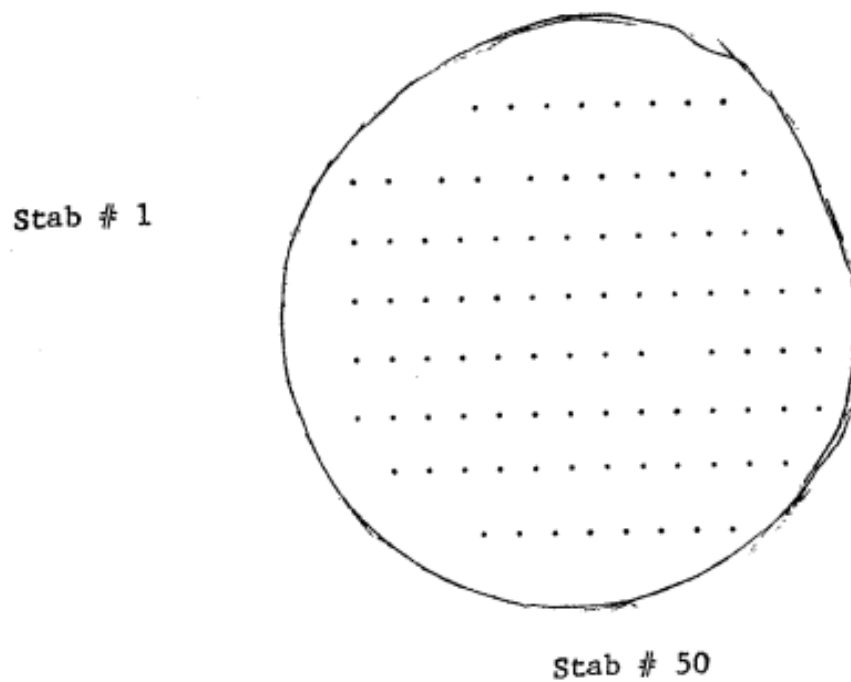


Stab Inoculation
different culture is inoculated to each section.



Radial streak
A different culture is inoculated in each section. Cultures are not allowed to merge with each other in the center of the plate.

FIGURE 3



7. Run the loop simultaneously a number of times in the areas marked B' C, & C'. This will bring a few of the bacteria from B' area and will spread them into the C and C' areas. Return the plate to its lid on the table.

Laboratory Session # 3 cont.

8. Without sterilizing the loop, streak the areas marked C' and D until you enter; area E. Without stopping, continue running the loop W back and forth in this area. Do not let the loop enter into any of the other areas. Return the plate to its lid on the table.
9. Sterilize the loop and put it away.
10. Mark this plate as #1. Also write on it your name and that of the culture being streaked. Show it to your instructor. Following his suggestions streak another plate and incubate it until the next laboratory session.

Next your instructor will demonstrate the stab and radial streak methods of inoculating agar plate. These procedures are detailed in Figure 2. Refer to this figure. Observe the demonstration and then inoculate 1 TS plate by the stab method and 1 by the radial streak plate method. Make 8 streaks and stabs per plate. Use the inoculating needle for stab inoculations. Incubate these plates upside down in the incubator.

Next your instructor will demonstrate the procedure for inoculation of an agar deep and a slant which also has a butt such as the Kligler's Iron agar. Then inoculate the TS agar deep and the Kligler's Iron agar. Use the inoculating needle for this purpose and stab the agar once only all the way to the bottom of the tube. Incubate at 37°C.

Even after making all of the above inoculations it will still be difficult to appreciate how few organisms you really need to make the inoculation.

Also you may wonder how often you may have to change the inoculating loop for making a series of inoculations. The only way to answer these points is by letting you find it out for yourself. You can do so very readily. Take the inoculating needle, sterilize it, and after it has cooled charge it from the broth culture of Escherichia coli. Stab it 50 times in a TS agar plates as shown in Figure 3. Also similarly inoculate another TS agar plate, but obtain your inoculum from a slant culture of Escherichia coli. Incubate both the plates upside down at 37° C. Next time see how many of these stabs have shown growth.

[Your instructor will demonstrate the procedure to incubate culture in an atmosphere containing 5-10% carbon dioxide and also under anaerobic conditions.

READING ASSIGNMENT

Methods of obtaining and studying Microorganisms from our Environment

By this time you should be fairly familiar with the microscope and methods of inoculation and incubation. With this background we can proceed to study the following two questions:

1. Where are the microorganisms like bacteria and fungi found?
2. How do we obtain and study them?

Our aim will be able to learn how to obtain the samples and what to do with them after they have been collected. We must realize that the sample must be obtained from the precise area that we are studying and it must be obtained aseptically, i.e. without contamination. This necessitates the use of sterile equipment such as sterile swabs and sterile tubes.

Once the sample is obtained, it is studied either by:

- I. Direct examination under the microscope and/or
- II. By growing the organisms present in the sample.

I. By direct examination under the Microscope:

This procedure will reveal the presence of microorganisms only if the sample contains a large number of organisms. Microorganisms such as bacteria are colorless and almost transparent. If they are viewed directly under the light microscope we see almost nothing unless we cut down the light to obtain some contrast. Even then, we see a very hazy picture. This, therefore, necessitates that the organisms are stained by using dyes.

Two types of dyes are used, one type is called basic dyes. These carry a positive charge. Bacteria carry a negative charge. The basic dyes are, therefore, able to stain the bacteria. Methylene blue, crystal violet, and safranin are examples of basic dyes.

The second type of dyes are called acidic. They carry a negative charge. Since bacteria have negative charges, these dyes do not stain the bacteria. They merely deposit around the bacterial cells and contrast the background. Bacteria, therefore, appear colorless.

Staining procedures where basic dyes are used are called direct staining procedures. There are two types of direct staining. In one a single basic dye such as methylene blue, crystal violet, or safranin is used. Such procedures are, therefore, simple staining procedures. In the second group of methods more than one stain is utilized. This enables one to differentiate either among different bacteria or different structures of a bacterial cell. Such procedures are called differential staining procedures. The Gram's staining procedure, which you will learn later, is a differential staining method.

Staining procedures where acidic dyes are used are called indirect or negative staining procedures. Since these stains layer around the outer most boundary of the bacteria cell, cells examined by the negative staining method appear larger. This method is, therefore, particularly useful for deciding the shape of the bacterial cell.

Laboratory Session # 3 cont.

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Laboratory Session # 6 cont.

The direct staining methods, on the other hand, do not stain the cell wall, but stain the cytoplasmic membrane which lies on the inside of the cell wall. Cells stained with the direct staining methods thus appear smaller than they actually are.

Another point to remember about the direct and indirect staining methods is that in the direct staining methods excess stain is removed by washing the slide after staining. Therefore, considerable number of bacteria also wash off the slide when this is done. In the indirect stain on the other hand, the slide is never washed. All the microorganisms contained in the sample, therefore, remain on the slide.

Now let's learn how to observe microorganisms by the direct and indirect staining methods. We will study the following:

1. A slant culture of *Escherichia coli*
2. A broth culture of *Escherichia coli* (these have already been provided).
3. A soil extract
4. Scrapping from your teeth.

You will use the following stains:

1. Nigrosin (for negative staining)
2. Methylene blue (for direct simple staining)

Slides for microscopic examination of bacteria must be grease-free. Flame slides. A good procedure to clean slides is by using scouring powder. First put a small amount of water on the slide. Then put the scouring powder on the slide. Make a paste and rub it on both sides of the slide. Then rinse the slide in tap water. Hold the slide under the tap water in such a way that the water runs from the slides towards your fingers. If you let the water fall on your fingers first and allow it to run on the slide, the oil from your fingers will make the slide greasy and unfit for use. Dry the slide with a paper towel and then use it. A drop of water placed on a properly cleaned slide will spread easily, but will bead up if the slide was not grease-free. Remember if you were not able to spread the bacterial culture on the slide you should not proceed any further. Wash the slide and start all over again.

Your instructor will demonstrate the procedure to clean slides and then will show you the negative staining procedure using the culture of *Escherichia coli*. After the demonstration, you prepare a negative smear from one of the two cultures and also from the scrapping from your teeth and the soil extract. Note the shape and arrangement of the microorganisms that you see. The negative staining procedure is as follows:

1. Place a small drop of nigrosin on the clean slide.
2. Transfer a very small amount of the culture or the sample to the drop of nigrosin. Remove the culture from the tube aseptically as you had learned during the last laboratory session. Mix and spread the culture on the slide covering an area approximately 1/2" X 1"
3. Let the film dry in air
4. Fix the film by passing it 2-3 times over the flame.
5. Examine under the oil immersion lens.

Laboratory Session # 3 cont.

Next your instructor will demonstrate the direct staining procedure. Then you prepare direct stains from broth and slant cultures of Escherichia coli. While making smears from the broth culture use 3-4 loopsful of the broth and spread evenly on the slide. The large amount of the culture used is necessary because bacteria from broth cultures do not stick to the slide well and wash off readily. The procedure for making direct stains requires that a smear of the culture is first made as follows:

1. From solid cultures:

- a. Pick a drop of water with the inoculating loop and place it on a clean slide.
- b. Aseptically obtain a very small amount of the culture and mix it in the drop of water and spreading it on a 1/2" X 3/2" area.
- c. Let the smear dry in air.
- d. Fix it by passing over the flame 2-3 times
- e. Let it cool and then proceed with the staining

2. From broth cultures:

- a. Place 3-4 loopsful of the broth cultures on the slide and spread it on a 1/2" X 3/2" area.
- b. Allow the smear to dry in air and then fix by heat and stain.

Stain smears as follows:

- a. Place the smear (after fixing) on the staining tray.
- b. Cover the smear with a generous amount of the stain.
- c. Stain for 1 minute
- d. Discard the stain in the staining tray.
- e. Wash the smear with a gentle stream of water from the wash bottle.
- f. Let the excess water drain from the slide and then let the slide dry in air.

Note: Drying in air is preferred when smears from broth cultures are stained. Smears made from solid media can be dried by blotting between two layers of paper towel.

- g. Examine the smear under the oil immersion lens.

(Methods to obtain and study Microorganisms from our environment (cont.))

II. Examination of samples by growing the organisms present in the sample:

As we have stated in the last laboratory session the sample can be grown in broth or on agar plate. In the following experiments we will use both types of media. All plates will be inoculated by the streak plate method.

Students will be supplied with the following samples:

Pasteurized milk

Normal blood

Urine

Body lotion or bath oil.

The following samples will be obtained by the student:

Material from: nose, throat, teeth, hair, palm of hands, table tops, and floor or handles of water faucets.

Laboratory Session # 3 cont.

Remember to use aseptic technique while collecting the samples. Obtain samples only from the area which is being studied.

You will also receive 11 tube of Thioglycollate broth (10 for inoculation and 1 for moistening the swab), 12 plates of Trypticase Soy Agar, 1 plate of Sabourauds agar, 6 sterile swabs, and 1 tongue depressor.

Procedures for obtaining and inoculating the samples:

Inoculation of Pasteurized milk, blood, urine, body lotion or bath oil in separate tubes of broth:

Your instructor will first demonstrate the procedure. Then you inoculate each of the above samples in separate broth tubes. The procedure is:

1. Sterilize and cool the bacteriological loop.
2. Inoculate the broth with 2 to 3 loopsful of the samples being tested.
3. Mark the tube with the name of the sample and your own name.
4. Incubate the tube at 37° C until the next laboratory session.

Inoculation of pasteurized milk, blood, urine, body lotion or bath oil on separate agar plates:

Although you have learned this method previously your instructor will demonstrate it again. Use the streak plate method. Inoculate 2-3 loopsful of the sample in the area A, A' (figure 1). Then streak the plate as before. Each student is to inoculate each of the samples on separate plates.

Inoculation of broth tube and agar plate with the sample obtained from nose swab):

Your instructor will demonstrate how to obtain and inoculate the sample from the nose. Then you obtain the sample and inoculate in broth and on agar plates. The procedure is given below:

1. Remove a sterile swab from the container and gently insert it into one of the nostrils. Rotate the swab within the nostril and remove it from the nostril.
2. Introduce the swab into the broth tube and gently stir the broth with the swab.
3. Remove excess fluid from the swab by squeezing it on the inside wall of the tube and remove it from the tube.
4. Using the same swab, inoculate the area of the petri plate marked A and A' (see figure 1). Discard the swab. Sterilize and cool the inoculating loop and streak the plate for isolation of bacteria.
5. Mark both the tube and the plate with your name and that of the sample. Incubate at 37° C until the next laboratory session.

Inoculation of broth tube and agar plate with the sample obtained from throat (throat swab):

Your instructor will first demonstrate how to obtain and inoculate the sample from your throat. Then you obtain the sample and inoculate in broth and agar plates.

1. Hold the sterile swab in your hand
2. Have the subject open his mouth and depress the anterior (front)
3. Introduce the swab in the mouth and proceed directly to the throat area. Touch the swab gently to the area being sampled and withdraw it from the mouth.

Laboratory Session # 3 cont.

4. Remove tongue depressor.
5. Inoculate broth tube and the agar plate following steps 2 through 5 as given under the inoculation of nose swab.

Inoculation of agar plate from hair:

Your instructor will first demonstrate. Then you inoculate plate from your hair. The procedure is:

1. Open one agar plate.
2. Bring the agar directly in contact with your hair a few times, pressing it gently against the hair each time.
3. Cover the plate and mark it with your name and that of the sample.
4. Place it in the incubator at 37° C until the next laboratory session.

Inoculation of broth tube and agar plate with a sample obtained from teeth:

Your instructor will demonstrate how to obtain and inoculate the sample from the teeth. Then you obtain the sample and inoculate in broth and on agar plates. The procedure is given below:

1. Remove one sterile swab from its container and rub it over the outer surface of your teeth.
2. Inoculate broth tube and the agar plate following steps 2 through 5 as given under the inoculation of nose swab.

Inoculation of broth tube and agar plate with samples obtained from palm of hand, table, tops, floor or handles of faucets:

Your instructor will demonstrate how to obtain and inoculate the samples. Then you obtain the samples and inoculate in broth tubes and agar plates. The procedure is given below:

1. Take out the sterile swab from its container. Moisten it with sterile broth and remove excess broth by squeezing the swab on the inside wall of the tube.
2. Select a one inch square area of the surface being sampled. Rub the swab in the entire area first from left to right and then from top to bottom.
3. Inoculate the broth tube and the agar plate following steps 2 through 5 as given under the inoculation of nose swab.

Growing organism from air:

1. Open one TS agar plate and leave it on your desk for 1/2 hour. Then cover it and incubate at 37° C.
2. Similarly, open and expose the Sabourauds plate for 1/2 hour. This is for growing molds. So incubate it at room temperature because molds will not grow at 37° c.

Laboratory Session # 7 cont.

Methods to obtain and study microorganisms from our Environment II: Today you will see what is growing from the various samples which you had inoculated during the last laboratory session. If the sample was sterile and you did not contaminate it, then you will see no growth from it either in broth tubes or on agar plates. Normal blood is sterile and you should not find any growth from it today. Nature has its way of keeping our blood sterile and you can realize why we should not contaminate it by injecting into people substances which are not sterile. We should also not use non-sterile instruments for the same reason.

What about body lotion? Did you get some growth from it? Most likely, you will see growth in broth culture, but not on agar plates. This is because most cosmetics contain chemicals as preservatives. These and the lack of food in the cosmetics suppress the growth of microorganisms. The organisms, however, may remain alive. These, when we inoculated the sample in broth, may be able to grow for two reasons. First, because they found food and, second because the chemicals got diluted with the broth and thus became less effective. You may, therefore, see some growth in the broth. On agar plates, on the other hand, the few bacteria that were in the body lotion did find food, but the chemicals inhibiting their growth did not get diluted. The bacteria therefore, may fail to grow on the agar plates.

Media inoculated with all the other samples should show very good growth. Broth tubes should be turbid or cloudy because the organisms have now multiplied to become billions in number; on the average each ml. of the broth now contains 500 million bacteria. The growth on agar plate will be very dense near the point where you had made the original inoculation. It will be less dense in other areas. You will also see small specks of growth well isolate from each other. These are called colonies. If they are well separated they are probably pure cultures of one type of bacteria. To be sure though, one should re-streak the colony on a fresh plate to further isolate the cells. Colonies which grow on this plate will then be regarded as pure culture.

Even though you got very good growth from different samples, not every organism present in the sample was able to grow in the medium that you used. If one wishes to grow as many of the organisms as the sample contains then one will have to use different types of media and incubate them under different temperatures and under different atmospheres, such as carbon dioxide and anaerobic conditions, so that most of the organisms could find conditions suitable for their growth. What you see on the plates today then is just a small part of what may actually be present in the various samples.

Now let's see if we can recognize what types of microorganisms are growing on the plates. Can you recognize which are the colonies of molds. These will be fluffy cotton-like colonies. What types of molds these are - one cannot be sure by just looking at the colony. One will have to mount a tiny part of the fungus in lactophenol cotton blue and look at it under the microscope. Then one must recognize the shape, type and distribution of spores and many other features before one can identify them. Although you may not be able to identify the mold this way just yet, you should nevertheless learn the simple technique of making the mount.

Laboratory Session # 3 cont.

Laboratory Session # 7 cont.

Take a clean slide. Put a small drop of lactophenol cotton blue stain on it. Remove a very small piece of the fungus with the forceps and place it in the drop. Put a coverslip on the slide and examine it under 10X and 40X objectives. Locate the hyphae. Also look for spores and make drawings of their shape and arrangements. Include the structures to which the spores are attached.

The cultures of bacteria which are growing on the plates cannot be identified by looking at their colonies or even by making smears from them. Shape and texture of the colony and the shape and arrangement of the cells in a smear made from the colony is nevertheless useful information and together with other features of the culture, which you will learn later, they serve to identify the organism.

Look at the colonies and try to describe their characteristics from the accompanying figure showing morphology. Then make a few smears from some of the colonies growing on your plates. You may use direct or indirect procedures and check the shape and arrangement of the cells. Also try to relate the shape and arrangement of the cells to the type of colony from which they were obtained. A fast laboratory technician can examine at least 20 different colonies in an hour. Let us see how many you can examine today. Be neat. Speed is of no use if your results are not reliable. Examine at least 10 colonies. Record all of today's results in your note book. Later copy them neatly on the assignment sheet and hand it in to your instructor. This assignment is worth 100 points.

Assignment Sheet II

Student Identification # _____

Sample	Growth in broth	Growth on agar plates		
		Amount of growth in broth	Description of selected colonies of	Labeled drawings
Hair, nose, throat, blood, urine, milk or	Positive (+) or Negative (-)			

Laboratory Session # 3 cont.

body lotion etc.	(heavy, light etc.)	bacteria and molds
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Laboratory Session # 7 cont.

As we have stated previously the colonies growing on the plates today may not represent pure cultures. In order to obtain pure cultures you should streak today three different well isolated colonies onto three agar plates. Your technique of streak plating should be perfect by now. If it is not, this is the last time to learn it well. Incubate the plates at 37° C until the next laboratory session.

Laboratory Session # 8 cont.

1. Today you should use isolated colonies on each of the three plates that you streaked last time. Transfer 1 colony from each of the three plates to an agar slant. Write your identification number and the colony number on the tube and hand them in to you instructor. These slant cultures will be incubated and then examined for purity as well as for your technique of inoculating them. You will receive a grade. This assignment is worth 30 points.

II. Classification Identification of Bacteria - I

Gram Staining Procedure

The Gram staining procedure was discovered by Christian Gram in 1894, and is the most important aid in the identification of bacteria. Gram found that some bacteria, when stained with a dye similar to crystal violet and then with iodine, were able to retain the color of the dye when he washed the smear with alcohol. Other type of bacteria when treated in a similar fashion rapidly lost the color and became colorless. He thus proposed that all bacteria can be divided into two categories. He named all those bacteria which did not become colorless after the alcohol wash as Gram positive, while those which became colorless as Gram negative. Now a somewhat modified form of his staining procedure is in general use. This procedure is given below:

1. On a clean glass slide, place a drop of water. Emulsify (mix) a small amount of the culture to be Gram stained in the water and spread it in the form of a thin smear. Allow the smear to dry in air and then fix it by passing it 2-3 times over the flame.
2. Stain the smear for 2 minutes with crystal violet.
3. Wash the smear with water and then stain it with iodine for 1 min.
4. Wash with water.
5. Wash the smear quickly with 95% alcohol. This is a very critical step and the washing must be done rapidly and quickly as soon as the violet color has stopped leaching out from the smear, the smear must be washed in water and carefully blotted with a towel. Follow this step as demonstrated.
6. Stain with safranin for thirty seconds.
7. Wash with water. Dry and observe under the oil immersion lens.

A Gram positive culture will be of violet color. Why? Because a Gram positive culture does not readily lose its color when washed with acetone-alcohol. It, therefore, stays violet throughout the procedure. The red dye safranin is not able to mask this color.

A Gram negative culture will appear red? Why? Because this culture loses the violet dye rapidly when washed with acetone-alcohol and thus becomes colorless. Being colorless now it readily picks up the red dye safranin and appears red.

Laboratory Session # 3 cont.

Laboratory Session # 8 cont.

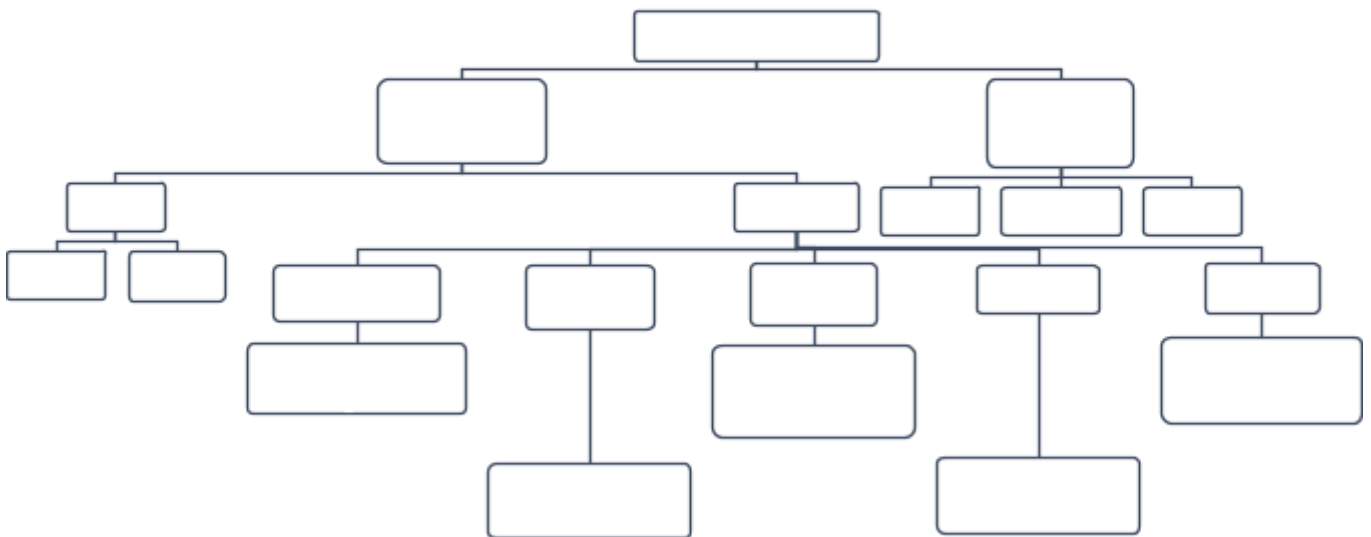
Laboratory work assignment:

Since the Gram staining procedure is the most important and most frequently employed procedure in the bacteriological laboratory, it is necessary that you familiarize yourself with the procedure thoroughly and memorize it. Three pure cultures, listed below, are provided for your use today.

1. Staphylococcus epidermidis - slant culture
2. Escherichia coli - slant culture
3. Bacillus subtilis - slant culture.

Make one smear from each of these cultures and determine their Gram reaction. Also select at least three colonies from the plates that you had streaked last time and stain these by the Gram's method. While examining these smears you will also be able to note the shape, relative size and arrangement of the cells. Also include any unusual things that you may see in the smear. Record all your findings in the table provided for this purpose. Later copy all the information neatly on the assignment sheet and hand it in to your instructor. This assignment is worth 50 points.

All bacteria on the basis of their Gram reaction, shape and arrangement can be



divided into groups as follows:

Laboratory Session # 3 cont.

While the information you have obtained today is helpful in the identification of the organism, notice that you are still not able to name the organism or establish its identity. Many more tests are to be performed for that. Such tests are:

1. Determination of cytological (the presence or absence of various structures of the bacterial cell) and functional features of the bacterial cell, such as the presence of capsules, spores, metachromatic granules and motility.
2. The ability of the organism to use carbohydrates, proteins, fats, and other food materials.
3. Ability to produce acid or acid and gas from certain sugars.
4. The amount of acid produced.
5. The ability to produce pigments.
6. The ability to withstand heat or certain chemicals such as sodium chloride or bile, and certain dyes such as crystal violet or a mixture of eosine and methylene blue.

In the experiments to follow you will be given pure cultures of various bacteria along with the necessary reagents and media so that you are able to learn how to determine these and many other features of the bacteria and eventually be able to identify unknown cultures. It is only after learning these aspects of bacteriology that you will be able to handle samples of patients and diagnose the cause of the disease and help the physician in his ultimate diagnosis and treatment of the patient.

Name or source of the culture	Results			Unusual things observed in the smear.
	Shape	Arrangement	Gram reaction	

Laboratory Session # 3 cont.

Assignment# III

Student identification
number _____

Lab. Session # 6

Part I, Hand in 3 slants each containing a pure culture of a different colony. Mark each tube as follows:

Your number _____

of colony _____

Laboratory Session # _____

Describe the features of the cultures below:

Cultures #	Description of colony
------------	-----------------------

Part II. Gram Reaction of Culture:

	Gram Reaction	Shape	Arrangement	Unusual features
Staphylococcus epidermidis				
Escherichia coli				
Bacillus subtilis				

Laboratory Session # 9 cont.

Classification and Identification of Bacteria - II

By this time you should be able to do the following: (1) obtain a pure culture of the bacteria, (2) make a smear (3) stain it with the Gram's method (4) observe it under the microscope using the oil immersion lens (5) determine its Gram reaction and (6) determine its shape (also called morphology), relative size, and arrangement. Note: If you are not yet able to do all these by yourself, you should backtrack a bit and catch up with the rest of the group.

Using the information so collected, you should be able to place a given bacteria culture in one of the following five groups.

1. Gram positive rods (also called Gram positive bacilli)
2. Gram positive cocci
3. Gram negative rods (also called Gram negative bacilli)
4. Gram negative cocci
5. Gram negative spirals

This grouping is very helpful in the identification of bacteria. Members of each group are further differentiated from each other by many tests. Such tests are called differential tests. In some of the differential tests, we look for the presence of certain structures in a bacterial cell. Spores, capsules, and metachromatic granules are such structures. In this session we will learn how to look for spores and capsules.

- I. Spores: Spores are hard, round, or oval shiny bodies which are produced by some members of the group "Gram positive rods". Only one spore is produced per cell. The spore forms within the cell and upon maturation, it is released from the cell; the cell in turn dies because all the vital parts of the cell are now enclosed in the spore. The spores are very resistant to heat and chemicals which are readily able to destroy the bacterial cell. Each spore, in the presence of proper nutrients and moisture is able to germinate and give rise to one bacterial cell. Spores, being resistant to chemicals, are also difficult to stain and two procedures are used for their demonstration:
 - A. Direct staining of spores:
 1. Prepare a smear, dry it in the air and fix it by passing the slide 6-7 times over the flame (this procedure of fixing will alter the spore in such a way that the stain will now be able to penetrate its surface and stain it).
 2. Stain the smear with malachite green for 20 to 30 minutes.
 3. Wash with water and stain with safranin for 30 seconds.
 4. Wash with water, dry, and examine under the oil immersion lens.

By this procedure, the spores will stain green and the bacterial cells red.
 - B. Negative staining of spores: In this procedure the spores will not be stained and they will appear as shiny bodies while the bacterial cell will be stained.
 1. Make a smear and after drying in the air, fix it by passing it 2-3 times over the flame.
 2. Stain it with the methylene blue stain for 1 minute.
 3. Wash, dry, and observe under the oil immersion lens.

Note: Instead of staining the smear with methylene blue you can stain it with the Gram's staining procedure. Spores will still remain colorless while the bacteria,

Laboratory Session # 3 cont.

depending on their Gram reaction will stain either violet or red. Thus by making just one smear one can know the Gram reaction, shape, size and arrangement of the cells as well as the presence of spores. Bacteriologists, in order to save time and to speed up the identification of the organism prefer to use those tests which are able to give a large amount of information in the least number of steps and in the least amount of time.

Note: Spores are most readily found in older cultures, a 24 to 48 hours old culture is, therefore, ideal for their demonstration.

Laboratory work assignment:

Your instructor will show you how to stain spores by both the procedures. Then stain the culture by both the procedures. Note the Gram reaction and also look for spores in this smear. Complete the assignment sheet and hand it in to your instructor. This assignment is worth 50 points. (Assignment # 4)

1. Capsules:

Certain bacteria when grown in the presence of media rich in sugars or when they are growing inside the body of man or animal have the capacity to produce an envelope which surrounds the bacterial cells. This envelope is called a capsule. The capsules are of great advantage to the bacterial cells because when they are present the white blood cells are not able to engulf and destroy the bacteria. As a result the bacteria are able to grow freely in the animal body and produce disease.

Since capsules are not produced by all bacteria, their presence is of differentiating value. Their presence or absence is determined while identifying certain Gram negative rods and Gram positive cocci.

Most capsules primarily consist of carbohydrates and as such they do not stain readily. Their presence, therefore, is detected by the negative staining procedure.

1. Place a very small amount of congo red on a clean slide. Mix a small amount of the bacterial culture in the congo red and spread it in the form of a thin smear. Allow the smear to dry in air. Do not fix by heat.
2. Wash the smear with a few drops of Maneval's solution. Dry in air and examine under the oil immersion lens. Look for the presence of a capsule. It is the colorless area in between the red stained bacteria and the blue background.

Your instructor will now show you how to do the capsule stain. Following the demonstration, stain all the three cultures by the capsule staining procedure. Note which of the three cultures have capsules. Complete the assignment sheet and hand it in to your instructor. This assignment is worth 30 points. (Assignment # 5).

Assignment # 4

Student identification Number: _____

1. Spores

Name of the organism	Gram Reaction	Name of the staining procedure	Presence of spores	Drawing
		Malachite green method		
		Simple stain		
		Gram stain		

Laboratory Session # 3 cont.

Certain members of which of the following 5 groups of bacteria would you expect to have spores?

Gram+ rods

Gram+ cocci

Gram - rods

Gram - cocci

Gram - spirals

Assignment # 5

Student identification Number: _____

Name of the organism	Capsules	Drawing
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Members of which of the following 5 groups of bacteria may have capsules?

Gram+ rods

Gram+ cocci

Gram - rods

Gram - cocci

Gram - spirals

Laboratory Session # 3 cont.

Laboratory Session # 10 cont.

Classification and identification of Bacteria - III

Your experience in the laboratory by now should have enable you to follow written directions. As far as possible demonstration will, therefore, be omitted and you will be allowed more and more to work on your own. Prepared slides and tubes showing the positive and negative reactions will be acceptable. These your instructor will set up for you to see so that you know how to judge your preparations.

In the laboratory experiments for today, you will learn to do the following:

1. Staining and recognizing metachromatic granules
2. The acid fast staining procedure and its importance
3. Motility of bacteria
- I. The Metachromatic Granules: Certain Gram positive bacilli, especially those belonging to the genus *Corynebacterium* contain within their cells small granules which when stained with methylene blue stain darker than the rest of the cell. Using the following two cultures you are to learn to recognize the presence of these granules. Report your results on the assignment sheet
 - a. *Corynebacterium*
 - b. *Escherichia coli*

Procedure:

1. On a clean slide prepare a smear from each of the two organisms
Heat fix
2. Stain with Acidified methylene blue solution for 1 minute.
3. Wash the smear gently, dry and examine

Another procedure to stain metachromatic granules employs the Albert's stain. This procedure is as follows:

1. On a clean slide prepare a smear from the each of two cultures. Fix with heat.
2. Stain with Alberts stain for 1 minute
3. Rinse with water and stain with Lugol's iodine solution for 1 minute
4. Rinse with water and examine

- II. The acid fast staining procedure: The acid fast staining procedure is another very important staining procedure in microbiology. It is used for the identification of *Mycobacterium tuberculosis*, the organism which causes tuberculos (also called TB). In the laboratory, the sputum from patients suspected to be suffering from tuberculosis is examined by this procedure. The bacteria belonging to the genus *mycobacterium* are difficult to stain, but once they are stained they are not decolorized with acid alcohol. Other bacteria present in the sputum are easily decolorized by this treatment. These are then stained with methylene blue so that they become blue. The initial stain used in the procedure is Carbol Fuchsin which is red. The organisms of tuberculosis, therefore, stat red.

Procedure:

1. Prepare a smear from the culture of *Mycobacterium smegmatis*
2. Fix the smear by passing it through the flame.

Laboratory Session # 3 cont.

3. Cover the slide with Kinyoun's carbol fuchsin for 10 minutes. Do not let the stain dry on the slide. Put more stain on as needed,
 4. Wash with water.
 5. Decolorize with acid-alcohol until no more red dye washes off the slide (follow this step as demonstrated).
 6. Wash with water and dry with a paper towel. Stain the Loeffler's methylene blue for 10 seconds.
 7. Wash with water. Dry and examine. Complete the assignment sheet.
 8. Obtain 2 smears made from the sputum of persons found suffering from Tuberculosis. These smears are prepared from sterilized sputum and thus carry no danger. Stain these by the above method and look for rod shaped red organisms.
- III. Determination of bacterial motility: Some bacteria are equipped with whip-like appendages called flagella. These bacteria are, therefore, able to move about in the medium in which they are growing. Since only certain bacteria are able to move about, determination of the capacity of bacteria to move about (called motility) has become an important differential test and aids in the identification of organisms.

There are two ways for determining bacterial motility:

- (1) By suspending the organisms in liquid in the form of hanging drop
- (2) By inoculating the organisms in the motility test medium.

a. The Hanging drop method:

Note: broth cultures are used for this purpose.

Your instructor will first demonstrate how to set up a hanging drop. Following the demonstration you check the motility of: Proteus vulgaris and Staphylococcus epidermidis. The procedure is as follows:

1. Obtain a cavity slide.
2. Using a tooth pick, put a very small amount of vaseline around the cavity making certain that the vaseline is at least 1/8 inches away from the edge of the cavity and has not entered in the cavity.
3. Now take a cover slip. Put it on a clean surface on your desk
4. With a sterile bacteriological loop remove a very small drop of the broth culture and place it in the center of the coverslip.
5. Now pick up the cavity slide and invert it so that the cavity with the vaseline around it is facing downwards. Place the slide over the coverslip in such a way that the cavity is completely sealed by the coverslip. Do not press hard
6. Now pick up the slide and the coverslip will come with it. Invert the slide so that the drop of culture on the coverslip is now hanging in the cavity.
7. Examine this preparation under the microscope. The slide is placed on the microscope stage in such a way that the coverslip is towards the lens. Use 10X objective first. Cut down the light by closing the iris diaphragm. Focus and observe. This will take patience. Next switch over to the 40X objective and observe.

Laboratory Session # 3 cont.

Laboratory Session # 10 cont.

NOTE: You will see hazy outlines of bacteria moving either from one place to the other in the field of view or just bouncing about. It is necessary that you learn to differentiate such movements. Bacteria are considered motile only when they are able to move from one place in the field to the other. Just bouncing about is not true motility. It is called Brownian motion and is due to the fact that the molecules of the liquid are pushing the organisms around.

b. The motility test medium method:

Motility medium is semi-solid. It is stab inoculated with the needle with a pure culture of bacteria. The tube is incubated until the next day. Cultures which are motile spread in the medium, Non-motile bacteria stay where they were inoculated and grow in that area only,

Test the motility of the following organisms:

Proteus vulgaris

Staphylococcus epidermidis

Procedure:

1. Inoculate the organisms separately in the semi-solid motility medium. Use inoculating needle and stab the media just once with the needle carrying the culture, Incubate at room temperature until the next laboratory session.
2. Observe and record results in the assignment sheet.

Assignment# VI

Student identification

Lab. Session # 8

number _____ Assignment sheet

Complete the following tables

Laboratory Session # 3 cont.

Name of the organism	Metachromatic granules	Color of cells	Color of granules	Hanging drop method	Culture method
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Name of the organism	Acid fast	Color if cell
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Name of the organism	Motility by Hanging Drop	Motility by Culture
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Consider that you have to teach somebody how to do the motility test. In your own words explain the procedure step by step to him. Indicate what step should be carried out carefully and what he should look for. Also include what difficulties he may encounter.

Special reading assignment for Lab. # 10: Your instructor will allow you time to read the following pages and a written examination will follow.

THE CYTOLOGY OF BACTERIAL CELL

The cytoplasm of a typical bacterial cell is enclosed in a cytoplasmic membrane and a cell wall which is on the outside of the cytoplasmic membrane. Certain bacteria: also contain a capsule which is located on the outer surface of the cell wall. Some bacteria also contain a slime layer which lies on the outside of the cell wall or the capsule.

The cytoplasmic membrane is a thin membrane responsible for selective permeability of the cell. It is this membrane which governs the entry of various food materials into the cell. It is thick and is shown to contain tiny pores size. From the chemical standpoint, the membrane is rich in protein, lipids, and nucleic acid and stains intensely with the basic dyes. In fact it is this boundary of the cell which is seen in the stained preparations.

The cell wall mainly consists of carbohydrates and is difficult to stain. It acts as a sieve but does allow free passage of nutrients to maintain the shape of the cell. When the cell wall is removed by mechanical means (grinding, sonication, etc.) or when it becomes defective due to interference with its synthesis brought about by antibiotics such as penicillin the cell is not able to retain its shape and in fact becomes spherical and if not maintained in a hypertonic environment (consisting of media containing 5% sodium chloride or 20% sucrose) will undergo spontaneous lysis and dissolution. The average size of bacterial cell wall is reported to be thick for Gram positive bacteria and thin for the Gram negative bacteria.

The capsule lies outside the cell wall in bacteria which retain it. It is considered to be composed of material excreted by the cell. Such material is produced by all cells, but it is generally soluble in the media and, therefore, does not accumulate as a capsule around the cell. Only in those bacteria where it is insoluble in the media, does the capsule develop. Slime layer is of similar nature but is not organized in the form of a definite structure.

The production of both the slime layer and the capsule is enhanced by growing the culture at a temperature lower than the temperature for optimal growth in media containing sugars. Their production is also potentiated in vivo (inside the body of man and animals). Here they play an important role. Capsulated bacteria are not ingested by the white blood cells (phagocytes) and, therefore, are able to grow and produce disease while the non-capsulated bacteria are ingested by the white blood cells and are destroyed.

The chemical composition of the capsule and the slime layer is mainly polysaccharide (carbohydrate). Only certain bacilli contain capsules which consist of proteins. Being mainly carbohydrates, the capsules are not readily stained in the laboratory. They are thus detected by the negative staining procedure. Some direct staining procedures employing Alcian blue as the stain however are available.

Within the cytoplasm, a bacterial cell contains a number of granules and structures. Some of which are easily visible under the light microscope. For instance, the lipid (fat) granules are readily seen by staining with sudan black dyes. Similarly

Laboratory Session # 3 cont.

the metachromatic granules contained within the cytoplasm of certain bacteria can be seen by staining the cells with alcoholic, methylene blue solution or with special stains such as the Albert's stain.

Other granules or structures present within the cytoplasm are not so readily detected. For instance, the nuclear material of the bacterial cell is masked by the proteins and ribonucleic acids of the cytoplasm. These stain intensely with the basic dyes and must be removed before the underlying nuclear structure can be stained and visualized for their interference must be minimized by suspending the cells in 20-30% gelatin and examining the preparation under the Phase Contrast Microscope.

The proteins and the nucleic acids of the cytoplasm can be removed by treating the cells with 1 N. hydrochloric acid at 60 C. for 10 minutes or by treating the cells with enzymes such as proteinase and ribonuclease. Such treatments break down (hydrolyze) the proteins and ribonucleic acids without destroying the deoxyribonucleic acid (the latter is the chemical substance making up the nuclear material of the cells). Such hydrolyzed cells can then be stained and their nuclear material examined under the light microscope.

The 20-30% solution of gelatin used for examining the nuclear material by Phase Contrast Microscopy has the same light transmitting capacity as the cytoplasm of most cells. Cells suspended in gelatin, therefore, appear to have clear cytoplasm, and the nuclear materials having different light transmitting ability stand out as a contrasting structure in the central part of the cell. Within the cytoplasm of bacterial cells also lies a network of membranes which appears to be continuous with the cytoplasmic membrane. These are called mesosomes and are demonstrated by examining thin sections of bacteria under the electron microscope.

Some species of bacteria have the ability to produce spores. There are hard retractile bodies which are produced only one per bacterial cell. They contain within them all the essential parts of the cells (such as the nuclear material) producing them. The cell eventually dies releasing its spore as a free and mature spore which is highly resistant to environmental influences such as heat, desiccation, (drying) and chemicals and often persists for years in its dormant form. The spore upon finding conditions favorable for its generation, gives rise to one vegetative cell.

Some species of bacteria are motile by means of flagella which originate from within the cytoplasm for basal granules called the blepharoplasts and pass through the cytoplasmic membrane and the cell wall to the outside of the cell. The number and distribution of flagella on the bacteria belonging to different groups is not the same. Following arrangements have been recognized:

Atrichous

Monotrichous

Peritrichous

Lophotrichous

Amphitrichous

Since flagella are difficult to stain, their presence on the bacterial cell is routinely demonstrated by detecting the motility of the organism. This is determined

Laboratory Session # 3 cont.

by examining a hanging drop preparation of the culture under the light microscope (unstained live bacteria are examined so the intensity of light while examining the suspension is reduced by partly closing the iris diaphragm of the microscope) or by cultural techniques. In the latter case the organisms are stab inoculated by means of a bacteriological needle in motility test medium. Motile bacterial culture will spread from the point of inoculation while the non-motile cultures will grow only within the line of inoculation.

Besides these appendages, certain bacteria are shown, by electron microscopy, to contain numerous small, stubby appendages. These are called Fimbria or pili. Their function has been suspected to be that of attachment to surfaces or to other bacterial cells. They are now shown to be evolved in the processes of bacterial conjugation.

Laboratory Session # 11 and 12 cont.

Skills Examination – I Assignment # 7

This examination will enable you to review the past work and to let you see how well you are prepared for what is to come. You will receive no assistance from your instructors today.

Part A:

1. Obtain 2 unknown cultures of bacteria from your instructor and determine their Gram's reaction, shape, and arrangement.
2. Based on the Gram reaction perform additional tests such as spore, capsule, and metachromatic granules stains.
3. Determine motility by inoculating the cultures in separate tubes of motility medium.
4. Streak each culture for isolation on separate TS agar plates. Write your ID # on each plate and hand it in to your instructor.
5. Transfer the cultures to new TS agar slants. Write your ID # on each of the new slants and hand in to your instructor
6. Mark each slide that you have prepared neatly and hand them in to your instructor.
7. Complete the assignment sheet and hand it in to your instructor

This part of the examination is worth 100 points

Part B:

Your instructor will dictate to you a list of 50 selected words (including cultures) taken from Laboratory Session 1 through 8 of this manual. This part of the examination is worth 50 points.

Part C:

You will also receive a multiple choice type examination worth 100 points. The questions will be from all the material covered until the last laboratory session.

			Assignment # 7				
Student identification			Lab. Session # 9				
number _____			Assignment sheet				
Unknown	Gram	Shape	If you were trying to identify this culture,				Acid Fast
number	stain	Arrangement	would you look for:				
			Spores	Capsules	Metachromatic	Motility	
			Granules				character

Laboratory Session # 3 cont.

Unknown number	Spores	Capsules	Motility	Acid Fast character
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Laboratory Session # 13 cont.

Completion of work begun last time. Check and record results of the motility medium inoculated with your unknown culture. Hand in the assignment sheet.

BIOCHEMICAL REACTIONS - I

The differential tests that you have learned to perform by now are the following:

- Gram's reaction
- Determination of shape and arrangement
- Determination of pores, capsules, metachromatic granule, and motility.
- Acid Fast staining reaction.

Notice that in all these differential tests you were concerned with the determination of the staining, morphological, and structural differences among bacteria. The differential tests which you are now going to learn will be concerned with the determination of the ability of different bacteria to use different substances as food materials and the ability to use certain and products. This category of differential tests are called biochemical differential tests or simply biochemical tests. Today in the laboratory you will learn how to perform the following biochemical differential test.

II.

- a. Ability to break down starch
- b. Ability to break down proteins such as gelatin
- c. Ability to break down nucleic acids (the deoxyribonucleic acid or DNA; the chemical material found in the nucleus of cells)
- d. Ability to break down fats such as butter fat

III. Ability to use sugars (fermentation reactions) such as sucrose, glucose, and lactose

You will receive the following cultures for these tests:

Bacillus subtilis

Escherichia coli

Sarcina lutea

Proteus vulgaris

Staphylococcus aureus

Alcaligenes Fecalis or

Pseudomonas aeruginosa

Procedure:

Ability to break down starch:

1. Obtain from your instructor 1 starch agar plate. With a marking pencil mark and divide the bottom of the plate into six sections
2. Write the name of 1 different culture in each of the six sections.
3. With a sterile and cooled needle obtain a small amount of the culture and stab it in the center of the area marked for that culture.
4. Incubate the plates at 37° C until the next laboratory session.

Ability to break down DNA, gelatin, and butter fat:

Follow the procedure you have just used for inoculating the starch agar plates and inoculate 1 plate each of the DNA, gelatin, and lipase media. Incubate plates until the next laboratory session.

Laboratory Session # 3 cont.

II. Ability of bacteria to use sugars (also called fermentation reactions)

In this experiment you will test the ability of bacteria to use (ferment) glucose (also called dextrose), sucrose, and lactose. Ability of bacteria to use many other sugars is similarly tested. These tests are also called sugar fermentation tests.

Bacteria, if they are able to use a particular sugar, will produce from it either acid or acid and gas.

Two kinds of media are commonly used for sugar fermentation tests. One of the media is called the BCP broth and the other is called the CTA medium

BCP Broth (Brom Cresol purple Broth): This medium contains all the nutrients which are needed for the growth of bacteria being tested. In addition to these ingredients the medium also contains Brom Cresol Purple as the indicator and the sugar being tested. If the bacteria are able to use the sugar then they produce acid from the sugar. This acid then changes the color of the Brom Cresol Purple indicator from purple to yellow. If the bacteria being tested cannot use the sugar, then the color of the medium remains purple. If we want to know that the bacteria, in addition to producing acid, also produces gas from the sugar then we also place a small glass tube inside the broth (this is done at the time the medium is prepared; during sterilization the air from the small inner tube gets forced out by the heat and the tube gets filled with the broth). Notice that the inner tube (also called gas vial) is placed in the tube in an upside down position and is completely filled with the broth. If bacteria produce gas from the sugar then the gas gets trapped in the inner tube. Presence of gas bubbles in the inner tube, therefore, constitutes a positive test for gas production by bacteria

CTA MEDIUM (CYSTEIN TRYPTICASE AGAR MEDIUM): This medium is better in the sense that it is richer than the BCP broth and is able to support the growth of many bacteria which do not grow in the BCP broth. This medium contains PHENOL RED AS THE INDICATOR AND ALSO THE SUGAR BEING TESTED. If the bacteria have the ability to use the sugar present in the medium then the medium changes color from red to yellow. This is due to the acid being produced from the sugar. If the bacteria also produce gas from the sugar, then the bubbles of gas get trapped in the medium. This happens because the medium is viscous and is able to trap the gas bubbles.

Laboratory Session work assignment:

For this part of the experiment work in pairs. One of you should test the following 3 cultures:

Escherichia coli

Proteus vulgaris

Alcaligenes faecalis or Pseudomonas aeruginosa

The other partner should test the following 3 cultures:

Bacillus subtilis

Laboratory Session # 3 cont.

Sarcina luteaStaphylococcus epidermidis

Procedure:

1. Obtain 3 tubes each of the BCP glucose, sucrose, and lactose broths. Notice that the contents of all tubes are purple. Mark the name of the sugar that they contain on the tubes. This is very important otherwise you will not be able to tell the tubes apart.
2. Inoculate the cultures separately in separate tubes of each of the three BCP sugars. Incubate at 37° C until the next laboratory session.
3. Similarly inoculate and incubate three tubes each of the CTA glucose, sucrose and lactose.

Laboratory Session # 14 cont.

Biochemical Reactions - II

Lab. Session# 14: Completion of work begun last time.

1 Ability to break down starch, DNA, gelatin and butter fat.

You will flood the plates with necessary reagents to see which of the substances are broken down (hydrolyzed) by the bacteria. Obtain the plates from the instructor.

Note that the bacteria inoculated last time have grown on the Plates. Then proceed as follows to determine if they have hydrolyzed the various substances.

Starch agar plates: Completely flood (pour generously, do not apply drops) the starch agar plate with iodine solution. Note that the media in the plate is slowly turning blue, while areas around some of the bacterial cultures have remain clear. The blue color is due to the reaction between starch and iodine. The clear areas around the bacterial growth indicate that there is no starch in these areas. Since the entire plate before inoculation contained starch, the absence of starch around the growth now indicates that these bacteria were able to break down starch. This they did because they had the capacity to produce the enzyme amylase which diffused into the medium and hydrolyzed the starch. Those bacteria which did not produce amylase did not break down starch. The blue color of starch and iodine complex, in such cases, extends all the way to the growth of the bacterial culture. The test is mostly used for the differentiation of Gram positive bacilli.

Gelatin agar plates: Flood the plate with acidified mercuric chloride solution. Note that the color of the media in the plate is slowly becoming opaque white while areas around certain bacterial cultures continue to remain clear. This is because the mercuric chloride precipitates the gelatin in the medium but where there is no gelatin (because it has been hydrolyzed by the enzyme gelatinase produced by these bacteria) the media remains clear. The test is mostly used for the differentiation of Gram positive bacilli and also Gram positive cocci.

DNA agar plates: Flood the plate with 1 normal solution of hydrochloride acid. Wait for 5 minutes. Notice that the medium is slowly turning to opaque white. This is because of the reaction of DNA with the hydrochloric acid. The areas around the growth where the DNA has been hydrolyzed by the DNase enzyme, have remained clear. The test is mostly used for the differentiation of certain Gram positive cocci especially staphylococci and certain Gram negative rods especially members of the genus *Serratia*.

Spirit Blue agar plates: These agar plates already contain the indicator spirit blue in the medium, so no indicator is needed to develop the reaction. Note the color of the zones around the growth. Cultures that have been able to break down butter fat would be surrounded by clear, dark blue zones. While the cultures which have not been able to hydrolyze butterfat would indicate no change around their growth. Record results in the table. The determination of lipolytic activities are useful in the identification of certain Gram positive cocci and Gram positive rods.

Laboratory Session # 3 cont.

Read the description of BCP and CTA media (see laboratory session 10). Observe the tubes of BCP and CTA sugars which you inoculated last time. Record results below. Use A for acid (yellow color) and AC for acid and gas

Group discussion:

- What is an indicator?
- What is a differential medium?
- What are sucrose, glucose, and lactose?
- How do food materials (nutrients) get into the bacteria?
- What does BCP stand for?
- What are the ingredients in the BCP broth?
- What are the ingredients in the CTA medium? What are their functions?

Laboratory Session # 3 cont.

- h. When bacteria grow in a broth tube, would the broth appear clear or turbid? Why?
- i. Draw a diagram of a bacillus which contains a capsule. Is the capsule found inside or on the outside of the cell? If you stained this bacterium by the Maneval's method, what would be the color of the capsule? What would be the color of the background?
- j. How would you determine in the laboratory if a pure culture of bacteria is able to break down starch? What is the name of the enzyme which breaks down starch? Do you have this enzyme?
- k. How would you determine in the laboratory if a bacterium is able to grow on starch.
- l. Read the pages entitled "the cytology of a bacterial cell. Then list the various parts of a bacterial cell and make a drawing showing the location of these parts in the bacterial cell.

Experiments for today:

Determination of the ability of bacteria to break down hydrogen peroxide:

Some bacteria produce an enzyme called CATALASE. This enzyme is able to break down hydrogen peroxide to oxygen and water. In order to test for the presence of this enzyme in bacteria, the bacteria are first grown in a medium such as Trypticase Soy Agar and then the growth is mixed with a small amount of hydrogen peroxide. If the enzyme catalase was present in the bacteria then bubbles of gas (oxygen) are rapidly produced in the mixture. If, on the other hand, the bacteria did not contain catalase then bubbles of gas are not produced.

Procedure:

The following cultures are provided for this experiment:

Staphylococcus aureus

Streptococcus lactis

Obtain one TS agar plate. Stab inoculate the two cultures in separate halves of the plate. Circle the location of the plate where the culture is inoculated and identify this

Laboratory Session # 3 cont.

area by the name of the culture. Incubate the plate at 37° C until the next laboratory session.

During the next laboratory session, we will complete this experiment. We will place a drop of 3% hydrogen peroxide on the bacterial growth. If gas bubbles appear we will record that the culture produced catalase. -Otherwise it will be recorded as negative for catalase.

Determination of the ability of bacteria to reduce nitrates:

Some bacteria are able to break down nitrates to nitrites and some can break the nitrites to nitrogen which appears as gas and escapes into the atmosphere.

In order to determine the ability of bacteria to use nitrates, the culture to be tested is inoculated in nitrate broth and then incubated for 12 to 24 hours. At the end of this time, the culture is tested for the presence of nitrites by the addition of 10 drops of sulfanilic acid and 1 to 2 drops of dimethyl-alpha-naphthylamine solution. If a red color appears in the medium then the medium is recorded to contain nitrites. This culture is thus recorded as positive for the ability to reduce nitrates.

If however, there is no change in color at this time, then it means that:

1. Either the nitrites have changed all the way to nitrogen gas and thus have escaped into the atmosphere
OR
2. Nitrates of the medium have not been changed, i.e. the organism not have the ability to convert nitrates to nitrites.

In order to determine which one of the above 2 possibilities have happened, add a small amount of zinc dust to the tube. Now if the red color appears then it means that the media contained the nitrates and bacteria have not been able to break them. This then indicates a negative test.

If, upon the addition of zinc, the color did not change to red, then it means that the nitrates have all been used up and have escaped into the atmosphere as the nitrogen gas. This then constitutes a positive reaction.

Procedure:

Obtain three tubes of nitrate broth and inoculate two of these tubes separately with Bacillus subtilis, Bacillus megatherium. Keep the third tube as control. Incubate all three tubes at 37° for 24 hours. During the next laboratory session you will add to each tube 10 drops of sulfanilic acid and 2 drops of dimethyl-alpha-naphthylamine solutions and watch for the appearance of red color. If red color appears you will record the culture as positive for the reduction of nitrates. If red color does not appear you will add a small amount of zinc dust (avoid contact with skin). If red color now appears then you will record the culture as negative for the reduction of nitrates. If color does not change even after the addition of zinc then you will record the culture as positive for the reduction of nitrates.

Determination of the ability of bacteria to break down red blood cells (hemolysis).

A number of bacteria have the ability to break down red blood cells or produce discoloration on the plates containing red blood cells. Medium containing blood, therefore, is a good differential medium.

Laboratory Session # 3 cont.

On blood agar:

Some cultures will produce clear zones around colonies because they hemolysis all the red blood cells around their colonies. Such cultures are called beta hemolytic.

Some cultures will produce green zones around their colonies. These are called alpha hemolytic.

Some cultures will produce no zones around their colonies. These are called ahemolytic or gamma hemolytic.

Use the following cultures for this experiments:

Streptococcus viridas, pneumococcus pneumonia, streptococcus pyogenes, and Streptococcus ahemolyticus.

Laboratory work assignment for # 14:

Determine which of the above cultures are able to break down red blood cells present in the blood agar medium. Obtain 4 blood agar plates and streak each of the organisms on separate plates. Mark the plates with the name of the organism inoculated. Incubate the plate at 37° C until the next laboratory session. Rub a swab in the inside of your mouth. Inoculate it on the plate and streak the rest of the plate to get isolated colonies.

During the next laboratory session determine which of the cultures are: alpha hemolytic, beta hemolytic, or gamma hemolytic. Also note the appearance of their colonies. Classify colonies growing on the plates inoculated with the material from your mouth on the basis of their hemolytic characteristics.

Continue discussion begun during the last laboratory session

Laboratory Session # 15 cont.

Biochemical Reaction III

Completion of the experiments begun last time.

Production of Catalase:

You had inoculated one TS agar plate with 2 different cultures of bacteria. Today obtain that plate from the incubator and proceed as follows:

Place a drop of 3 per cent hydrogen peroxide on the bacterial growth. Mix gently and watch for the appearance of bubbles. If bubbles appear rapidly then record the culture as positive for catalase production. If bubbles do not appear then record the culture as negative for catalase.

Culture	Catalase Production

Nitrate Reduction:

Last time you had inoculated 2 tubes of nitrate broth separately with different bacteria. You had also kept one tube of nitrate broth as control. In order to complete the test, proceed as follow:

Add to each tube 10drops of sulfanilic acid and 2 drops of dimethyl-alpha-naphthylamine solutions. Watch for the appearance of red color. If red color appears record the culture as positive for the reduction of nitrates. If red color does not appear, add a small amount of zinc dust (avoid contact with skin). If red color now appears then record the culture as negative for the reduction of nitrates. If color does not change even after the addition of zinc, then record the culture as positive for the reduction of nitrates.

Laboratory Session # 15 cont.

Culture	Nitrate Reduction

Ability of bacteria to break down blood agar:

Interpret results as: alpha hemolytic, beta hemolytic, or gamma hemolytic.

Record results below:

Culture	Hemolytic Reaction	Size, color, and general appearance of the colonies

Experiments for today:

Laboratory Session # 3 cont.

Production of Indole from Tryptophane:

All proteins are complex substances and are made of about 20 different amino acids. One of these 20 amino acids is called tryptophane. Some bacteria have the ability to change tryptophane to a substance called Indole. In order to determine the ability of the bacteria to change tryptophane to indole, a pure culture of the bacteria is inoculated in tryptone broth. The medium is then incubated for 12 to 24 hours and then Kovac's reagent is added to the medium. If the culture had been able to change tryptophane to indole, then a red color will develop.

Procedure:

The following cultures are provided for this experiment:

Escherichia coli, Aerobacter aerogenes:

Obtain three tubes of Tryptone broth and inoculate them separately with a pure culture of different bacteria. Incubate the tubes until the next laboratory session. During the next laboratory session add a few drops of Kovac's reagent to the tubes and watch for the appearance of a red color. This indicates that the media now contains indole and the culture is recorded as positive for the production of indole from tryptophane. If the color does not change to red then, the culture is recorded as negative for the production of indole.

Methyl red reaction:

Certain bacteria when grown in a medium containing peptone (partly broken down proteins) and glucose produce more acid than other bacteria. The large amount of acid produced by bacteria is detected by adding a few drops of Methyl red to the broth culture. In the presence of sufficient acid, the methyl red indicator color the medium red. Otherwise the color changes to yellow.

Procedure:

Inoculate two tubes of the MR VP medium separately with Escherichia coli and Aerobacter aerogenes. Incubate these tubes until the next laboratory session. During the next laboratory session obtain these tubes and add to them a few drops of the methyl red indicator. Note the change of color of the culture. If it becomes red, then mark the culture as Methyl red positive. Otherwise mark it as negative.

Voges-Proskauer Reaction:

While certain bacteria such as Escherichia coli can produce sufficiently large amount of acid from the sugar provided in the MRVP medium, others like the Aerobacter aerogenes cannot do so. Instead they produce another substance called Acetyl methyl carbinol. In the culture of Aerobacter aerogenes, therefore, this material accumulates in the form of acetone. Its presence is detected by adding 10 drops of potassium hydroxide and 20 drops of alpha naphthol to the culture of the organism. If the acetone is present in the medium then the potassium hydroxide converts it to acetyl methyl carbinol which reacts with the alpha-naphthol and produce a red color. The presence of red color, therefore, determines a positive Voges Proskauer (or VP) reaction. The absence of this red color determine a negative reaction.

Procedure:

Inoculate two tubes of MRVP medium separately with Escherichia coli and Aerobacter aerogenes. Incubate these tubes until the next laboratory session. Then obtain these tubes and add to them 10 drops of potassium hydroxide and 20 drops of

Laboratory Session # 3 cont.

alpha naphthol. Shake the tubes and wait for the appearance of red color. The presence of red color indicates positive reaction.

Ability to grow on citrate alone (citrate Utilization test):

Certain bacteria are so versatile that they are able to grow on citrate alone. Others require many more substances. The ability to utilize citrate as the sole source of food, therefore, is an important differential test. Simmon's citrate agar is commonly used for determining the ability to use citrate. This medium besides containing citrate (in the form of sodium citrate) also contains an indicator Brom Thymol Blue. If the bacteria grow on this medium then the color of the medium changes from light green to dark blue. This happens as the result of the growth of the bacteria causing the medium to get alkaline.

Procedure:

Inoculate tubes of citrate agar with the cultures of Escherichia coli and Aerobacter aerogenes. Incubate these tubes until the next laboratory session. At that time note and record the color of the medium. Dark blue color will indicate that the culture has grown on the medium and, therefore, it will be recorded as citrate positive. The culture which did not change the color will be recorded as citrate negative.

The above four test are collectively called IMViC reaction. The word is made by taking I from Indole, M from Methyl red, V from Voges Proskauer, and C from Citrate. The small i is added so that the word can be pronounced.

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Laboratory Session # 16 cont.

Biochemical Reactions IV

Completion of experiments begun last time:Production of Indole:

You have inoculated two different cultures separately in Tryptone broth. Obtain these tubes and proceed with the test as follows:

Add a few drops of Kovac's reagent to the tubes and watch for the appearance of a red color. This indicates that the media now contains indole and the culture is recorded as positive for the production of indole from tryptophane. If the color does not change to red, then the culture is recorded as negative for the production of indole.

Methyl red reaction:

You have inoculated 2 tubes of MRVP medium separately with Escherichia coli and Aerobacter aerogenes. Obtain these tubes and add to them a few drops of the methyl red indicator. Note the changes of color of the culture. If it becomes red, then mark the culture as methyl red positive. Otherwise, mark it as negative.

Voges-Proskauer Reaction:

You have inoculated two tubes of MRVP medium separately with Escherichia coli and Aerobacter aerogenes. Obtain these tubes and add to them 10 drops of potassium hydroxide and 20 drops of alpha naphthol. Now gently shake the tubes and place them in the rack. Watch for the appearance of a red color. This indicates a positive reaction. If no red color appears within 15 minutes, mark the test as negative.

Citrate Utilization test:

Last time you had inoculated 2 tubes of citrate agar with Escherichia coli and Aerobacter aerogenes. Today note the color of the medium. If it has become a dark blue color, it is because it was able to use citrate and grow. Mark this culture as positive for the utilization citrate. The tube which shows no change of color is negative for citrate utilization.

Record results of all 4 tests below:

Culture	Indole	Methyl red	Voges Proskauer	Citrate
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Experiments for today:Production of Pigments:

Certain bacteria when grown primarily at room temperature are able to produce colored materials called pigments. Some of the bacteria produce pigments which are soluble in the medium. Therefore, these are able to color the entire medium. For instance the bacterium Pseudomonas aeruginosa is able to produce a greenish color pigment which makes the pigments stay confined to the bacterial cell. These bacteria, therefore, do not color the medium, but their colonies are variously

Laboratory Session # 3 cont.

colored. Presence of pigments is useful in the identification and classification of bacteria.

Production of pigments by bacteria is determined by growing the culture on suitable medium. Generally medium containing sugar which is utilized by the bacteria is helpful and the incubation of the culture at room temperature also enhances the production of the pigments. Certain special media have also been designed which enhance the production of pigments by certain bacteria. For instance, medium containing milk of glycerol enhances production of pigments by Staphylococcus aureus. A medium called Pyocyanin agar enhances the production of pigments by Pseudomonas aeruginosa. Temperature also affects the production of pigments.

You will receive broth cultures of:

Pseudomonas aeruginosa, Staphylococcus epidermidis, Staphylococcus aureus, and Serratia marcescens:

Laboratory work assignment:

Determine at what temperature and on which of the following two media the above culture produce the maximum amount of pigment.

Procedure:

Inoculate each of the cultures separately on 2 TS agar slants and 2 milk agar slants. Incubate 1 set of TS agar slants and 1 set of milk agar slants at 37° C. incubate the other set at room temperature. Observe the color of the growth during the next laboratory session and record results.

Determination of hydrogen sulfide production:

Certain bacteria have the ability to produce hydrogen sulfide from proteins or from their break down products. One of the medium used for determining this ability of bacteria is the Lead Acetate agar. In the presence of hydrogen sulfide, this medium turns brown around the growth.

Procedure:

Inoculate two tubes of lead acetate agar separately with Escherichia coli and Proteus vulgaris respectively. Incubate them at 37 ° C until the next session. Note the brown color. Record results.

Determination of the break down of urea:

Certain bacteria, especially those belonging to the genus *Proteus* have the ability to break down urea to ammonia. The ammonia is alkaline and brings about a color change in the medium from light yellow to bright red. The indicator in the medium is phenol red.

Procedure:

Inoculate 2 tubes of urea broth separately with the cultures of Escherichia coli and Proteus vulgaris respectively. Incubate until the next laboratory session. Note the color and record results. Bright red color indicates positive reaction because the indicator, phenol red, changes to an intense red color in the presence of alkaline conditions produced by the presence of ammonia.

Laboratory Session # 3 cont.

Oxidase Reaction:

When oxidase reagent (a freshly prepared 1% solution of tetramethyl para-phenylenediamine) is poured on bacterial colonies, colonies of certain bacteria; turn first pink and then black. This is .due to the enzyme oxidase produced by these bacteria. Such colonies, if needed for further tests should be transferred to fresh medium while still in the pink stage because when they have turned black the cells have died.

Procedure:

Inoculate cultures of Staphylococcus aureus, Escherichia coli, and Neisseria catarrhalis in separate areas of a TS agar plate. Inoculate these cultures by the radial streak method. Incubate the plate at 37° C until the next laboratory session.

Laboratory Session # 17 cont.

Biochemical Reaction V

Production of pigments

Completion of experiments be n last time:

Production of Pigments:

You had inoculated different cultures on agar slants and had incubated them at 37° C and at room temperature.

Observe the color of the growth and that of the medium. Record results. Indicate the pigments production by a + sign. If more pigment is produced at one temperature than the other indicate by a ++, +++ or a ++++ sign.

Culture	Pigment production				Color of medium	Color of growth
	TS		Milk			
	37° C	Room temperature	37° C	Room temperature		

Based on your results which of the cultures produced soluble pigments and which ones produced the insoluble pigments. Also which ones produces both types of pigments?

Culture	Soluble	Insoluble	Both
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Production of Hydrogen Sulfide:

Note and record the name of the culture which has been able to change the lead acetate agar to a brownish color. This culture has produced hydrogen sulfide. You will, therefore, record it as positive for the production of hydrogen sulfide

Laboratory Session # 3 cont.

Culture	Hydrogen Sulfide Production

Urea Broth:

Note the color of the urea broths inoculated during the last session. The organism which is able to change the color of the medium from light yellow to bright red today has been able to break down urea to ammonia. Note which of the cultures have done this. Record your results.

Laboratory Session # 17 cont.

Culture	Urea Breakdown

OXIDASE TEST:

Place a drop of the oxidase reagent on the growth of each of the three organisms. Observe the change in color of the growth. The organisms which changed color are oxidase positive. Record results.

Culture	Oxidase Reaction

Experiment for today:

The Coagulase Test:

Some bacteria have the ability to clot plasma. This is done because they are able to produce a substance called coagulase. Detection of coagulase production is used for identification of Staphylococcus aureus. The test is performed as follows:

1. Add 1/2 ml of plasma to a sterile tube
2. Add a small amount of bacterial culture.
3. Mix and incubate the tube at 37° C.
4. Observe the tube every hour. If the bacteria produced coagulase, the plasma which was previously liquid would become clotted. Some members of Staphylococcus aureus are able to clot the plasma very quickly and the clot is firm. Others are slow and take as long as 24 hours, and the clot is sometimes not very firm. Any amount of clotting within 24 hours however constitutes a positive test.

Laboratory work assignment:

Obtain two sterile tubes (10mm X 80mm). Place 1/2 ml of sterile plasma in each of the tubes.

Inoculate heavily one tube with Staphylococcus aureus and the other tube with Staphylococcus albus.

Place the tube at 37 ° C for 1-2 hours and look for the formation of clot. Record your results.

Culture

Coagulase Reactions

Notes: This test is routinely continued for 24 hours, but for the purpose of the class, 4 hours of incubation is considered sufficient.

Blood consists of 2 parts: a liquid portion called the plasma and the solid portion which is the various cells. When blood is obtained from the body, it tends to clot. This clotting is prevented by collecting the blood in a solution of sodium citrate.

Plasma: Plasma from the blood is obtained by centrifuging it. This process makes the cells settle to the bottom of the tube leaving the clear plasma on top. See demonstration by instructor.

Serum: If we allow the blood to clot, it will eventually release a small amount of fluid. This fluid does not have the capacity to clot and is called serum.

Kligler's Iron Agar:

Up till now you have been using differential media to perform individual tests. You used glucose broth containing brom cresol purple indicator for determining the breakdown of glucose. For lactose you used lactose broth containing the same indicator. For determining hydrogen sulfide, you used lead acetate agar.

From the practical standpoint bacteriologists feel that routine use of such test is time consuming and is not economical. So they have devised special media in which they can perform many tests together.

Kligler's Iron Agar is one such special medium. By using this media we can determine if:

1. bacteria use glucose and produce acid
2. bacteria use glucose and produce acid and gas
3. bacteria use glucose and lactose and produce acid
4. bacteria use glucose and lactose and produce acid and gas
5. bacteria produce hydrogen sulfide also

This medium contains:

Beef extract, yeast extract, peptones (these are for the growth of the bacteria).

0.1% glucose and 1% lactose (these are to determine if the bacteria being tested can use glucose or glucose and lactose).

Ferrous sulfate and sodium thiosulfate (these are to detect the production of Hydrogen sulfide; they are the indicators of hydrogen sulfide production)

Phenol red (as indicator of acid production)

Agar (to make the medium solid).

This medium is dispensed in tubes in such a way that it provides both a slant surface for inoculation and a butt for stabbing (stab inoculation).

In order to learn the mechanism of the Kligler's Iron Agar, we should know the following:

1. When bacteria grow in the presence of a sugar which they can use and they also have oxygen available to them (as on the surface of the slant), then from the sugar they produce acid which in the presence of oxygen changes to carbon dioxide and water. This means that the indicator, phenol red will first change to

Laboratory Session # 3 cont.

yellow (because of the acid) and as all the acid is converted to carbon dioxide and water, the phenol red will return to its original red color. This will happen on the surface of the slant.

2. The medium contains only 0.1% glucose and 10 times as much lactose. If the bacteria use only glucose then the acid produced from it will be converted to carbon dioxide and water within a short period of 12 to 18 hours. The color of the slant portion will return to its original red color within this time, but if the bacteria use lactose also then there will be a good deal of acid present to keep the indicator yellow for periods much longer than 12 to 18 hours.
3. When the bacteria are growing in the presence of a sugar that they can use, but there is no oxygen available to them (as in the butt of the Kligler's Iron Agar) then they are only able to produce acid or acid and gas from the sugar. The phenol red indicator will thus only change to yellow and will not return to its original color. This happens in the butt portion of the slant.
4. If the bacteria produce hydrogen sulfide, the butt portion will turn black.
5. The production of gas is indicated by the bubbles that get trapped in the medium or by the appearance of cracks in the medium. This happens in the butt portion.

Various combination of reaction on Kligler's Iron Agar and their meaning

Color Reaction of Slant	Butt	Meaning
Red	Red	Neither glucose nor lactose was used
Red	Yellow	Glucose was used and only acid was produced
Red	Yellow; gas	Glucose was used and acid and gas produced
Red	Yellow with or without gas but black also	Glucose was used with acid or acid and gas production; hydrogen sulfide was also produced.
Yellow	Yellow	Glucose and lactose were used and only acid produced.
Yellow	Yellow; gas	Glucose and lactose were used with acid and gas production.
Yellow	Yellow with or without gas but black also	Glucose and lactose were used with acid or acid and gas production, hydrogen sulfide was also produced.

Inoculation of Kligler's Iron Agar:

Procedure:

Use inoculating needle. Sterilize and pick the culture with it. Inoculate the surface of the slant and then stab the butt. Incubate at 37° C for 12 to 18 hours.

Laboratory Session # 3 cont.

Note: If for some reason you are not going to be able to record reactions immediately after the incubation period is over, refrigerate the tubes.

Laboratory assignment:

Your instructor will first explain how the Kligler's Iron agar works. Then you inoculate tubes of Kligler's Agar (both slant and butt) separately with (1) Escherichia coli, (2) Aerobacter aerogenes, (3) Pseudomonas aeruginosa, (4) Alcaligenes faecalis, and (5) Proteus vulgaris. Incubate all tubes at 37° C for 12 to 18 hours.

Laboratory Session # 18 cont.

BIOCEMICAL REACTION VI

Completion of work begun last session # 17:

Kligler's Iron Agar: Note the color of the slant and the butt of this medium. Record your results as per details given in the last session. A table for this purpose is provided below:

Name of the culture	Color of slant	Color of butt	Interpretation
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At the completion of this section, your instructor will provide you with a prepared summary of procedures for biochemical reactions.

Experiments for today:

Selective media:

You have by now learned that a number of different kinds of bacteria are found in various samples that are received in the microbiology laboratory for testing. Most of these bacteria are normal inhabitants of these samples and the sites from which these samples are obtained. These are generally not harmful, and therefore, are considered unimportant. From the medical point of view, the physician is interested in knowing if the sample which he sends to the laboratory for analysis contains the harmful bacteria, i.e., the bacteria which have been shown conclusively to produce certain diseases.

We can isolate these bacteria from the sample by streaking the culture on solid agar medium. Microbiologists, however, have found that quite frequently, the harmful bacteria which microbiologist is trying to isolate are few in number and that the normal inhabitants of the sites from which the sample was obtained have the tendency to overgrow these bacteria. The isolation of the bacteria that the physician is interested in, therefore, becomes a bit difficult.

In order to overcome these problems, microbiologists have devised special media which they have named Selective Media. These media are so designed that the ingredients contained in them are toxic to most or all of the bacteria that are not sought for. While the bacteria that are being searched for are not harmed to any great extent and these are able to grow out on the medium. For instance, if one wishes to isolate Staphylococcus aureus from the nose of healthy individuals, it can be readily done by growing the specimen obtained from the nose of the individuals

Laboratory Session # 3 cont.

on medium containing 7.5% sodium chloride. This concentration of sodium chloride is readily tolerated by Staphylococcus aureus while other organisms are inhibited or even killed. Addition of 7.5% sodium chloride, therefore converts an ordinary medium such as Trypticase Soy agar or Brain Heart Infusion agar into a selective medium for Staphylococcus aureus. There are many more such media designed to isolate specific bacteria or specific groups of bacteria.

Selective and Differential Media:

Since the identification of bacteria depends on the isolation and biochemical reactions of bacteria, microbiologists have designed media wherein not only the desired organism is isolated from the rest of the organisms by selection (due to the presence of a selective agent in the medium), but some of its biochemical characteristics are also determined simultaneously. Such media are called selective and differential media and there are many such media that are used in the bacteriology laboratory. Their preparation is fairly simple. For instance, by adding the sugar mannitol to the Brain Heart Infusion containing 7.5% sodium chloride and also including in the medium an indicator such as phenol red, we are not only able to isolate the cultures of staphylococci, but are also able to simultaneously determine the ability of the culture to use mannitol and produce acid from it.

Laboratory work assignments:

Your instructor will explain the selective and differential media. Then you obtain 2 plates of Phenol red mannitol salt agar (PRMS agar) and 2 plates of Eosine methylene blue agar (EMB agar). Streak for isolation each of the following 4 cultures separately on one half of the EMB and PRMS agar plates. (1) Escherichia coli, (2) Staphylococcus aureus, (3) Staphylococcus albus, and (4) Aerobacter aerogenes. Your instructor will show you how to streak a culture on one half of a plate. The procedure is the same as for streaking the whole plate.

Incubate the plates at 37° C until the next laboratory session. Then observe.

(NOTE: Escherichia coli & Aerobacter aerogenes on EMB plates

Staphylococcus aureus, Staphylococcus albus on PRMS agar)

Laboratory Session # 19 cont.

Biochemical Reactions VII

Completion of work begun last session:

Note which of the cultures are growing on the PRMS and the EMB agar plates. Especially note the color of the growth and the color of the media around the growth.

Your instructor will discuss the usefulness of both the media. Record all information in the following table:

Culture	Medium	Growth and color of growth	Color of medium around growth	Usefulness of the medium
---------	--------	----------------------------	-------------------------------	--------------------------

USEFUL SELECTIVE AND DIFFERENTIAL MEDIA*

Selective and Differential Media

1. Eosin Methylene Blue agar (EMB agar)
2. Salmonella-Shigella (SS) agar

Used for

1. Isolation and differentiation of Gram negative enteric bacilli.
2. Isolation and differentiation of Gram negative enteric pathogens, e.g. Salmonella and Shigella.

Laboratory Session # 3 cont.

- | | |
|---|---|
| 3. MacConkeys Agar | 3. Isolation and differentiation Gram Negative enteric bacilli. |
| 4. Bismuth Sulfite agar | 4. Isolation of Salmonella |
| 5. Phenol red mannitol salt (PRMS) agar | 5. Isolation of coagulase positive staphylococci |
| 6. Vogel Johnson agar | 6. Isolation of coagulase positive staphylococci |
| 7. Tomato juice agar | 7. Isolation of lactobacilli |

*As you come across additional selective and differential media, add them to this list.

Experiments for today:

Skill Examination (Assignment# 8)

You will receive 2 unknown broth cultures of bacteria. Set up appropriate experiments to determine the answers to the following questions. Report your findings to your instructor in a table form.

1. Do they break down (hydrolyse) starch? DNA? Gelatin? Butter fat?
2. Do they ferment glucose? Sucrose? Lactose?
3. Do they break down nitrate? Is the end product nitrite or nitrogen?
4. Do they produce gas from lactose?
5. Are they hemolytic? If so, what type of hemolysis do they produce?
6. Are they motile?
7. What are their IMViC reactions?

Laboratory Session # 3 cont.

8. Are they catalase positive?
9. What type of reactions do they produce on Kligler's Iron Agar?
10. Streak the cultures for isolation on TS agar plates. Hand in these plates to your instructor for grading.
11. Which of the 2 media, CTA or BCP with a gas vial would you prefer for studying the fermentation reaction of bacteria?

Assignment # 8

Student Identification Number _____

Lab. Session# 19

Complete and hand in during laboratory Session # 20

Test	Culture # _____	Culture # _____		
	Interpretation	Color observe	Interpretation	Color observe
Starch				
DNA				
Gelatin				
Fat				
Glucose				
Sucrose				
Lactose				
Nitrate				
Hemolysis				

Laboratory Session # 3 cont.

Motility

Indole

MR

VP

Citrate

Catalase

Kligler's

Laboratory Session # 20 cont.

Completion of work begun last session:

Complete and interpret all results of the skills examination (Assignment # 8).
Hand in your results neatly arranged on the assignment sheet to your instructor. This examination is worth 100 points.

Experiments for today:

Identification of Unknown Organisms

You have been learning to perform and interpret a number of differential staining and biochemical tests. The purpose of these tests was to prepare you so that you could identify unknown bacteria.

Today you will begin the identification of medically important bacteria. Initially, we will confine ourselves to bacteria belonging to the groups: GRAM POSITIVE COCCI and GRAM NEGATIVE COCCI. The medically important bacteria included in these groups are:

GRAM POSITIVE COCCI:

Staphylococcus aureus

Staphylococcus albus (also called Staphylococcus epidermidis)

Streptococcus pyogenes (commonly called beta strep)

Streptococcus faecalis (and other enterococci found in the intestinal tract of man)

Diplococcus pneumoniae:

GRAM NEGATIVE COCCI:

Disease producing (pathogenic) organisms such as:

Neisseria meningitidis

Neisseria gonorrhoeae

Non-disease producing Neisseria such as:

Neisseria Gattarrhalis

Neisseria sicca

Neisseria flava

Laboratory work assignment:

First three unknown cultures:

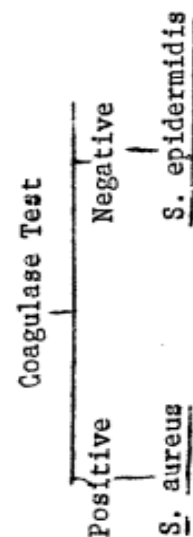
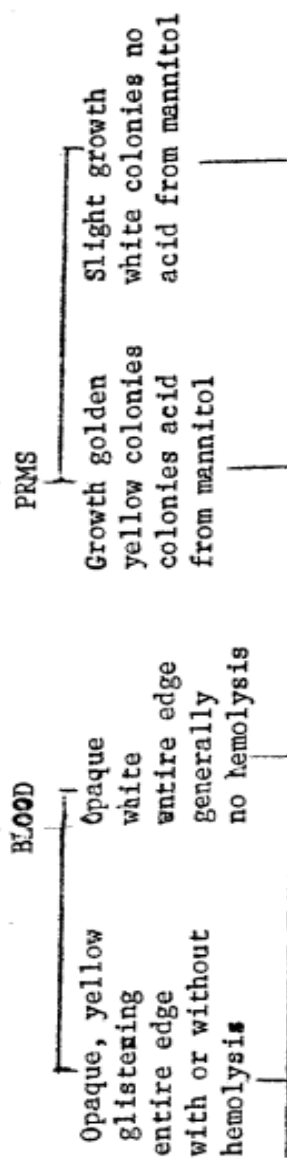
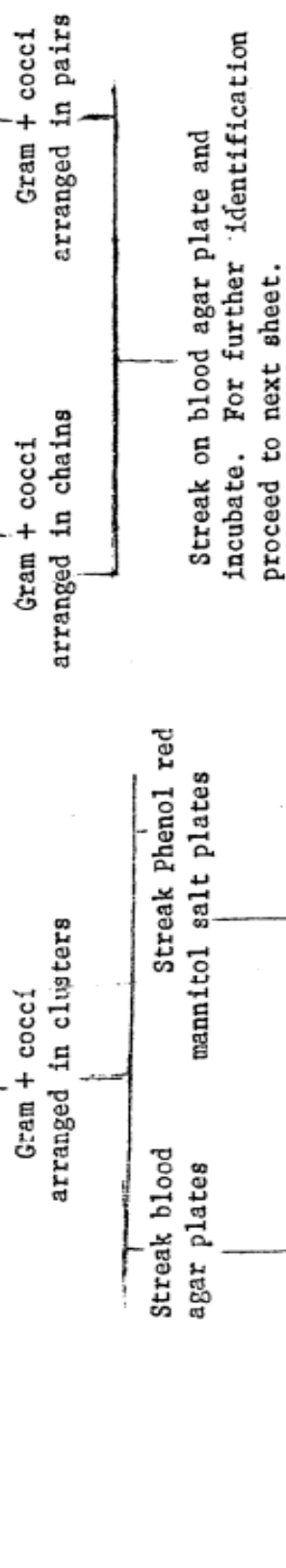
Identify the three unknown cultures of cocci which are made available to you. Note the number written on the culture in your notebook. Keep record of all results. Write summary of your results and conclusions and hand it in to your instructor. This assignment is worth 100 points.

The schemes for identifying these culture are given on the following pages.

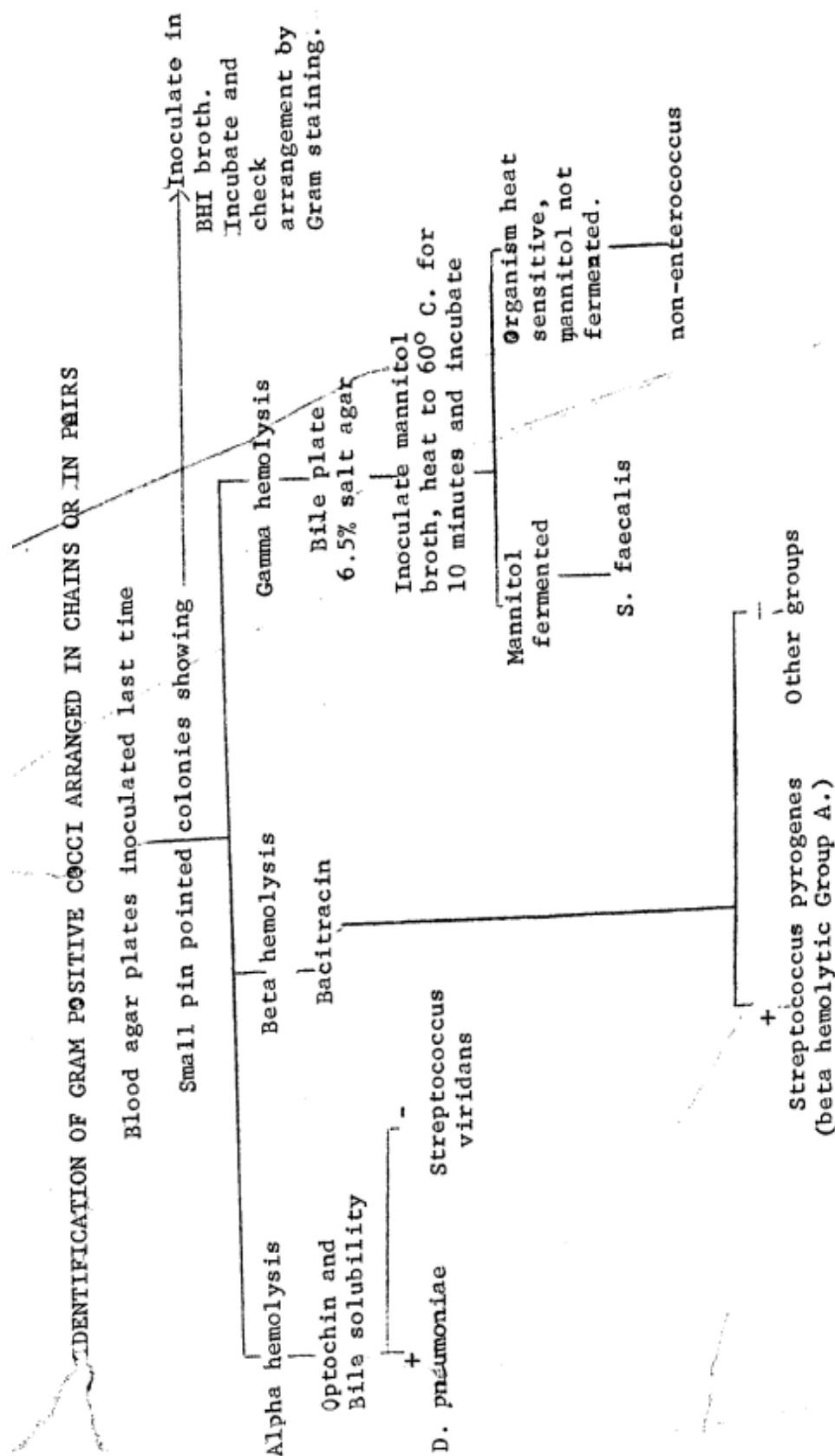
Laboratory Session # 20 cont.

IDENTIFICATION OF GRAM POSITIVE COCCI

Stain smear by the Gram's Method



Laboratory Session # 20 cont.



NOTE: See next page for differential tests which you have not previously done.

Laboratory Session # 20 cont.

The differential tests included in the scheme of identification which you have not previously learned are given below:

Optochin Test: With a sterile swab, spread the culture on the surface of a blood agar plate and then place a filter paper disc containing optochin on the surface of the agar plate (on the area just swabbed). Incubate until the next laboratory session (usually 12 hours are sufficient). The optochin will inhibit the growth of Diplococcus pneumoniae around the disc. This is a positive reaction. If the culture grows all the way up to the paper disc then the test is recorded as negative.

Bile Solubility Test: Inoculate the culture in BHI broth tube and incubate it. After 12 to 24 hours add 0.5 ml of a 10% solution of bile or sodium taurocholate. Incubate for 30 minutes. The culture of D. pneumoniae will lyse (break up and dissolve) and the broth will become clear. This represents a positive test. If the broth stays turbid then the culture is not soluble in bile. It is thus not D. pneumoniae.

Bacitracin test for Beta Streptococcus Group A: Set up as for optochin test, but use a bacitracin disc instead of optochin disc. Absence of growth around the disc indicates a positive test. The culture is thus reported as belonging to group A of streptococci.

Streptococcus faecalis: Growth on 40% bile agar plates and on 6.5% salt agar plates indicates positive tests and identifies Streptococcus faecalis. Production of acid from mannitol after withstanding heating for 1/2 hour at 60° C is another test used for identifying Streptococcus faecalis.

IDENTIFICATION OF GRAM NEGATIVE COCCI

Gram stain the specimen. If Gram negative cocci arranged in pairs are found, grow the specimen on Chocolate agar. Incubate under 10% carbon dioxide (see instructor). After incubation perform oxidase test. Pick out suspected colonies while still pink. Transfer to Chocolate agar slants. Also stab into following sugars and incubate.

Dextrose

Laboratory Session # 3 cont.

Lactose

Sucrose

Maltose

Identify the organisms from the reactions given in the following table.

Identification of the Members of Genus *Neisseria*

Name of the organism	Fermentation of			
	Dextrose	Lactose	Sucrose	Maltose
<i>N. catarrhalis</i>	-	-	-	-
<i>N. sicca</i>	+	-	-	-
<i>N. flava</i>	+	-	-	+

Laboratory Session # 20 cont.

Assignment # 9

Student Identification Number _____

First Unknown # 1

Culture Number	Tests performed	Results	Interpretation	Conclusions
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Summary

Laboratory Session # 20 cont.

Assignment # 9

Student Identification Number _____

First Unknown # 1

Culture Number	Tests performed	Results	Interpretation	Conclusions
-------------------	--------------------	---------	----------------	-------------

Summary

Laboratory Session # 20 cont.

Assignment # 9

Student Identification Number _____

First Unknown # 1

Culture Number	Tests performed	Results	Interpretation	Conclusions
-------------------	--------------------	---------	----------------	-------------

Summary

Laboratory Session # 21 cont.

Completion of work begun last session: (lab session #20)

Continue with the identification of the three unknown cultures. Today work with the isolated colonies and proceed as shown in the flow sheets for the identification of Gram positive and Gram negative cocci.

Laboratory Session # 22 cont.

Experiments for today:

Isolation of Selected Organisms from among your own normal Flora.

Directions for isolating selected organisms from samples are given below. For identification follow the flow sheets pertaining to identification of Gram positive cocci. Complete assignment sheet (100 points).

Laboratory work assignments:

- I. Determine if you are a carrier of Staphylococcus aureus. Test samples from skin or hair, nose, and throat.

Procedure:

Staphylococci from skin or hair:

Rub your fingers on a PRMS agar plate or press the agar against your hair. Incubate plate at 37°C until the next laboratory session.

Staphylococci from nose:

Blow your nose using a tissue paper or paper towel. Gently insert a sterile swab in your nose and heavily inoculate in the upper 1/2 inch portion of the plate. Save the other half for streaking the throat swab. Incubate until the next laboratory session.

Staphylococci from throat:

Have another member of the class obtain a throat culture from you. Inoculate in 1/2 inch area of the still unused half of the PRMS agar plate. Streak for isolation as for the nose swab. Incubate until the next laboratory session.

- II. During the next laboratory session your instructor will help you identify the colonies of staphylococci. Then you perform the coagulase test to confirm if there are Staphylococcus aureus.

Determine if you are a carrier of Streptococcus pyogenes (group A beta hemolytic streptococci).

Procedure:

Have another member of the class obtain a throat culture from you. Streak for isolation.

Laboratory Session # 22 cont.

During the next laboratory session your instructor will help you locate the colonies of beta hemolytic streptococci. Perform the Bacitracin sensitivity tests on two of these colonies (swab each colony on 1/2 of the blood agar plate). Determine if they belong to group A.

III. Determine if you are carrying Pneumococcus pneumoniae in your oral cavity.

Procedure:

Moisten a sterile swab with saliva and plate it on one end of the blood agar plate.

Streak for isolation and incubate until the next laboratory session.

During the next laboratory session your instructor will help you look for small alpha or gamma hemolytic colonies, Test for such colonies by the Optochin sensitivity method and see if they are Pneumococci. To do so, suspend the colonies separately in BHI broth tubes and then swab the suspension in separate quarters of a blood agar plate. Place optochin discs in each of the 4 quarters. Incubate until the next laboratory session.

Isolation of Selected Organisms from Among Your Normal Flora

ASSIGNMENT #10

To determine if you are a carrier of Staphylococcus aureus:

[illegible]

Laboratory Session # 3 cont.

Laboratory Session # 23 cont.

Identification of Unknown Organism

Completion of work begun in Laboratory session # 20.

Complete the identification of the three unknown cultures. Hand in your report.

Continuation of work begun in Laboratory session 22.

Work with isolated colonies obtained from the samples of skin or hair, nose, throat, and saliva. Set up needed differential tests and incubate until the next laboratory session.

Since only coagulase test is needed to identify Staphylococcus aureus this part of the assignment will be completed today. Hold on to your results until the next session though. Hand them in with the rest of your results.

Experiment today:

Second two unknown culture:

You have previously identified medically important bacteria belonging to the Gram positive cocci and Gram negative cocci groups. Today you will receive two unknown cultures belonging to the group Gram negative rods for identification. This assignment is worth 100 points.

Follow closely the key given below and work patiently.

First day: Prepare Gram stained smears and note Gram reaction and morphology.

Streak the unknown cultures on EMB agar plates. Incubate at 37° C until the next laboratory session

Second day:EXAMINE EMB PLATES

Colonies with
green sheen

These organisms are lactose fermenters
and are members of the coliform group.
Likely possibilities are:

Escherichia coli
Aerobacter aerogenes
Klebsiella pneumoniae

Colonies pink
without or with
dark centers

Pink viscous
colonies

These organisms are non-lactose
fermenters. Likely possibilities
are:

Proteus

Colorless
spreading
growth

Colorless
colonies

Pseudomonas
aeruginosa
Alcaligenes
faecalis
Proteus

Laboratory Session # 23 cont.

If results of growth on EMB agar plates indicate that the organism is a lactose fermenter and belongs to the Coliform group, then proceed with further identification of this organism by inoculating:

- Kligler's Iron Agar
- Lactose broth with vial
- Indole reaction - Tryptone broth
- Methyl red reaction - MR VP broth
- Voges-Proskauer reaction - MR VP broth
- Capsule stain

If results of growth on EMB and SS agar plates indicate that the organism is a non-lactose fermenter, then proceed with further identification by inoculating:

- Urea broth
- Kligler's Iron agar slants
- Motility Test medium
- TS agar slant for pigment

Identify the organism from the tables given on the next page.

Laboratory Session # 23 cont.

Identification of Lactose Fermenters

Name of Organism	Kligler's Iron Agar			Lactose broth	Indole	MR	VP	Cit	Capsule
	slant	butt	H ₂ S						
<u>Escherichia coli</u>	Y	YG	-	YG	+	+	-	-	-
<u>Aerobacter aerogenes</u>	Y	YG	-	YG	-	-	+	+	-
<u>Klebsiella pneumoniae</u>	Y to R	Y	-	NC or YG	-	-	+	+	+
								or -	

Mucous like growth on EMB plate

Identification of non-lactose fermenters

Name of Organism	Kligler's Iron Agar			Urea	Motility	Lactose	Pigment
	slant	butt	H ₂ S				
<u>Proteus vulgaris</u>	R	YG	+	+	+	NC	-
<u>Pseudomonas aeruginosa</u>	R	R	-	-	+	-	green
<u>Alcaligenes fecalis</u>	R	R	-	-	+	-	-

Symbols: Y = yellow; acid
R = red; alkaline
MR = methyl red

YG = Yellow gas
acid gas
VP = Voges-Proskauer

NC = no change
H₂S = Hydrogen s
Cit = Citrate
utilizati

Laboratory Session # 23 cont.

ASSIGNMENT #13 Gram Negative Rods

Student Identification Number _____

Second Unknown #1Culture
NumberTests
Performed

Results

Interpretation

Conclusions

Summary

Laboratory Session # 23 cont.

ASSIGNMENT # 13 (gram negative rods)

Student Identification Number _____ Second Unknown #2

Culture Number	Tests Performed	Results	Interpretation	Conclusions
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Summary

Laboratory Session # 24 cont.

Continuation of work begun last session #23

Examine the EMB and SS agar plates and continue with the identification of the organisms by inoculating the needed media.

Experiments for Today:

SPECIAL READING ASSIGNMENT

CLINICAL MICROBIOLOGY I

By following the principles of identifying organisms that you have learned microbiologists have been able to establish that different infectious diseases are caused by different bacteria. The signs and symptoms of these diseases are recognized by the physician when he examines a patient. On the basis of the symptoms the physician realizes what may be the cause of the disease, but he is never sure unless he sends appropriate samples from the patient to the bacteriology laboratory for testing. The laboratory, first of all, streaks the sample for isolating the organisms and then looks for too suspected disease producing organism. Organisms which are normally expected to be present in the sample are ignored.

The disease producing organisms are first tentatively identified on the basis of reactions noted on selective and differential media. Such a tentative diagnosis is helpful to the physician who, on that basis, proceeds with treatment. In the meantime, the laboratory completes the identification of the organisms and submits the final report. On the basis of this report, the physician is able to continue or modify the treatment already prescribed.

GRAM POSITIVE COCCI

Staphylococcus aureus

Staphylococcus epidermidis

Streptococcus pyogenes beta hemolytic group A streptococci

Pneumococcus pneumoniae

GRAM NEGATIVE COCCI

Neisseria gonorrhoeae

Neisseria meningitidis

GRAM POSITIVE RODS

Bacillus anthracis

Corynebacterium diphtheriae

Clostridium tetani

Clostridium botulinum

GRAM NEGATIVE RODS

Hemophilus influenza

Borrelia recurrentis

Pasteurella pestis

Brucella melitensis

Escherichia coli

Salmonella typhosa

Shigella dysenteriae

Laboratory Session # 3 cont.

Klebsiella pneumoniae

Proteus vulgaris

GRAM NEGATIVE SPIRALS

Vibrio cholera

SPIROCHETES

Treponema pallidum

Borrelia recurrentis

Leptospira icterohaemorrhagiae

OTHER IMPORTANT BACTERIA

Mycobacterium tuberculosis

In forthcoming laboratory sessions you will look for the presence of certain disease producing organisms in various samples. This will be just like working in a hospital diagnostic laboratory.

THE SOURCE OF DISEASE PRODUCING ORGANISMS

The majority of the organisms which produce disease in humans are actually carried in the human population. The person carrying them does not necessarily have to be ill. Many normal looking, healthy persons carry disease producing organisms. Such persons are called carriers. Carriers can thus be described as healthy, but carrying disease producing organisms in or on their bodies. Besides carriers disease producing organisms are, of course, present in persons suffering from the disease. From these two sources, i.e., carriers and patients, the organisms transmit to other persons.

Certain organisms are found in the nose and throat of patients and carriers. Such organisms are thus transmitted via coughing and sneezing. Others are present in the digestive systems. These are excreted in the feces and are generally transmitted via food, water, and milk. Certain other organisms like the ones which cause syphilis and gonorrhea are transmitted by direct contact only because the organisms are very fragile and do not stay alive outside the body.

What determines that a disease producing organism will produce disease in its new host?

Once the organisms are transmitted to a new person he does not necessarily become ill. His chances of becoming ill depend on:

- 1) how many organisms he has received
- 2) did they get in through the right route, i.e., the route via which they are able to establish in the host
- 3) his immunity to these organisms.

This last part we should understand. A person becomes immune to a disease producing organisms in two ways:

- 1) by acquiring the disease and then recovering from it, or
- 2) by getting vaccinated.

Vaccination is the artificial way of immunizing persons by injecting small quantities of the organism known to have caused the disease. Generally the organism is killed prior to injection or made weaker by growing it away from its natural environment. Such organisms are alive, but they do not produce disease. We call them attenuated cultures.

When a person receives an injection of the vaccine his body produces antibodies. These have the capacity to tie-up the organism and eliminate it from the body. When a vaccinated person receives a second injection of the vaccine he produces far more antibodies far more quickly. That's why people are given more than one injection of the vaccine. Repeated injections of a vaccine given to build a large amount of antibodies quickly are called booster injections. When a vaccinated person receives the organism from a patient or a carrier has body already having the antibodies fights off the organism. Not only does the disease fail to develop in such person, but the organism acts as a booster injection with the result that the person ends up having more antibodies in short period of time and stays protected from the disease.

Laboratory Session # 3 cont.

Laboratory Session # 24 cont.

DETECTING THE PRESENCE OF DISEASE PRODUCING ORGANISMS IN
SELECTED SAMPLES

All samples viewed in the hospital diagnostic laboratory are recorded in the log book. A record sheet is provided for your use. Record all samples that you receive on this sheet. When completely filled, hand in to your instructor. The assignments for throat, nose, stool, and urine cultures are worth 100 points.

EXPERIMENTS FOR TODAY (throat and nose sample)

Throat culture:

You will receive a swab obtained from the throat of a patient. Check if it contains Group A beta hemolytic streptococci.

Procedure:

Streak the sample for isolation on blood agar plate. Incubate until the next laboratory session at 37°C. Next time look for small pin-pointed colonies which are surrounded with complete zones of lysis. Consider these as colonies suspected to be beta hemolytic streptococcus. Make a Gram stain to check Gram reaction, morphology, and arrangement. Perform the bacitracin sensitivity test on two selected colonies to check if the organism is a group A streptococcus. If your throat swab was negative for beta hemolytic strep, get a beta hemolytic colony from the plate of someone else in the class and test it by the bacitracin test.

Nose culture and phage typing of staphylococci:

The sample you received today is a nose culture obtained from one of the nurses assigned to the hospital nursery. There appears to be an outbreak of staphylococcal infection in the nursery. The second sample which you received is a sample from the infected area of one of the infants. Check if these samples contain Staphylococcus aureus.

Procedure:

Streak the sample for isolation on Phenol red mannitol salt agar plates. Incubate until the next laboratory session. Look for golden yellow colonies which are surrounded with a yellow zone. Check if they are coagulase positive by inoculating one of the colonies in 1/2 ml. of sterile rabbit plasma. Incubate the tube at 37°C. If the plasma turns into a clot report the culture as Staphylococcus aureus. The above procedure would only tell us if both the samples contain Staphylococcus aureus. It, however, does not tell us if both the staphylococci are alike. To find that out we perform a test called the Bacteriophage typing. Bacteriophages (called phages for short) are viruses which have been isolated from staphylococci and which have the ability to infect and lyse certain strains of staphylococci. Identical strains of staphylococci will always be lysed by the same phages. The test is done as follows:

Suspend the colony to be Ophage typed in a TS broth tube. Then heavily swab a TS agar plate with the suspension. Let the surface of the plate dry. Mark the back of the plate in four sections (in practice the plate is marked in 21 sections and 21 different phages are used). In each section place 1 drop of a different phage. Let the drop be absorbed by the agar and then incubate the plate upside down in the

Laboratory Session # 3 cont.

incubator until the next day. Areas where drops of phages were placed will show no growth if the phages they contained were able to lyse the staphylococci. Note the code number of such phages. This information represents the phage type of the organism.

Check if the phage type of both the cultures is identical. If they are identical, the patient was most likely infected by the nurse who was the carrier. Carriers of those strains of staphylococci which are known to produce severe diseases in hospitals among patients are not assigned to work in high risk areas such as nurseries and operating wards. They are also treated and until shown to be consistently free of the dangerous staphylococci are not allowed to work on wards where their presence is likely to be hazardous.

RECORD SHEET

Specimen Number and Date	Patient's Name Number or Name of Physician	Specimen (Describe)	Work Requested	Results	Date Results Sent

Student Identification Number _____

Clinical Microbiology

Detecting the Presence of Disease Producing Organisms in Selected Samples

Throat Culture

Specimen Number	Tests Performed or Media Inoculated	Description of Suspect Colonies	Possible Identity of Suspect Colonies	Confirming Tests Performed	Results Con

Student Identification Number _____

Clinical Microbiology Detecting the Presence of Disease Producing Organisms in Selected Samples

"Nose Culture"

Specimen Number	Media Inoculated or Tests Performed	Description of Suspect Colonies	Possible Identity of Suspect Colonies	Confirming Tests Performed	Results	Conclusions

Laboratory Session # 25 cont.

CLINICAL MICROBIOLOGY II

Completion of work begun in Laboratory Session #23

Complete the biochemical reactions of the two Gram negative rods given to you as unknowns. Interpret your findings and report results on assignment sheets (100 points) (assignment #13)

Continuation of work begun during the last laboratory session #24

Throat culture:

Look for beta hemolytic streptococcus and set up the bacitracin sensitivity test.

Nose culture:

Set up coagulase test. Suspend the coagulase culture in TS broth and set up phage typing as per details given the last laboratory session. Your instructor will guide you in how to do this experiment in a systematic way. Wait until the entire group is ready for phage typing then proceed.

Experiments for today: (Staph and Urine samples)

Stool cultures:

The stool sample which you received today is from a patient suspected to be suffering from Salmonella infection. Determine if the sample contains organisms belonging to this genus.

Procedure:

Streak the sample heavily on an EMB on an SS agar plate. Incubate the plates until the next laboratory session. Look for the following types of colonies on the SS media.

EMB plates: colorless colonies

SS plates: colorless colonies

Note: Pink dark colored colonies are not of Salmonella or Shigella

Since the suspected organisms in the stool samples are expected to be very few in number, simultaneously on the first day of the test inoculate the sample in Selenite broth. Incubate this tube at 37°C, This media is enrichment medium for Salmonella. It will allow the few organisms to multiply and thus make their detection easier. Next day streak the material and look for the types of colonies described above.

Report that colonies suspected to be Salmonella were found or not found. In the hospital, these colonies would be further identified by inoculating into differential media. In the class, however we will not proceed any further because you have already learned the principles involved in the identification of bacterial cultures.

Urine culture:

The urine sample is from a patient who has been complaining of irritation during urination. It is being sent to the laboratory to determine if the patient has an urinary infection. Proceed as follows:

1. Using a sterile swab and inoculating loop, inoculate an EMB plate and streak for isolation. Check at 24 and 48 hours. Identify any non-lactose fermenters.
2. Make dilutions of the urine as follows. Transfer 0.1 ml. of urine to a tube of 9.9 ml. of sterile broth. Mix and using the same pipette transfer 1 ml. of the dilution

Laboratory Session # 3 cont.

to the surface of a blood agar plate and spread it over the entire surface of the plate with the loop.

3. Read the dilution plate at 24 and 48 hours. Count the colonies and multiply by 1,000. Use the table below to determine the significance of the count.

<u>Colonies on Plate</u>	<u>Organisms/ml.</u>	<u>Significance</u>
100 or more	over 100,000	considered a significant number of organ
10-100	over 10,000	suspicious
under 10	under 10,000	not significant

4. If count is over 100,000 the predominant organisms are identified and the Antibiotic Sensitivity tests will be performed.

ASSIGNMENT # 17

Clinical Microbiology

Detecting Presence of Disease Producing Organisms in Selected Samples

Urine Cultures

Specimen Number	Media Inoculated or Tests Performed	Description of suspect colonies	Possible identity of these colonies	Confirming Tests Performed	Results	Comments

Laboratory Session # 25 cont.

Questions:

Your instructor will explain the following in class.

1. Why do we attach significance to the number of organisms found in a urine sample?
2. How is a urine sample collected?
3. What is the best way of collecting a urine sample?
4. Is there any danger of infecting the patient when a catheter is used for collecting urine. How can this danger be avoided? What is a catheter?
5. What other samples are processed in a bacteriology laboratory?
6. How are blood cultures made?
7. What is a blood culture bottle?
8. What is bacterimia?
9. What is bactiurea?

The procedure to process blood culture is given below. It will not be carried out in the class.

Blood culture:

1. The blood, aseptically obtained from the vein with sterile syringe, is indirectly inoculated into a blood culture bottle and the bottle is incubated at 37°C.
2. The culture is then examined for unusual appearance (foaming after shaking; greening of top layer of blood; hemolysis; cotton ball colonies in clot). If culture looks unusual the sub-culturing protocol below is followed. Blood cultures should be examined every day and sub-cultured if they appear unusual. Otherwise, they are routinely sub-cultured at 48 hours and at 7 days. If subculturings are negative and cultures appear sterile, they may be discarded on the 9th day and a negative report sent to the physician.

Blood culture sub-culturing technique:

1. Sterilize top of blood culture bottle by placing gauze or cotton soaked with 70% alcohol on the top of the stopper. Allow to remain for two minutes.
2. Withdraw 1 ml of broth into the syringe. Drop 2 drops onto one side of a chocolate agar plate and dispense the remainder into thioglycollate broth. Streak plate minimally. Examine at 24 and 48 hours, discard if negative. If positive, identify the isolate.

Laboratory Session # 26 cont.

CLINICAL MICROBIOLOGY III

Completion of work begun during Laboratory Session #24.

Read the final results of the nose and throat culture. Write your conclusions neatly on the assignment sheets and hand them to your instructor.

Continuation of work begun last session #25.

Stool culture:

Refer to directions of stool culture and streak an SS and an EMB plate from the Selenite broth. Incubate at 37°C until the next session.

Urine culture:

Read the results of the urine culture. Write your conclusions on the assignment sheet and hand it in to your instructor. If the count is over 100,000 and the organism is identified, perform antibiotic sensitivity tests in lab session #27 and hand the assignment sheet in during lab session #28 upon completion of results.

Experiments for Today:Determination of Sensitivity of Bacteria to Antibiotics

Since the discovery and availability of penicillin for the treatment of infections, antibiotics have become the preferred drugs. Penicillin, however, is not effective against all bacteria and since many bacteria have also become resistant to penicillin and other antibiotics, search for additional antibiotics is being continued. A large number of antibiotics have, therefore, become available to the physician.

The physician always desires to administer the antibiotic which is most effective against the organism causing the disease provided, of course, the patient is able to tolerate the antibiotics, i.e., the patient is not allergic to the antibiotic.

The bacteriology laboratory helps the physician in selecting the most effective antibiotic. This is done by performing an antibiotic sensitivity test. Information regarding the allergic state of the patient is obtained first by merely asking the patient if he ever had any adverse reactions to the antibiotics. If the patient is not certain, very small amount of the antibiotic is injected into the skin of the patient. If there is no adverse reaction, the patient is regarded as not allergic. Such a patient can receive the antibiotic. If, on the other hand, the patient reacts to the antibiotic, either an alternate is given in very small doses first (these desensitize the patient) and then in large doses. All this is done under strict supervision.

Laboratory work assignment:

You will be provided with 4 sets of various antibiotic discs along with the following 4 cultures:

- 1) Escherichia coli
- 2) Staphylococcus aureus
- 3) Pseudomonas aeruginosa
- 4) Proteus vulgaris

Your instructor will first demonstrate how to set up the test. He will also show you how to handle the antibiotic discs. If available, he will show you multi-discs and disc dispensers. Following the demonstration you determine the antibiotic sensitivity of the cultures provided to you.

Laboratory Session # 3 cont.

Procedure:

1. Spread the culture evenly over the surface of an agar plate by means of a sterile swab.
2. Allow the surface of the plate to dry.
3. Place (one inch apart) filter paper discs separately containing different sulfonamides or antibiotics on the surface of the plate.
4. Incubate the plate until the next laboratory session.

Next Laboratory session:

Note the size of zones of inhibition around various discs. Use the following scale to determine the sensitivity or resistance of the bacteria tested.

Definite zone of inhibition ----- The organism is susceptible to the drug.

No zone to a trace of a zone of inhibition ----- The organism is resistant to the drug.

Laboratory Session # 27 cont.

Completion of work from last session #25.

Stool culture (continued)

Read the various plates streaked from the Selenite broth during the last session. Look for non-lactose fermenting colonies. Record your results and hand in your assignment sheet.

Antibiotic sensitivity testing:

Note to which of the various antibiotics tested the organism was sensitive and to which ones it was resistant. Record your results in a table form and hand it in to your instructor.

Urine Culture (cont.)

If the count of the predominant organism is over 100,000 and the predominant organism is identified on (BMB plate), perform the antibiotic sensitivity test for urine sample.

IMMUNOLOGY I

(See also pages 144, 145, and 146)

Experiments for Today: Record the results of antibiotic sensitivity testing of urine sample and hand the assignment sheet #13 to your instructor.

Today you will also begin the study of immunity. This subject is explained in the section also entitled "Immunology". That section is a special assignment. Your instructor will begin explaining it to you today; continuing during the next 2 sessions. Your instructor will first explain the terms antigen and antibodies and will also demonstrate the slide agglutination and the tube precipitation reactions. Following this demonstration you practices, both the slide agglutination and tube precipitation tests, and learn to recognize a positive from a negative reaction.

The Procedure for carrying out these reactions are:

A. Agglutination reaction

1. Place a drop of the antiserum on the slide and then add a drop of the bacterial suspension. Mix gently with an applicator stick. If the serum contained antibodies, bacterial cells will clump. This is a Positive reaction.

Repeat the Procedure using

- a) Antiserum A with Bacterial cells A.
- b) Antiserum B. with Bacterial cells B.
- c) Antiserum A with Bacterial cells C.
- d) Antiserum A with the unknown bacterial cells
- e) Antiserum B with the unknown bacterial cells

Record results in your note book.

B. Precipitation reaction

1. Obtain a Precipitant tube
2. with a capillary pipette fill the lower one half inch of the tube with the anti-human serum.
3. with another capillary pipette carefully layer human serum over the anti-human serum. (do not let the two solutions mix as you layer them.)
4. watch for the development of a white ring of Precipitate at the junction of too two liquids. This represents a positive reaction and indicates that the anti-human serum contains antibodies to the antigens contained in human serum.

Repeat this procedure using the anti-rabbit serum and the rabbit serum. Also using anti-rabbit serum with human serum and anti-human serum with rabbit serum.

Also test the unknown serum and determine if it is human or rabbit serum or none of these.

Record results in your note book

- C. The Precipitation test is also conducted in agar gel. This is called the Ouchterlony test. With this test, we can determine:
 - a) how many antigen-antibody systems are present in a sample

Laboratory Session # 3 cont.

b) if antigens obtained from different sources are alike

Set up the Ouchterlony test as follows: cut out wells in a Saline agar

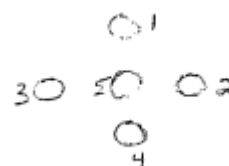
Mark the wells as shown

Fill well #5 with the anti-human serum

Fill well #1 and #3 with the human serum

Fill well #2 with the rabbit serum

Fill well #4 with the unknown serum

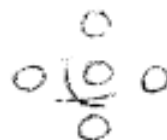


Hand in the plate to your instructor for refrigeration.

Observe the plate during the following laboratory sessions and note the lines of precipitate which will develop between well #5 and serum of the peripheral wells. Count these lines. This number represents the minimum number of antigens contained in the samples.

To determine if the antigens contained in 2 samples are alike, note the areas of the Precipitates that they form with the unknown antiserum. If the areas cross over as shown below:

Then the antigens are not alike.



If they do not cross over but merge with the antigens



each other as follows are alike.

Show your results to your instructor. Make drawings in your notebook. Show the number and shape of Precipitin arcs that you have obtained.

Next your instructor will type certain samples of blood by the slide agglutination reaction. Then you type the samples of blood given to you. If you wish to type your own blood you can do it with the help of your instructor. The procedure is as follows:

1. Wipe the tip of your index finger of your left hand with a gauze soaked with 70% alcohol.
2. Press the finger so that the blood is retained in the tip.
3. With a sterile lancet quickly and firmly prick the finger. Your instructor will demonstrate first.
4. Wipe the pricked area with the gauze soaked in 70% alcohol and then place a drop of blood on 2 separate halves of a slide.
5. Add a drop of Anti A antibodies to one drop and a drop of Anti B antibodies to the other drop.
6. Mix each drop separately with a piece of application stick. Observe for agglutination. Interpret your blood type from the table below:

Blood Agglutinated with	Blood Type
Anti A	A
Anti B	B
Anti A and Anti B	AB

Laboratory Session # 3 cont.

None	O
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During the remaining of the laboratory session your instructor will explain the useful and the harmful aspects of the antigen-antibody reactions. You will then read the pages entitled "immunology" and answer the following questions:

1. What are antibodies?
2. Where do the body of animals and man produce antibodies?
3. In what part of the blood are the antibodies found?
4. What is the primary response?
5. What is the booster response?
6. Have you ever been vaccinated?
7. What are the various vaccines which you have received so far?
8. Why are you required to be vaccinated against small pox when you are travelling to other countries?
9. What is your blood type?
10. What is hay fever? How is this produced? Why does everybody not get it?
11. What is erythroblastosis fetalis? What is now done to ensure that the mother does not develop the conditions which cause this disease in the fetus?

IMMUNOLOGY II
(see also pages 147 and 148)

TITER

Let us suppose that you were given two vials containing a certain liquid and you were told that these liquids are able to hemolysis (break down) red blood cells, and you were asked to determine the extent of hemolytic activity contained in these liquids. How would you go about obtaining this information?

In the laboratory this information is readily obtained by separately making doubling dilutions of the liquids and determining the highest dilution which demonstrates the hemolytic activity. Let us suppose that one of the liquids could be diluted 1: 10240 and still show the hemolytic activity, while the other liquid could only be diluted to 1: 640 and on further dilution lost the ability to hemolysis red blood cells. This means that one of the liquids is 16 fold stronger than the other liquid in its ability to hemolysis red blood cells.

The procedure is a convenient way to describe the extent of activity contained in solutions. It is called titration. The highest dilution of the solution, which is still capable of performing the activity being studied, is called the titer of the solution. In the above example the titer of the two vials in terms of their hemolytic activities will be reported as 1: 10240 and 1: 640 respectively. It is obvious that in order to obtain the titer the series of dilutions shall be carried out far enough to obtain an end point, i.e. the dilution beyond which the effect is not demonstrable.

Titration is a very important and convenient technique in the laboratory and is applied to quantitate the effects of a variety of different substances.

Laboratory work assignment:

Your instructor will demonstrate how to titrate the sample. Then you titrate and determine the hemolytic titer of the two solutions supplied you. This assignment is worth 50 points.

Procedure

1. Arrange 10 tubes in a row in a test tube rack. Mark these 1 to 10.
2. carefully pipette 0.5 ml. of saline in each of the tubes. Pipette 0.5 ml. of the test solution in tube #1. This tube now represents a dilution of 1:2 of the test solution. Mix the contents of this tube by drawing the liquid up into the pipette and gently blowing it out again. Repeat this procedure 2 more times. Then transfer 0.5 ml. of the mixture to tube 2. The tube #2 now represents a dilution of 1: 4 of the test solution. Mix the contents as you did for tube #1 and then transfer 0.5 ml. from tube #2 to tube #3.
3. Continue making doubling dilutions until the 10th tube. Discard the 0.5 ml. portion of the last dilution.
4. Similarly make 2 fold dilutions of the second sample.
5. Add 0.5 ml of 1% suspension of red blood cells to each tube
6. Determine the highest dilution showing lysis (clearing) of the red blood cells. These dilutions are the titer of your 2 samples. Write this information in your note book.

Laboratory Session # 3 cont.

Next you will receive 2 samples of antisera taken from a patient 7 days apart. These will be marked A and B. This patient is suspected to be suffering from typhoid fever. Titrate the 2 samples for Anti *Salmonella typhosa* antibodies by the agglutination reaction.

Procedure:

1. Prepare 10, 2 fold dilutions of each of the serum in 0.5 ml. quantities.
2. Separately place a drop of each of the various dilutions on 2 or more slides.
3. Also place a drop of saline and a drop of the positive antiserum separately on a slide.
4. Add a drop of the antigen in each of the drops. Mix the drop with a piece of the applicator stick. Use a new piece of stick for the next drop
5. Note the dilution which agglutinates the antigen. This is the end point. Report this dilution as the titer.

NOTE: The saline control must not agglutinate, otherwise the results are not valid. Record your results in your notebook.

Laboratory Session # 30 cont.

Special Reading Assignment, Lab Session 30: Your instructor will first explain the concepts contained in the following pages (a few concepts at a time and in the given order.) He will then allow you time to read the portion explained and will give you a learning examination.

IMMUNOLOGY
NON-SPECIFIC AND SPECIFIC DEFENSES
Antigens and Antibodies

Concepts for Lab Session 28

All living things have the ability to guard their bodies against the entry of foreign materials and of disposing those that may enter. The mechanisms by which the body accomplishes this are called the defense mechanisms of the body and are studied as a separate branch of science called Immunology. The overall mechanisms of defense that the body employs are placed in two categories: (1) the non-specific defenses and (2) the specific defenses.

The non-specific defenses.

In man and animals, skin covering the outside of the body and membranes lining the various openings of the body effectively prevent the entry of foreign particles. Those that do find their way through cuts, etc. are engulfed by the white blood cells and are generally eliminated. These mechanisms are so efficient that the blood streams of healthy animals and man are kept sterile. The reason this system of defense is called non-specific is because it operates against all types of foreign materials.

The specific defenses.

Despite the non-specific safeguards mentioned above, infections do occur and foreign materials occasionally find their way into the blood stream. Fortunately, living things are not entirely defenseless against such an occurrence. Our knowledge, however, is mainly confined to what happens in higher animals and man (animals with backbones). In their case, specialized cells of the body produce certain protein molecules which specifically bind the foreign material and help the body in its disposal. These special molecules of protein which are produced in response to the foreign material and which specifically recognize the foreign material and bind with it are called antibodies. The foreign material against which the antibodies were produced by the body is called an antigen.

An antigen, then is any substance against which the body of a man or animal will produce antibodies.

What kind of a substance behaves as an antigen?

Large molecules which are foreign to the body will behave as antigens. Antigens generally have a molecular weight of over 10,000. They are mainly proteins and polysaccharides and they are always foreign to the body. This is both important and fortunate. The body does not produce antibodies to its own molecules because if it did, it would destroy itself.

Do small foreign molecules ever behave as antigens?

Laboratory Session # 3 cont.

Yes. Certain small molecule become attached to proteins and then become antigens. Such small molecules are called Haptens or incomplete antigens.

How much time is needed by the body to produce antibodies?

When the antigen enters the body for the first time it takes about 7-10 days before antibodies can be shown in the blood stream of man or animal.

The body, however, has a remarkable memory. When it encounters the same antigen the second time, it produces antibodies very quickly. Within 24 hours, an amount of antibodies much larger than the amount produced after the first injection are found in the blood.

The response of the body when it encounters the antigen for the first time is called a primary response.

The response of the body when it encounters the same antigen for the second time is called the secondary response or the booster response. Since the body has responded as if it had remembered its first encounter with the antigen it is also called an anamnestic response (opposite of amnesia).

In which part of blood do the antibodies appear?

They appear in the liquid part of the blood which is the plasma. If we allow the blood to clot and collect the serum, most of the antibodies will be found in the serum.

What do we mean by the term specificity?

That the antibodies recognize and react with the antigen against which they were produced and not with any other antigen.

Is this specificity useful?

Very much so and in a number of ways:

- (1) Inside the body the antibodies due to their specificity, seek out the antigen against which they were produced and help eliminate it. Antibodies produced against microorganisms and their products thus bring the disease under control and restore health.
- (2) Specificity of antigen-antibodies and the phenomenon of booster response has enabled us to develop vaccines against various diseases. In this process, man and animals are injected with small quantities of those antigens which are important in the production of disease. Such small quantities are harmless or they are made harmless by heating or by treating with formalin. Heat will kill the organisms which will now not grow in the body, but still act as antigens. Formalin changes the toxins so that they are not poisonous, but they still behave as antigens. Such altered toxins are called toxoids. Antibodies produced against toxoids are able to bind with and neutralize the toxins. Vaccines give the body the first exposure to the antigen and ensure that if and when actual infection occurs, it will act as a booster dose. Large amounts of antibodies will thus become available and will not allow the infecting agent to establish in the body and produce disease. The process of vaccination is also referred to as

Laboratory Session # 3 cont.

active artificial immunity because due to vaccination, the body actively produces antibodies. Another system of vaccination, is called passive artificial immunization. In this, blood or serum containing antibodies produced in another animal or individual is injected into the patient drastically in need of antibodies. The antibodies so received by the patient remain in the body about three weeks, but they do tie the patient over the critical period of disease. Also in the meantime, the patient responding to the infecting agent produces his own active immunity.

Sometimes both active and passive immunizations are used simultaneously. For instance, an injured person whose wound is suspected of having gotten contaminated with dirt is likely to come down with tetanus. To safeguard against tetanus, antibodies are injected for immediate protection (passive immunization) and tetanus toxoid is injected to enable the patient to produce his own antibodies (active immunization).

(Concepts for day 2, Session 29.)

- (3) Specificity enables us to divide a species of microorganisms into groups and types. Members of a given species do not all have the same antigen. On the basis of antigens which are shared by some members, the species is divided into groups. All members of a group will thus have at least one common antigen. Members of a group are further divided into types, again on the basis of common antigens.
- (4) Specificity of antigen-antibodies enables us to diagnose a particular disease. The most direct way of diagnosing a microbial disease, of course, is to isolate and identify the organism causing the disease. This, however, is not always possible. An alternate approach is to look for the antibodies in the serum of the patient. Mere presence of antibodies, however is not sufficient as these may be due to some past exposure to the antigen. In practice, the laboratory examines two samples of blood drawn 3-7 days apart. If the sample drawn later has more antibodies against the suspected organism then the patient is regarded to be suffering from the disease caused by that organism. If, however, the amount of antibodies in various samples of blood remains the same then the disease is regarded as due to some other cause.
- (5) Specificity of antigen-antibodies helps us determine the member of different antigens present in extracts of organisms or in body fluids. Since an antibody recognizes and reacts with the antigen against which it was produced, we have been able to develop methods by which we can determine how many different types of antigens are present in a particular fluid, say blood or an extract of an organism. All we have to do is to inject the material to be tested into an animal to which it is foreign and produce antibodies. The serum containing the antibodies is then reacted with the material. This reaction is conducted in agar gel. Two Wells are punched in a thin layer of agar. Antigen material is placed in one well and the antiserum in the other. The gel is then refrigerated for a few days. During this time different antibodies and antigens will diffuse towards each other and upon

Laboratory Session # 3 cont.

encountering each other will form a precipitate which can be seen as a white line located at a point somewhere between the two wells. Ideally, each antigen-antibody system is expected to give one line of precipitation and various lines are expected to be separate from each other. In practice, however, the lines are not so separate; some overlap. Nevertheless, by trying different distances between two wells and by diluting the antigen material and the antiserum, lines well separated from each other can be obtained. The procedure is, thus helpful for determining the number of different antigens contained in an antigenic material. It is, however, more useful for determining the parity of an antigenic material.

- (6) Specificity of antigen-antibodies also enables us to recognize the presence of disease producing organisms in a sample. Highly specific antibodies which will react only with a particular disease producing organism are tagged (combined) with a fluorescent dye (a dye which will shine when viewed under ultraviolet light). Such tagged antibodies are then used to treat a smear of the sample obtained from the patient. The antibodies will combine with the organisms if present in the sample. When examined under the ultraviolet light using a fluorescent microscope, such organisms are seen as brightly shining objects against a dark background. A disease can thus be diagnosed within hours.
- (7) Specificity of antigen-antibodies has enabled us to determine blood types of different individuals. We can thus match blood of donor and recipient before transfusion.

Laboratory Session # 30 cont.

CONCEPT FOR DAY 3, Session 30How do we determine that a serum has antibodies to an antigen?

This is done by means of antigen-antibody reactions of which there are many types (see Summary of Antigen-Antibody reactions.) In all cases, however, when antibodies encounter their antigen, they bind with the antigen. This part of the reaction is called sensitization and is not visible. The visible reaction occurs after sensitization and depends on the antibody and the state that the antigen is in. The following are the two types of visible reactions that occur:

The agglutination reaction: If the antigen is located on a particle such as a bacterial cell or a red blood cell, then in the presence of a salt solution, the antigen-antibody complex aggregates and forms a clump. This is called the agglutination reaction.

The precipitation reaction: If, on the other hand, the antigen was in the form of a solution, and the molecules of the antigen and the antibody are in equivalent proportions, i.e., neither one is in excess, then in the presence of a salt solution a visible precipitate is formed. This is called the precipitation reaction.

The presence of antibodies can also be recognized in a number of other ways. For instance:

- (1) The neutralization reaction: Antibodies will neutralize a toxic antigen and will make it inactive and harmless. Toxic substance produced by bacteria and fungi, and the snake venoms are thus neutralized by the antibodies produced by the body against such substances. Viruses are likewise neutralized by their antibodies.
- (2) The complement fixation reaction: Serum and plasma of most animals and man contain a set of proteins which are collectively called complement (abbreviated as C'). Together, these proteins have the ability to combine with a specific antigen-antibody system and, if the antigen is on such as a red blood cell, the particle will be lysed. This ability of the specific antigen-antibody system to bind complement is used for detecting the presence of specific antibodies. The reaction is carried out in steps as follows:
 - a) Serum is mixed with a known antigen and the mixture is incubated for 30 minutes. If the serum had antibodies they will combine with the antigen. No visible reaction, however, will occur.
 - b) Now the complement is added and the mixture is incubated for 30 minutes more. Complement will combine only if the antigen-antibody were specific for each other. Still, however, no visible reaction will occur.
 - c) To see if the complement has in fact combined with the antigen-antibody system we will now add a known specific antigen-antibody system in which the antigen is a particle: sheep red blood cells and anti-sheep red blood cells systems is ideal and is mostly used.

Results are interpreted as follows:

Laboratory Session # 3 cont.

This system being specific must bind the complement and if so the red blood cells will be lysed. This will mean that the complement was free, i.e., it had not combined with the first system. The first system was, therefore, not specific. The serum, therefore, had no antibodies to the antigen.

If, however, the red blood cells did not lyse this will mean that the complement had already combined with the first system. The first system was, therefore, specific and the serum had antibodies.

The complement fixation reaction is very useful in detecting incomplete antibodies. These are antibodies which do not have the capacity to precipitate or agglutinate their antigens.

- (3) Hemagglutination inhibition reaction: Certain viruses have the ability to agglutinate red blood cells. Antibodies to these viruses are able to block this hemagglutinating ability of the viruses. Presence of antibodies to viruses can thus be detected.
- (4) Fluorescent anti-antibody reaction (Coomb's test): Antibodies which fail to give a visible reaction with their antigens can be detected a clever sandwich technique. Antibodies are protein molecules of a type called the gamma globulin. If gamma globulin from human is injected into rabbits or goats, the animal will produce anti-human gamma globulin antibodies. These are then labelled with a fluorescent dye and are used for detecting antibodies which have combined with the antigens, but have not given a visible reaction. For instance, antibodies to the organism of syphilis combine with the organism, but show no visible reaction. But this antigen-antibody complex when treated with the fluorescent anti-gamma globulin fluoresce: when viewed under the ultraviolet microscope.

How can we determine the amount of antibodies that a sample of serum contains?

By titration as follows: Make a series of 10 fold, but preferably 2 fold dilutions of the serum in saline. Test each dilution to see if it still reacts with the antigen. The highest dilution giving a positive reaction is regarded as the end point, or the titer, i.e., beyond this dilution the antibodies are far too few to give a positive reaction. The highest dilution giving a positive reaction is recorded as the titer of the serum. The results of titration are interpreted as follows:

- (a) If two samples of serum have the same titer against a given antigen then they are regarded to have equal amounts of antibodies.
- (b) If the titer of two samples of serum show only a one tube difference they are still regarded to have the same amount of antibodies unless the difference shows up on repeating the titration 2-3 times. Thus titers of 1:20 and 1:40 are not regarded as significantly different unless they repeatedly come out that way. This is because the titration procedure is not very accurate.
- (c) If two samples have two or more tubes difference in titers then the samples are regarded to contain different amounts of antibodies to a given antigen.

NOTE: The above rules of interpreting results of antibody titrations apply to all tube titration procedures.

Laboratory Session # 3 cont.

Are antibodies always useful to the body?

No, not always. Under natural conditions of infection they are very useful, but occasionally certain persons become hypersensitive. Such persons react violently to the antigen, not because the antigen in any way is toxic, but because the antigen-antibody complexes inside the body react with the tissues. Histamine is released in the process which brings about discomfort and even death in severe cases.

Fortunately, the state of hypersensitivity in individual can be readily detected by injecting in the skin very small amounts of the antigen. Hypersensitive persons will immediately show a reddening of the area of injection. In such cases it is best to avoid giving that antigen, but if it is essential that the antigen need be given then such persons are first desensitized by injecting progressively increasing doses of the antigen. Desensitized persons then tolerate large doses without any ill effect, but eventually become hypersensitive again. Hypersensitivity to penicillin is a good example of this phenomenon.

Another example is that of allergic individuals who react to pollens and weeds and suffer from hay fever when these weeds are in season. Here the antigen (pollens) first enters the system via inhalation. The person produces antibodies. As antigens are still being inhaled, these react with the antibodies and the release of histamine brings about the symptoms. Next season, the antigens act as booster dose, and the symptoms are severe.

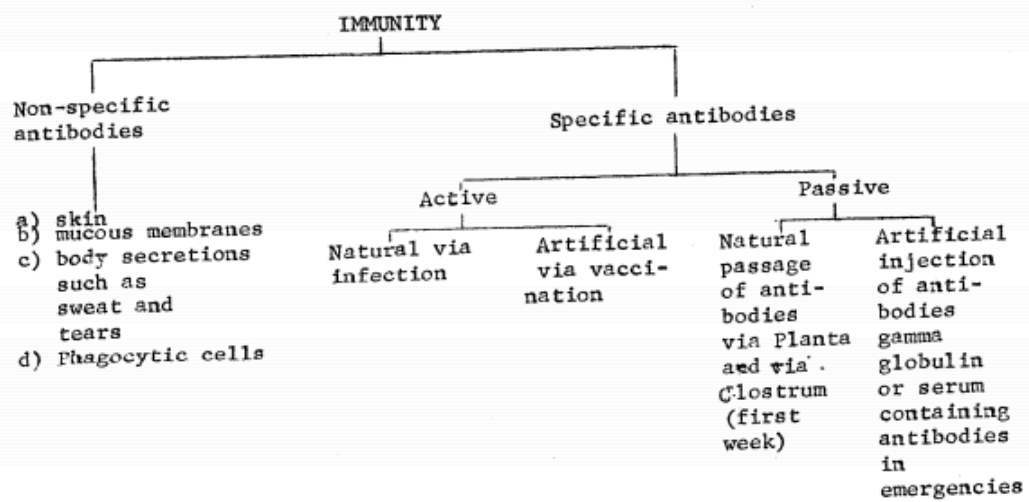
Another example where antibodies are harmful is that of Rh positive infants born to Rh negative mothers. Rh factor is an antigen which was first discovered in Rhesus monkeys, hence the name. It is also present in humans. Red blood cells of an Rh positive baby have this antigen. As they enter into the blood circulation of the mother, she produces antibodies to this antigen because it is a foreign material to her system. These antibodies, during the next pregnancy enter the blood system of the fetus and react with the antigen on the red blood cells and lyse the red blood cells. The disease so produced is called Erythroblastosis fetalis and is now brought under control, ironically, by the use of Rh antibodies. It was found that the Rh positive red blood cells of the fetus enter the blood stream of the mother at birth. Hence, if antibodies were given to the mother at the time of delivery, the antibodies will bind and eliminate the antigen. The mother will thus not have any Rh antigens left to produce antibodies against. This system works and the disease is not so much a threat any more.

Antibodies are also considered to be involved in preventing pregnancies. This drawback should perhaps be looked upon as a blessing in disguise as it seems to offer a plausible solution to the birth control problem.

Laboratory Session # 3 cont.

Laboratory Session # 30 cont.

Immunity at a Glance



Laboratory Session # 31 cont.

Skills and Comprehension Examination II

You will receive:

1 Stool Swab 111

1 Throat Swab 111

1 Urine Specimen 111

Each of the above samples will accompany an order form filled by the Physician.

Process these samples and obtain the information required by the physician.

Record results on the order form and hand in the completed form to your instructor.

You are allowed a total of 3 laboratory sessions including today to complete this assignment (100 points).

Laboratory Session # 32 cont.

Continuation of work begun last session# 31:

INDUSTRY AND SANITARY MICROBIOOOGY

Microbiological Quality Control

The industrial and sanitary microbiologists have two main functions.

Their first function is to isolate and maintain cultures used for producing antibiotics and other useful products. They also ensure that the cultures are pure and remain so upon inoculation in the huge fermenters used for the production of various products. In sewage treatment plants the microbiologist ensures that the desirable type of organisms involved with the digestion of sewage (sludge) are available in the tanks where the sewage is being processed.

The second function of the industrial and sanitary microbiologist is to ensure that the product is microbiologically safe. For instance, it is the job of the microbiologist working in a pharmaceutical company to ensure that all injectable drugs are sterile and that all the drugs which are to be taken by the mouth are free from the disease producing organisms, especially those organisms which produce disease via the oral cavity. Similarly, the food and dairy microbiologist ensures that the food is not only safe, by that it is of such a quality that when purchased by the consumer it will remain safe and usable for a certain period of time.

In order to ensure such a quality the industrial and sanitary microbiologist also keeps a check on the various steps involved in the production of every product. He ensures that the workers are not unduly contaminating the raw material or the product during the various stages of its production. He checks this readily by knowing the number of viable organisms (bacteria) that the product has during the various stages of its production, Steps causing more than a certain amount of contamination are then studied and modified.

The tests which are most commonly performed in industrial and sanitary laboratories are:

- a) Sterility testing,
- b) The number of viable bacteria present per ml or gram of the sample called the viable count
- c) The quality of milk and the effectiveness of pasteurization. Methylene blue reduction test. This test is based on the fact that the larger the number of bacteria that a sample contains, the faster it will reduce a given amount of methylene blue. Reduced methylene blue is colorless.
- d) The presence and number of coliform organisms. This test indicates that a sample which contains Escherichia coli has been contaminated with human feces via sewage or via other means. Such a product could thus also have those organisms which are found in the human feces and which cause disease via the oral-intestinal route. Such a product is, therefore, unfit for human consumption. Water and milk are tested in this way.

Laboratory Session # 3 cont.

Microbiology Quality Control I Sterility testing

In the experiments to follow you will perform each of the preceding tests.

Sterility Testing:

One of the most frequently performed tests by the bacteriological laboratory in the industries is the sterility test. All substances which have to be administered by injection or instilled into the eyes must be sterile. Likewise, certain cosmetics should also be sterile. The responsibility of assuring the sterility of the product lies with the manufacturer who, by law, is required to assure the safety of the product.

The test for sterility should ensure that the product is free from not only bacteria, but also fungi and other microorganisms. Routine tests, however, consist mainly of testing for bacteria and fungi and rarely for viruses.

Fluid thioglycollate and Sabouraud media are inoculated for detecting viable bacteria and fungi, respectively. The thioglycollate medium is used because in this medium most of the aerobic and anaerobic organisms are able to grow. The medium is dispensed in appropriate containers so that there is a low surface area to depth ratio. Thus for 15 ml media use 20 X 150 mm tubes, for 40 ml media use 25 X 200 mm tubes, and for 75-100 ml media use 38 X 250mm tubes. This ensures that both aerobic and anaerobic conditions are existing in one tube. A larger variety of organisms are thus able to grow.

Sterility testing is not an easy procedure. Mere inoculation of the sample into a medium and incubating it for a length of time is not sufficient. The inhibitory effect of the preservative contained in the product must be considered otherwise false negative results will be obtained. Products pronounced sterile on the basis of such results will be unsafe because when administered these will get diluted inside the body. As a consequence the preservative will be ineffective and the organisms present in material injected will be able to grow and probably produce disease.

In order to avoid such false negative sterility tests, the product is first variously diluted with the medium and then the mixture is inoculated with an organism sensitive to the preservative contained in the product. Medium without the product is also similarly inoculated. The dilution where the organism grows just as well as in plain medium is then determined. For sterility testing the product is diluted with the medium to achieve this dilution and then incubated for 7 days. Absence of growth at 7 days indicates that the product can be regarded as sterile. If, however, growth occurs within 7 days the product is reported as not sterile. In addition, the culture grown is also identified.

In practice, the sterility test is done in three steps:

1. Step one is designed to determine if the sample in itself is inhibitory. For this the sample (minimum of 1.0 ml of a liquid sample and 50mg of a solid sample is added to 10 ml of thioglycollate broth. The mixture is inoculated with 0.1 ml of a 1/10,000 dilution of an overnight broth culture of *S. aureus*. If the fungistatic activity of the sample is to be tested, an overnight broth culture of *Candida albicans* is used. A tube of thioglycollate broth is similarly inoculated. The tubes are then incubated at 37° C for 12-24 hrs.

Laboratory Session # 3 cont.

After incubation, one compares the growth (turbidity) of the culture in two tubes.

If the growth of the organism in the tube containing the product is less than the growth in the control tube, then the product is considered inhibitory. It then becomes necessary to determine the amount of broth that will dilute out the inhibitory effect of the sample. This is then the second step. To do this one adds the sample (1 ml if it is liquid and 50 ml if it is a solid),

- 1) To 10; 40; and 100 ml quantities thioglycollate broth. These are then inoculated with the test organisms and incubated. Similar quantities of broth without the sample are also inoculated and incubated. Later determine the dilution where the test organisms grew to the same extent as in thioglycollate broth without the sample. This is the dilution where the inhibitory effect of the product has been nullified. This is the dilution which is then used for actual sterility.

The third step is the actual sterility test. For this, the sample added to that amount of broth which was able to dilute out the inhibitory effect of the sample (as previously determined in step 2). The tube is then incubated at 37° C for 7 days. It is examined daily. If no growth occurs, the sample is regarded sterile. If growth occurs, the sample is reported as non-sterile and the culture is isolated and attempts are made to identify it.

Sterility testing of antibiotics and other such chemotherapeutic agents is more difficult. Previously dilution of the sample beyond its inhibitory concentration was the only method available. Now membrane filter techniques have simplified the sterility testing of such products. In this method, the sample is filtered through the membrane filter. This traps the bacteria. The filter is then placed in the broth and incubated. If growth results the sample is regarded as non-sterile.

Test the sterility of the sample provided to you.

Procedure:

First determine the bacteriostatic nature of the product:

1. Obtain 4 thioglycollate broth tubes.
2. Aseptically add 1.0 ml. of the sample to two of the tubes.
3. Inoculate all 4 tubes with 0.1 ml. of the 1/10,000 dilution of *S. aureus*
4. Incubate at 37° C. for 24 hours
5. Determine the extent of growth by comparing the turbidity of the tubes. This can be done by 1) holding the tubes against a host of printed paper and trying to read the print through the test and the content tubes. Or 2) by measuring the turbidity of the tubes using a photometer,

Then determine the amount of broth which will be needed to dilute the bacteriostatic effect:

1. Add 1.0 ml of the sample to 15, 40, and 100 ml. quantities of thioglycollate broth.
2. Add 0.1 ml of the 1/10,000 dilution of *S. aureus* to each of the tubes. Also inoculate 1 tube of the thioglycollate broth as a control

Laboratory Session # 3 cont.

1. Incubate all tubes at 37° C for 12-24 hours. Then determine the extent of growth in all tubes and find the dilution where the culture grew just as well as in the thioglycollate broth which did not contain the sample.

Finally set up the sterility test:

Add 1.0 ml of the sample to that amount of thioglycollate broth which had diluted in the bacteriostatic effect of the sample. Incubate the mixture for seven days. Observe daily for growth. Record your results in your note book.

Laboratory Session # 33 cont.

MICROBIOLOGY QUALITY CONTROL II

Viable Count

Continuation of work begun last session #31. Hand the results to your instructor.

Continue sterility testing Lab session #32.

Experiments for Today:

Determination of the Number of Viable Bacteria present in a Sample

If we enable each viable bacteria which is present in a sample to grow it will become a colony. The number of these colonies will thus be equal to the number of viable bacteria present in the sample.

In practice, however, all the viable bacteria present in a sample are not able to grow on any one medium. Thus only a portion of the viable bacteria grow and form colonies. The number of colonies thus represent the minimum number of bacteria present in the sample. Also each chain cluster or a clump of bacteria gives rise to one colony.

Because of this factor also, the number of colonies appearing on the media are less than the total number of viable cells present in the sample.

The viable count is thus represented as the "minimum viable" count of a sample. It is reported as the number per ml or per gram of the sample.

How the viable count is performed:

Since samples generally contain too many bacteria, a portion of the sample (generally 1.0 or 0.1 ml) is serially diluted and portions of the various dilutions are plated and incubated. The number of colonies appearing on plates showing 30-300 colonies are then counted and the figure is multiplied by the dilution plated. The answer is reported as the minimum viable count per ml or gram of the sample.

The procedure of making serial dilutions is very important. Each student should, therefore, learn and understand it thoroughly. Your instructor will demonstrate this procedure which is given below:

1. Arrange 5 sterile tubes in a row.
 2. Place 9.0 ml of sterile saline in each of the tubes.
 3. With a sterile 1.0 ml pipette add 1 ml of the sample (methylene blue) to the first tube. Discard this pipette.
 4. Take a new 1.0 ml pipette and mix the contents of the tube #1 by sucking about one half of its fluid with the pipette and then by letting it run into the tube. Make sure that the tip of the pipette is immersed well below the surface of the fluid in the tube. Otherwise, the fluid will rush into the pipette and you may get a mouthful. With the same pipette transfer 1 ml of the mixture to tube #2. Discard the pipette.
 5. With a new pipette mix the contents of tube #2 and transfer 1.0 ml to tube #3. Discard the pipette.
- Repeat as above with tubes #3, #4, and #5.

The dilution of the samples so obtained are:

Tube #1 - 1/10

Tube #2 - 1/100

Tube #3 - 1/1000

Tube #4 - 1/10,000

Laboratory Session # 3 cont.

Tube #5 - 1/100,000

Since each dilution is 10 times more dilute than the previous one such dilutions are called serial 10 fold dilutions.

Also note that since 1.0 ml of the sample was added to tube #1, tube #1 now has 0.1 ml of the original sample.

Similarly each ml of fluid in tube #2 has 0.01 ml of the sample. Each ml of the fluid in tube #3, 4, and 5, have 0.001, 0.0001, and 0.00001 ml of the original sample respectively.

The serial dilution method thus is a way to obtain very small volumes of the original sample.

The serial dilution method, however, is not very accurate. The accuracy further falls when we make a larger number of serial dilutions. There is, however, a way to get around this problem. Since 1.0 ml of each dilution represents a certain portion of the original sample, 0.1 ml of this dilution will represent a 10 times lesser amount. Therefore, to increase accuracy and also to cut down on making excessive number of dilutions 100 fold dilutions of the sample are generally made and a 1.0 ml and a 0.1 ml portion of the dilution is used in tests. This is the procedure that you will use for determining the viable count of the broth culture given to you.

Laboratory work assignment:

Your instructor will demonstrate how to prepare the serial 10 fold dilutions and how to plate the sample. Following the demonstration, you prepare the dilutions of the broth culture and determine the viable count per ml. This assignment is worth 100 points. The procedure is as follows:

1. Arrange 5 sterile tubes (16 X 150 mm) in a row. Pipette 9.9 ml of sterile saline in each of the tubes.
2. Using a sterile 1.0 ml pipette, add 0.1 ml of the sample to the first tube. Discard the pipette and mix the contents of this tube with another sterile 1.0 ml pipette. You now have a 1/100 dilution of the sample.
3. With the same pipette, transfer 1.0 ml of the dilution to an empty sterile Petri plate. Also transfer 0.1 ml to another empty sterile Petri plate. These plates now contain 1/100 and 1/1,000 dilutions of the sample respectively. Mark the plates as such.
4. With the same pipette transfer 0.1 ml of the dilution to the second tube. Discard the pipette and mix the contents of the tube with another sterile 1.0 ml. pipette. You now have a 1/10,000 dilution of the sample in tube #2.
5. With the same pipette transfer 1.0 ml and 0.1 ml quantities separately to 2 sterile empty Petri plates. These plates now contain 1/10,000 and 1/100,000 dilutions of the sample respectively. Mark these plates as such.
6. With the same pipette transfer 0.1 ml of the dilution from tube #2 to tube #3. Discard the pipette. With a fresh 1.0 ml pipette mix the contents of the tube #3 and transfer 1.0 ml and 0.1 ml quantities separately to 2 sterile empty Petri plates. These plates now will contain 1/10,000 and 1/10,000,000 dilutions of the sample.
7. Similarly continue to tube #4 and tube #5. At the end of this procedure you will have 10 plates separately containing:

1/100
1/1,000
1/10,000
1/100,000
1/1,000,000
1/10,000,000
1/100,000,000
1/1,000,000,000
1/10,000,000,000
and
1/100,000,000,000 dilutions of the sample.

8. Now obtain a bottle containing melted TS agar. Make certain that the agar is not too hot. The bottle when touched should be just warm to the touch.
9. Aseptically pour 10-12 ml of the agar in each of the 10 Petri plates containing the serial dilutions of the sample.
10. Mix the contents of each Plate by gently moving the plate in a circle on the table. DO NOT LET THE LIDS OF THE PLATES GET SMEARED WITH THE AGAR.
11. Allow the agar to solidify and then incubate the plates in an upside down position in the incubator.
12. During the next laboratory session obtain these plates and examine.
13. The plates containing the low dilutions of the sample will have too much growth. Record these plates as having confluent growth which is too many to count (TMTc). Count the actual number of colonies on plates showing between 30 and 300 colonies. Record results in your note book. Also complete the assignment sheet and hand it in to your instructor.

[illegible]

Laboratory Session # 33 cont.

VIABLE COUNT TESTING

ASSIGNMENT # 18

Student Identification Number _____

Laboratory #33

Viable Count

Dilution Number of Colonies (Number of colonies x reciprocal of the dilution)

Average _____

The minimum Viable Count is _____ Bacteria/ml.

Laboratory Session # 34 cont.

MICROBIOLOGY QUALITY CONTROL III MILK TESTING

Continuation of work from the last session: Finish lab session #31. Hand the results in to your instructor.

1. Continue sterility testing lab session #32
2. Calculate and report the viable count of the sample which you had processed last time lab session #32

Experiments for Today:

Bacteriological Examination of Milk

Milk is examined to determine if it is safe for human use. The bacteriology laboratory is mainly concerned with the following:

1. How many viable bacteria are in the milk. This number is expressed as number of viable bacteria per ml of milk. Based on this information the milk is graded as follows:

Certified milk: Less than 10,000 viable bacteria per ml

Grade A Raw milk: Not over 50,000 viable bacteria per ml

Grade B Raw milk: Not over 200,000 viable bacteria per ml

Grade C Raw milk: Not over 1,000,000 viable bacteria per ml

Grade D Raw milk: Not over 5,000,000 viable bacteria per ml

Grade A Pasteurized milk: Not over 10,000 viable bacteria per ml

Grade B Pasteurized milk: Not over 100,000 viable bacteria per ml

2. Presence of coliform bacteria: Fresh raw milk may contain a few coliform bacteria, but pasteurized milk should not have any coliform bacteria. If coliform bacteria are found in pasteurized milk then it means (1) incomplete pasteurization, or (2) contamination of milk with fecal matter (from persons, sewage, or polluted water) after pasteurization.
3. Presence of disease producing bacteria in milk: Their presence is not routinely looked for in pasteurized milk. Occasionally, however, the bacteriological laboratory may be asked to look for those disease producing bacteria which have been known to be transmitted through milk. These are:

Mycobacterium tuberculosis: cause tuberculosis

Salmonella typhosa : cause typhoid fever

Streptococcus pyogenes: cause septic sore throat

Corynebacterium diphtheriae: cause diphtheria

Brucella species: cause undulant fever

Shigella dysenteriae: cause dysentery

Routine Tests performed for Grading Milk

Methylene blue reduction test: As most bacteria grow in milk they use up oxygen. The larger the number of bacteria present in the milk, the more rapidly will they use up the oxygen. Depletion of oxygen can be determined by adding small amount of methylene blue to the milk. Methylene blue colors the milk blue when it is added to the milk, but as the bacteria grow and use up the oxygen the color of the milk begins to turn back to its original white color. The time required for the methylene blue to

Laboratory Session # 3 cont.

turn colorless is noted and it is correlated with the number of viable bacteria present in the milk:

Class I good milk: Not decolorized in 5 and half hours (will have a viable count of less than 500,000 per ml)

Class II fair milk: Decolorized in less than 5 and half hours, but not less than 2 hours (will have a viable count of 500,000 to 4,000,000/ml.)

Class III unsatisfactory milk: Decolorized in less than 2 hours, but not less than 20 minutes (will have a viable count of 4,000,000 to 20,000,000/ml.)

Laboratory work assignment:

Test the quality of the pasteurized milk by performing the methylene blue reduction (also called the reductase test). This assignment is worth 50 points.

1. Place 10.0 ml of milk sample in a sterile test tube. Add 1.0 ml of the methylene blue thiocyanate solution. Close the tube with a rubber stopper. Mix the contents of the tube and place the tube in the water bath at 37°C (for the class purposes, place the tube in the incubator). Observe change of color after every hour and note the time it took for the color of methylene blue to disappear. Report the quality of milk, i.e., the class to which it belongs.

Test the quality of the milk by performing the viable count: This assignment is worth 50 points.

1. Obtain the sample of milk. Shake it 25 times (each shake should be an up and down excursion of about 1 foot). Also pipette 9.0 ml of saline into 5 sterile screw capped tubes.
2. Add 1.0 ml of the sample to the dilution tube #1; this will give a 1 to 10 dilution. Discard the pipette and mix the dilution just prepared by shaking up and down 25 times.
3. Transfer 1.0 ml of the dilution with a new pipette to a sterile Petri plate. Also transfer 1.0 ml of the dilution to the next tube containing 9.0 ml of saline. Discard the pipette and mix the dilution by shaking. Mark the plate with the dilution plated.
4. Using a new pipette transfer 1.0 ml of the dilution to the Petri plate and also 1.0 ml to the next tube. Mark the plate with the dilution plated.
5. Using this procedure continue making dilutions until you have obtained and plated the following set of dilutions:
1:10 1:100 1:1,000 1:10,000 1:100,000
6. Pour 10-12 ml of melted and cooled agar (approximately 45° C) in each of the plates. Mix the contents by moving the plate in a circular motion. Incubate all plates at 37° C until the next lab session.

Laboratory Session # 34 cont.

MILK TESTING

ASSIGNMENT # 19

Student Identification Number _____

Laboratory Session #34

Quality of MilkReductase Test

The sample of milk decolorized in _____ hours. The milk is therefore classified as class _____ .

Viable Count of the sample of milkDilution#of ColoniesViable Count

Average: _____

This sample of pasteurized milk is thus of grade _____ .

MICROBIOLOGY QUALITY CONTROL IV WATER TESTING

Completion of work from the last laboratory session:

Continue the sterility testing lab session #32

Determine and report viable count of the sample of milk lab session #34

Experiment for Today:

Bacteriological Analysis of Water

Most bacteria present in rivers, lakes, spring, and oceans are harmless. These bodies of water, however, occasionally get contaminated with raw sewage and the water, therefore, becomes unsafe for drinking as it now contains bacteria which cause such diseases as dysentery, and typhoid fever. Water supplied to cities for drinking purposes is, therefore, first purified and the bacteriological laboratory is called upon to test that the water is safe for drinking.

Bacteriologists generally perform two tests on water: One is the standard viable count which determines the number of viable organisms present in the water and the second is the test designed to detect the presence of coliform groups of bacteria in water.

Why does the bacteriologist look for the coliform group of bacteria in the water:

The bacteriologist is mainly concerned with the fact that the water is not polluted with raw sewage, and if it was once polluted, it has been sufficiently purified to be safe for drinking. He reasons that if the water was polluted with sewage it will contain those bacteria which are commonly found in the sewage and which have come from the discharge of human feces into the sewage. The bacteria most abundant in the feces of humans are Escherichia coli. He, therefore, look for these bacteria in the water and argues that if this species of organisms are present then the water is polluted with human excretions and it may also contain bacteria capable of producing disease through the oral (mouth) route. The presence of Escherichia coli is, therefore, regarded as an index of pollution of water and the water is considered unsafe for human consumption.

How is the water tested for the coliform group?

There are two methods. One is the conventional method, the other method is the newer method, involving the use of membrane filters. In the class you will use both of these methods.

1. In the conventional methods, water sample is inoculated into lactose broth containing gas vials and the tubes are incubated at 37° C. for 24 hours. If gas is produced from lactose then the test is reported as a presumption because bacteria besides the coliform group may be present in water and these may produce gas from lactose. The second part of the test is called Confirmed test and in this part a loopful of the lactose broth from tube showing gas production is streaked on EMB plates and the plate is incubated at 37°C for 24 hours. Presence of typical colonies showing green sheen is regarded as a positive confirmed test. The third part of the test is the completed test. This part consists of inoculating a tube of lactose broth from an isolated colony obtained from the EMB plate. Production of gas in the tube constitutes a positive completed test.

Laboratory Session # 35 cont.

In the membrane filter methods, 50-100 ml sample of the water is filtered through a sterile membrane filter (pore size 0.45 micron, diameter of filter 47 mm.). Bacteria present in the sample are thus retained on the surface of the filter. The filter is then placed on an absorbent pad saturated with a selective and differential medium and the assembly is incubated. Coliform organisms grow on the surface of the filter. These are counted and the results are accordingly reported as # of coliform organisms per 100 ml of water.

Laboratory work assignment:

Test the sample of water for possible fecal pollution by both the conventional and the membrane filter methods.

Procedure:

Conventional Method:

First day:

Inoculate the following amounts of water in lactose broth tubes. Prepare dilution of water as needed.

10.0 ml of water in each of the three double strength lactose tubes

1.0 ml and 0.1 ml of a 1:100 dilution of water in two single strength lactose broth tubes respectively.

1.0 ml from a 1:10,000 dilution of water to a single strength lactose broth tube.

Second day:

Transfer one loopful of broth culture from the tube showing the production of gas to EMB plate and streak for isolation. Incubate plate at 37° C. until the next session. This is the confirmed test.

Third day:

If pink colonies with dark centers or dark colonies with a green sheen developed on EMB plate, transfer one colony to lactose broth and incubate at 37° C. Tests for citrate, indole, methyl red, and Voges-Proskauer reactions may also be performed, but will not be done in the class.

Fourth day:

Examine the broth tubes for gas production. If present, the water is reported to contain Escherichia coli. It has somehow been polluted with fecal matter and this is not safe for drinking.

Membrane Filter Method:

1. Obtain a sterile membrane filter assembly containing a membrane filter. Your instructor will demonstrate how to filter the sample of water and place the filter on top of the absorbent pad for incubation. Then you test the sample of water provided. The procedure is as follows:

1. Attach the filter assembly to a source of vacuum via a trap so that there is no chance of water entering into the pump or the vacuum line.
2. Aseptically filter a 25 ml sample of the water through the filter.
3. Disconnect the assembly from the vacuum.
4. Place a sterile absorbent pad in the small (15 X 60 mm) sterile Petri plate. Soak the pad with 2-3 ml of Endo's medium.
5. Using a sterile forceps (sterilized by flaming and cooled) remove the membrane filter and place it, bacteria side up, on the absorbent pad.

Laboratory Session # 35 cont.

6. Incubate in a humid incubator until the next laboratory session.

Second day:

Observe the color of colonies. Count red colonies and report your results as the number of coliforms per 100 ml of water. Since you have filtered 25 ml of water, obtain the count per 100 ml by multiplying the count by 4.

Class Discussion:

1. Escherichia coli is not pathogenic for man. Then why should the presence of this organism in water make the water unsafe for drinking?
2. What is double strength lactose broth and why is it used for testing water?
3. Why couldn't we directly plate water on the EMB plate and demonstrate the presence of Escherichia coli?

Laboratory Session # 35 cont.

WATER TESTING

ASSIGNMENT # 20

Student Identification Number _____

Bacteriological Analysis of Water

Sample

Conventional Method

Membrane Filter

MICROBIOLOGY QUALITY CONTROL V

FOOD TESTING

Sanitary Quality of a Food

Processing Plant

Food processing plant employs a considerable amount of employees who process raw food material in large quantities. Raw food material contains its own flora. Handling of this food material provides opportunities of further contaminating it.

Poor handling and storage of food and lack of hygienic conditions further add to the microbial load. There are two disadvantages if steps are not taken to control contamination and microbial growth.

- 1) the larger the number of microorganisms, the harder it is to can or otherwise preserve the food and
- 2) the poorer, the hygienic conditions the more chances of contaminating the food with disease producing microorganisms from workers.

These two drawbacks become more serious where frozen and ready to serve foods are concerned.

To overcome these problems, the microbiological laboratory of the food industry maintains a surveillance on the produce from start to finish. This is done by performing viable counts on samples obtained at various stages of production. Results of this surveillance determines the points where procedures have to be modified for manufacturing a quality food product.

In the laboratory session you will receive 4 samples of food obtained at various stages of manufacturing and retailing a frozen ready to serve food. Perform viable counts on these samples and see if the various steps in the overall manufacturing process are being carried out satisfactorily.

Sample 1 reports the mixture of raw dry ingredients. Sample 4 reports the product just prior to shipment to the retail outlets.

Record your results in your note book. Also complete the assignment sheet and hand it in to your instructor (100 points).

FOOD TESTING

Student Identification Number _____

ASSIGNMENT # 21

Sanitary Quality of a Food Processing Plant

<u>Sample</u>	<u>Viable Count</u>
A	
B	
C	
D	

From the above would you conclude that the growth of the product at various stages of its production is satisfactory or unsatisfactory. List and discuss your reasons. What steps in the process are likely to increase the microbial load. List suggestions that will improve these steps.

STUDY OF VIRUSES

Viruses are strict parasites and do not grow until they are able to infect live compatible cells. Sources of such cells are:

- 1) animals, plants, or bacteria
- 2) embryonated eggs (eggs in which the embryo is developing) and
- 3) tissue culture

The tissue culture which is a culture of cells grown in bottles mostly as a single layer of cells, is the most widely used. It is not too difficult to set up the cells for tissue culture are generally obtained from animals which have died just recently. Another advantage is that once the cells are obtained and cultured in the laboratory, they can be maintained and propagated almost indefinitely. There is thus virtually no need to ever again obtain the cells from the animal.

Laboratory work assignment:

The method to set up a monkey kidney cell culture is detailed at the end of this laboratory session. You will not do this experiment in the class. Your instructors, however, will explain its various steps and will also show you tissue culture bottles or plates where viruses have been grown.

While examining these bottles or plates look for plaques and also see the monolayer of cells under the microscope. Your instructor will explain the meaning of the term cytopathic effects. These are the changes that the viruses produce in cells. These range from the death of the cell to mere changes in the shape and structure of the cells.

In the class the principles of virus propagation and quantitation will be illustrated by enabling you to propagate and quantitate a bacteriophage using the bacterial culture which it can infect (its host cells).

Propagation of Bacteriophages:

NOTE: For best results set up this experiment as the first thing in the morning. Your instructor will first demonstrate how to propagate the phage. Following his directions, set up 2 plates. The procedure is as follows:

1. Melt the semi-solid TS agar deep.
2. Cool the deep, but make certain that it is still liquid.
3. Add 0.1 ml of the bacterial suspension and 0.1 ml of the phage sample to this tube of melted semi-solid agar,
4. Mix and spread the contents of the tube on the surface of the TS agar plate. Let this layer solidify and then incubate the plate for 4-5 hours at 37° C.

THE STUDY OF VIRUSES II

Harvesting the bacteriophage:

Your instructor will first demonstrate this procedure. Then you harvest both of your plates. The procedure is given below:

1. With a 10 ml pipette place 2-3 ml of sterile TS broth on the surface of each of the 2 plates. Scrape the surface of the plates and suspend the layer of the semi-solid agar in the broth. Add more broth (up to 10 ml per plate if needed, but avoid excess).
2. Pool the suspensions from both plates in a plastic centrifuge tube and centrifuge it for 10 minutes.

Note: The centrifuge will not rotate smoothly unless the tube is exactly balanced. To do this balance 2 small beakers by placing them separately on the 2 pans of a double pan balance. Then place the tube in one of the beakers. Place an empty centrifuge tube in the other beaker and add enough water to this tube so that it exactly balances the tube to be centrifuged. Now place both tubes opposite each other in the centrifuge and turn on the centrifuge. If the centrifuge is equipped with a switch to regulate its speed, first start it at the low speed and then slowly raise speed to its minimum limit.

After centrifugation, pour the clear liquid from the top (called the supernatant) into a clean tube. This liquid still contains a considerable number of bacterial cells. Therefore, filter it through a membrane filter of 0.45 micron size. Your instructor will first demonstrate this step. Then filter the liquid and collect the filtrate in a sterile test tube. The procedure of filtering is:

Remove the plunger from a 30 ml syringe and attach the syringe to a 47 mm membrane filter holder which has been previously assembled and sterilized with a 0.45 micron filter in place. Pour the liquid to be filtered in the syringe and place the outlet part of the filter in the empty sterile test tube. Attach the plunger and force the liquid with a steady gentle pressure. Collect the filtrate.

NOTE: The filter will retain a considerable amount of the liquid. If you wish to filter and collect this liquid also, remove the syringe and fill it with air. Attach the air filled syringe to the filter and force air through the filter. Repeat if needed. This procedure will enable you to collect almost all of the liquid that you are trying to filter.

Titration of the bacteriophage preparation:

This is done to determine how many bacteriophage particles per ml are present in the preparation. Your instructor will first demonstrate the steps. Then you titrate the filtrate that you have collected. The procedure is:

1. Place 0.9 ml of TS broth in 5, 13 X 100 mm or equivalent test tubes.
2. Using 0.1 ml of the filtrate prepare 10 fold serial dilutions. You may use the same 1 ml pipette to prepare all the dilutions.
3. Swab the propagating strains for the bacteriophage on a TS agar plate.
4. Divide the plate into 5 sections. With a sterile loop place one drop of the dilutions in separate sections.

NOTE: You may also use a pipette to place the drops. If you do use a pipette, place the drops from the highest dilutions first. This way you can use a single

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pipette to place all the drops.

5. Let the drops be absorbed by the agar and then incubate the plate.
6. During the next laboratory session note the highest dilution which has lysed all the bacterial cells in the area where a drop of that dilution was previously placed. Report this as the titer of the bacteriophage preparation.
7. You may also count the plaques (moth eaten areas) produced by one of the higher dilutions of the phage. Each plaque is a viral colony. Multiply the number of plaques by the dilution and report the results as the number of virus (bacteriophage) particles per ml of the preparation.

Procedure to set up tissue culture of kidney cells:

1. Obtain the kidney aseptically. Place it in Hank's balanced salt solution.
2. Remove the outer covering of the kidney (called decapsulation).
3. Cut kidney lengthwise in half and remove the cortex.
4. Place the cortex in the balanced salt solution and cut or mince it into small pieces.
5. Wash the cut or minced cortex with the balanced salt solution. Several washings will be required. Add balanced salt solution and let the pieces settle down. Then pipette off the balanced salt solution. Wash until the salt solution remains clear. Transfer pieces to a 50 ml Erlenmeyer flask.
6. Add about 250 ml of a 0.25% solution of trypsin (0.25% trypsin dissolved in the balanced salt solution) to the washed pieces of cortex.
7. With a magnetic stirrer agitate the pieces of the cortex fairly fast. Place an asbestos pad on the magnetic stirrer so that the flask is not over heated. Continue stirring for 1 hour.
8. Filter the contents through cheesecloth. The filtrate will contain the cells for culturing.
9. Centrifuge the filtrate and wash it a few times with the balanced salt solution. Pool all cells so washed.
10. Measure the volume of the packed cell mass. This is done by centrifuging the cells in a graduated centrifuge tube.
11. Make a 1/100 dilution of the packed cells in Hank's growth medium. This can be regarded as containing 10,000,000 (10^7) cells per ml

OR

Count the cells using a hemocytometer and then prepare a final suspension of the cells in Hank's growth medium so that it contains 10 cells per ml

12. Distribute the cell suspension in 0.8 ml quantities in 16 X 150 mm sterile culture tubes or in 10 ml quantities in 3 ounce prescription bottles.
13. Incubate cultures at 37° C. Replace medium 3-5 days later with Earle's maintenance medium and continue replacing medium every week until the cells are to be used.

Using such a tissue culture, viruses which can grow in it can be:

- 1) propagated and
- 2) quantitated

This same tissue culture is also used for determining the presence of antibodies in the patient serum by the viral neutralization technique.

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