# LABORATORY GUIDE

IN

# BACTERIOLOGY.

BY

J. G. FITZGERALD.

UNIVERSITY PRESS
TORONTO

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## PREFACE.

The exercises included in the "Laboratory Guide" are those at present prescribed in the bacteriological course, in the Medical Faculty of the University of Toronto.

Acknowledgement is due to the authors of several current text-books on the subject. Among these are Park's "Pathogenic Bacteria and Protozoa;" Jordan's "Bacteriology;" and A. Besson's "Technique Microbiologique." The author is also indebted to the excellent monograph of Jungano & Distaso, "Les Anaérobies," for suggestions in regard to anaerobic methods.

J. G. F.

TORONTO, SEPTEMBER 1910.

# University of Toronto

FACULTY OF MEDICINE.

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## BACTERIOLOGICAL LABORATORY.

The key of a laboratory locker and the following supplies, will be furnished each group of two students. Students are responsible for return of same, in good condition. The microscope is to be kept clean; the oil immersion lens must be carefully dried with Lens paper, at the end of each exercise.

LOCKER NO. — SUPPLIES.

- 1 Microscope and Cedar Oil.
  Absorbent cotton.
- 9 Dropper bottles with stains, etc.
- 1 Tripod.
- 1 Bunsen burner and rubber tubing.
- 6 Doz. Test tubes and 1 Test tube rack.
- 1 Tin cup.
- 2 Glass tumblers.
- 1 Burette stand and holder.
- 1 Sheet of blue litmus.
- 1 Sheet of red litmus.
- 10 Petri dishes.
- 2 Blotting Books.
- 6 Fermentation tubes.
- 1 Glass stirring rod.
- 4 Wire baskets.
- 1 Glass funnel, with rubber tubing and clip.
- 3 Erlenmeyer flasks — 500 c.c. capacity; 200 c.c. "; 100 c.c. "
- 2 Saucepans.
- 1 Sheet of Filte-paper.
- 1 Porcelain dish.
- 1 Burette.
- 1 Test tube brush.

TO BE OBTAINED BY THE STUDENT.

| | |
|---|---|
| 1 Note Book. | 1 "China" Pencil. |
| 1 Hand lens. | 1 Platinum "loop." |
| Slides. | 1 Platinum "straight." |
| Cover glasses. | 1 Apron. |

# LABORATORY COURSE—BACTERIOLOGY.

## EXERCISE I.

1. Read laboratory rules, and enter them in the first page of the note-book.

2. Check the list of supplies. If anything is missing, report at once.

3. Clean all glassware—test tubes, Petri dishes and flasks.

First wash in hot water and soap; rinse with HCl 1%. Then wash in sterile water, and allow to drip. When dry plug 2 dozen tubes and place in wire baskets; also 2 flasks; and put 6 Petri dishes in the paper bag (supplied). Write name on each article, and sterilize for *one hour* in hot air at 150° C.

N.B.—*To plug a test tube.* Take a small piece of cotton, and, with a glass rod, insert it into the tube to a depth of ¾ of an inch. Have the smooth side of the cotton in contact with the wall of the tube. Leave at least half-an-inch of the cotton projecting from the top of the tube.

4. Describe the

### THREE METHODS OF STERILIZATION.

(*a*) *Hot air.* Exposure—once for one hour, to dry air at 150° C.

Used for all glassware, etc.

(*b*) *Arnold Steriliser* (by streaming steam).

Exposure—three times for thirty minutes, on successive days, to streaming steam.

This is the *Intermittent* method of Sterilization. In the intervals the articles are left *at room temperature* in order that any remaining spores may germinate and be killed at the next sterilization.

Used for any media.

(*c*) *Autoclave* (by steam under pressure).

Exposure—once, from 20 to 30 minures to steam under 15 lbs. pressure (temperature 250° F).

This will kill all forms of life.

Used for Media, dressings, etc.

N.B.—To Sterilize *scissors*, *knives*, *needles*, etc.

Boil for 5 minutes in water, containing 1% of a 6% sodium carbonate solution.

5. Make drawings of the three sterilizers.

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## EXERCISE II.

1. Conclude work of previous exercise.

2. *Preparation* of *Culture Media*: Nutritive substances on which bacteria will grow; must be sterilized after being prepared; are usually contained in test tubes; for special purposes flasks, plates (Petri dishes), etc., are used.

First medium to be prepared is *Bouillon*.

*Formula* is

| | |
|---|---|
| Lean Beef | 500 grams |
| or | |
| Extract of Beef (Liebig) | 3 " |
| Peptone | 10 " |
| Sodium Chloride | 5 " |
| Water | 1000 " |

Extract of Beef, Peptone and Salt will be given out.

### PREPARATION.

(*a*) Thoroughly cleanse two saucepans.

(*b*) In one put 1000 c.c. of tap water; add the salt; add the Extract of Beef, on a piece of paper (after a few minutes the Extract will leave the paper, which may then be taken out).

(*c*) Mark the level of the fluid on the inside of the saucepan with a china pencil.

(*d*) Boil gently until Salt and Extract go into solution; stir constantly to avoid burning, and, as boiling progresses, add tap water to make up for the loss from evaporation.

(*e*) Add the Peptone slowly, and continue the boiling until it goes into solution.

(*f*) Restore to original volume, and test the reaction of the medium with *phenolphthalein* as indicator, using N/20 NaOH *to titrate*.

## TWO METHODS OF TITRATING MEDIA.

1. With litmus paper.

2. Using phenolphthalein as an indicator, and N/20 NaOH or N/20 HCl to bring the medium to the desired reaction point. Take 5 c.c. of medium and place in a white porcelain dish. Add 45 c.c. of distilled water; boil for 2 minutes to drive off any free $CO_2$ and then add 2 or 3 drops of phenolphthalein; then add N/20 NaOH—a drop at a time—until the first appearance of a faint pink color. Read the result and, if necessary, add N/1 NaOH. Stir thoroughly. Medium should be "+1.5."

(*g*) Place bouillon in "Arnold" for 10-15 minutes (counting from the time the steam is up). By this procedure the phosphates are precipitated. Subsequent sterilization does not result in the medium becoming turbid.

(*h*) Prepare a cotton filter.

(*i*) Filter medium through cotton into the second clean saucepan; and filter again through filter-paper.

(*j*) Fill one dozen test tubes to a depth of 5 c.m., and put the remaining medium in flasks.

*To tube medium.* Pour it into the glass funnel, on the end of which has been attached the piece of rubber with the glass tubing and pinch-cock. Hold the tube in the left hand, and, with the right hand, remove the cotton plug and manipulate the pinch-cock. Do not allow the media to come in contact with the neck of the tube.

(*k*) Sterilize tubes for 30 minutes, and flasks for 45 minutes, in "Arnold."

3. *Prepare 6 tubes of "water-blanks."*

(*a*) Plug six tubes, and into each put 5 c.c. of distilled water, by means of a pipette.

(*b*) Sterilize in "Autoclave" for 20 minutes, at 15 lbs. pressure.

4. *Prepare 100 c.c. of Glucose Bouillon.*

(*a*) To 100 c.c. of bouillon, add 1% of glucose.

(*b*) Heat gently till glucose is dissolved.

(*c*) Fill fermentation tubes.

(*d*) Sterilize in "Arnold."

5. Clean Saucepans and return to Lockers.

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## EXERCISE III.

1. Continue sterilization of media prepared on previous day.

2. *Preparation of Nutrient Agar:* Bouillon, containing 2% of Agar (a Japanese sea-weed).

(*a*) Take 300 c.c. of the bouillon prepared in previous exercise; filter; place in saucepan; mark level.

(*b*) Break up agar into small pieces, and wash in a glass tumbler full of water (this softens and cleans it), pour off the water; add the washed agar to the bouillon.

(*c*) Boil the mixture carefully, until the agar goes into solution. It is necessary to stir the mixture constantly, to prevent burning, and to add water, as the boiling progresses, to make up for the loss from evaporation. The agar goes into solution very slowly, and will have to be boiled for a considerable time. When it is quite dissolved no fine jelly-like particles will remain on the bottom of the pan. Be quite sure of this before restoring to original volume (300 c.c.) by adding tap water.

(*d*) Titrate with phenolphthalein, as in Exercise II., to the same reaction point.

(*e*) Prepare a cotton filter, and wet it down with hot water (this is essential before using, otherwise the agar will solidify).

(*f*) Filter the medium. If it is not quite clear after the first filtering, repeat the process.

(*g*) Have 18 tubes plugged, and tube them to a depth of 3 c.m. with media.

(*h*) Sterilize in "Arnold" (for the *first* time) for 30 minutes; or in Autoclave (once) for 20 minutes at 15 lbs. pressure.

3. To 100 c.c. of filtered and titrated agar, add 1 c.c. of glucose.

(*a*) Dissolve by heating, (*b*) carefully tube six tubes, (*c*) sterilize this *Glucose Agar* in "Arnold."

4. Any excess of Nutrient Agar to be put in a flask, and sterilized in Autoclave for 20 minutes.

5. Clean Saucepans and return to Lockers.

---

## EXERCISE IV.

1. Conclude work of previous exercise.

2. Continue sterilization of media already prepared.

3. *Prepare Nutrient Gelatin.*

(*a*) Put 100 c.c. of filtered bouillon in a saucepan, marking level on the inside with a china pencil.

(*b*) Add 10 grams of "Gold Label" Gelatin (given out), and boil the mixture carefully until the gelatin goes into solution. Stir continually to avoid burning, and, as the boiling proceeds, add tap water to make up for the loss from evaporation.

(*c*) Titrate with phenolphthalein, as in Exercise II. & III., to the correct reaction point ("+1.5").

(*d*) Prepare a cotton filter; wet it down with hot water (this is essential, before using, otherwise the gelatin will solidify).

(*e*) Filter the medium; repeat a second time if the gelatin is not quite clear.

(*f*) Tube 12 tubes, that have been plugged, to a depth of 5 c.m.

(*g*) Sterilize in "Arnold" (for first time) for 30 minutes.

4. *Prepare Litmus Milk.*

(*a*) Take 100 c.c. of milk, and heat in "Arnold" (to separate the fat) for 40 minutes.

(*b*) Remove the fat-free milk with a pipette, and add to it sufficient blue Litmus solution to color the mixture a pale blue.

(*c*) Fill 6 plugged tubes to a depth of 5 c.m.

(*d*) Sterilize in "Arnold" (for first time) for 30 minutes.

5. *Prepare Potato Medium.*

(*a*) Six pieces of prepared potato will be given out.

*Method of preparation:* Solid cylinders about 5 c.m. in length were cut out of old, carefully-washed potatoes, by means of a potato cutter. These, in turn, were cut obliquely in a longitudinal direction, to obtain the slanting surface required, and the pieces washed over night in running water.

(*b*) In the bottom of 6 test tubes, that have been plugged previously, place a small quantity of absorbent cotton, soaked in water.

(*c*) Into each tube insert one piece of potato, the larger, lower end resting on the cotton (which prevents drying out).

(*d*) Replace plugs, and sterilize in "Arnold" (for first time) for 30 minutes.

6. Clean all Saucepans, etc., and return them to the Locker.

## EXERCISE V.

1. Conclude work of previous exercise.

2. Continue sterilization of media on hand.

3. *Preparation* of *Dunham's Peptone Solution.*

*Formula* for one litre

| | |
|---|---|
| Peptone | 10 grams |
| Sodium Chloride | 5 " |
| Distilled Water | 1000 c.c. |

*Prepare* 100 c.c.

(*a*) Place 100 c.c. of distilled water in a saucepan ; add 0.5 grams of salt, and one gram of peptone.

(*b*) Boil carefully until these substances have gone into solution.

(*c*) Restore to original volume.

(*d*) *Do not* titrate, as reaction is non-essential.

(*e*) Filter (preferably through paper) until quite clear.

(*f*) Plug 6 tubes, and fill each with the solution to a depth of 5 c.m.

(*g*) Sterilize in "Autoclave," for 20 minutes at 15 lbs. pressure.

4. *Preparation of Löffler's Blood Serum Mixture.*

*Formula*

| | |
|---|---|
| Glucose Bouillon | 1 part |
| Beef or Pig Serum | 3 parts |

(*a*) Blood is collected at the slaughter-house. (When the serum has separated, chloroform may be added to preserve it.)

(*b*) After twenty-four hours the serum is pipetted off, and thoroughly mixed with the Glucose Bouillon in the proportions given above.

(*c*) Tube the medium, and put the tubes in an oven at 95°C for 5 or 6 hours.

(*d*) Sterilize in "Arnold" on 3 successive days.

*Care of Media:* Keep in a cool dark place; examine daily for the first week, then every few days; if spots, cloudiness, or turbidity appear at any time, re-sterilize, as the medium has probably become contaminated.

*Platinum Needles:* "Loop" and "Straight" are generally used for planting media. Always sterilize before and after using, by passing through the flame and heating to a dull red glow.

*Culture Media:* Are used for the artificial cultivation of micro-organisms, and certain facts (cultural characteristics) are also learned regarding the life history of bacteria, by observing their behaviour on various media.

After micro-organisms are planted in media, masses appear which can be seen with the naked eye. They are known as "colonies." A colony arises as a result of the multiplication of the bacteria.

Cultures in test tubes may be made either in the form of *streaks* or *stabs*; or in Petri dishes, when they are known as *plate cultures.*

Tubes are seeded, or planted with platinum "loops" for *slants* and *liquid media*; with platinum "straights" for *stab* cultures.

In planting, tubes are held slanted to prevent contamination from dust particles in the air. Hold tube in left hand (as shown); flame platinum "loop" or "straight" with right hand, and remove cotton plug (be careful not to contaminate it); flame mouth of tube; carry over a small amount of the culture material, and either rub it over the surface of the slant, or carry to the depth of a "straight" culture tube. In the latter instance the growth appears along the line of the needle puncture.

*Cultures when made are incubated in the thermostat* at 37° C for variable periods, usually 24-48 hours. *Gelatin* cultures, however, *are grown at* 22° C, that is, at room temperature. Most pathogenic bacteria grow best at about 37° C. The thermostat is kept at this temperature by means of a thermo-regulator.

5. *Examine the Thermostat.* Make drawings, and indicate the principle of the thermo-regulator.

*Film, or Smear Preparations:* Bacteria are studied microscopically by means of these films.

They are made as follows:—

(*a*) Prepare a clean glass slide.

(*b*) With a *sterile* "loop" place on it a drop or two of distilled water, also a small amount of the culture material to be examined.

(*c*) Mix the two together, and spread well over the surface of the slide in the form of a thin film.

(*d*) Allow the film to dry, and "fix" it, by passing through the flame—film side up—three times.

(*e*) Apply stains as directed below.

*Stains:* The dyes used for staining bacteria are the basic aniline dyes. These are used to bring out the (*a*) *Morphology*, and (*b*) *Staining Characteristics*, of the micro-organisms.

6. *Prepare films from culture supplied.*

(1) *Stain one with Löffler's Methylene Blue.*

(*a*) Flood the film with the stain, and leave on from 1½ to 2½ minutes.

(*b*) Wash off, and dry with blotter. Do not put a cover-glass over the film.

(2) *Stain a second with Aqueous-Gentian Violet:*

Use the same method.

(3) *Stain a third by Gram's method:*

(*a*) Apply Aniline Gentian Violet for 1½ minutes.

(*b*) Pour off the stain without washing, and

(*c*) Apply Gram's Iodine Solution for 1½ minutes.

(*d*) Pour off, and apply 95% Alcohol until the drippings do not show a violet tinge.

(*e*) Wash off with water.

(*f*) Counterstain with Safranin for 1½ minutes, when necessary. That is, when the Gentian Violet has been bleached by the Iodine Solution and subsequent washing with Alcohol.

If the smear, therefore, is white and decolorized, counterstain as directed. If violet, or black color remains in the smear, no counterstaining is necessary.

*Gram Positive* describes those micro-organisms which *retain* the stain.

*Gram Negative* describes those which *do not* retain the stain.

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## EXERCISE VI.

1. Continue sterilization.

2. Continue preparation of film (smear) preparations.

3. *Stain a fourth film by the Acid Fast Method.*

(*a*) Pour on Carbol-Fuchsin; heat the stain until it steams; keep film well flooded with stain; be careful not to burn.

(*b*) Steam from 3-5 minutes.

(*c*) Wash off stain with water.

(*d*) Decolorize with Acid Alcohol until film is only a faint pink color.

(*e*) Wash off with water, and apply Löffler's Methylene Blue for 1½ minutes.

(*f*) Wash off, blot, and examine.

N.B.—By the use of this stain it is possible to determine whether or not a micro-organism is acid-fast. If it retains the *Carbol-Fuchsin* stain, and appears pink, when so treated, the micro-organism *is acid-fast;* otherwise not. B. Tuberculosis is the best example of an acid-fast micro-organism.

4. Make drawings of smears so prepared. Indicate, in text (1) whether smears show Gram positive or negative Bacteria; (2) whether or not they are acid-fast.

Make drawings, showing what is seen in 3 or 4 different "fields" of the preparation.

5. *Make swabs from cotton and other material supplied.*

(*a*) Take a small pledget of cotton and bind it about the straight end of a copper wire or stick so tightly that it cannot rub off.

(*b*) Plug a test-tube; put in swab, cotton end down.

(*c*) Sterilize in hot air sterilizer for 1 hour at 150° C.

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## EXERCISE VII.

1. Continue sterilization of Media.

2. Conclude study of film preparations made in previous exercise.

*Methods of obtaining material for Bacteriological diagnosis.*

(1) By smears.

(2) By inoculation of culture media (agar, bouillon, blood-agar, blood-serum).

(3) By swabs rubbed over an infected surface, and then over the surface of a slanted tube of some culture medium.

(4) By the inoculation of laboratory animals (to determine pathogenicity).

3. Each member of the class must make a "swab-culture" by swabbing the tonsil of some other member.

(*a*) Rub the swab over the surface of an agar slant.

(*b*) Incubate agar-tube for 24 hours at 37° C.

4. In obtaining a pathological fluid for examination, it is best to use a sterile syringe. Draw off the fluid.

(*a*) Inoculate culture media, and animals, if necessary. (Be careful not to contaminate).

(*b*) Make film preparations, to study the morphology, and staining reactions.

5. *Inoculate Agar slant, Bouillon tube, Litmus milk, Potato and Gelatin* with cultures of unknown micro-organisms (given out).

*Melt two Agar tubes.*

(*a*) When cooled to 40° C, inoculate these with unknown micro-organisms.

(*b*) Flame mouths of tubes, and "pour plates," having slightly warmed the lower part of the Petri dish.

(*c*) When medium has solidified in the plate, invert.

N.B.—Put all tubes in the thermostat for 24 hours at 37° C. Put gelatin in lockers.

6. *Make three film preparations* of each of the unknown micro-organisms.

(*a*) Stain one with Loffler's Methylene Blue; one with Gram's method; one with the Acid-Fast method.

7. Make drawings in book, showing the morphology of the unknown micro-organisms, and indicate their staining reactions.

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## EXERCISE VIII.

1. Conclude work of previous exercise; make drawings, in books, of smears from the culture planted.

2. Examine colonies that have appeared on the various media planted. At the top of the chart note the *number of hours* grown, and *temperature* at which growth occurred.

*Also note in Bouillon Cultures:*

(*a*) Condition of fluid, whether clear or turbid.

(*b*) Character of sediment, if any.

(*c*) Presence, or absence, of a membrane.

(*d*) Characteristic odor.

*Note in Agar Slants:*

(*a*) Form of growth.

(*b*) Size.

(*c*) Surface elevation.

(*d*) Consistency.

(*e*) Color.

(*f*) Effect on media, whether physically changed.

(*g*) Characteristic odor, if any.

*Note in Gelatin Stab Cultures:*

(*a*) Effect on media, whether liquifying or not.

(1) *When non-liquifying:* note the appearance on the surface, and along the line of puncture.

(2) *When liquified:* (*a*) note the shape of the liquified area.

(*b*) Condition of fluid.

(*c*) Character of sediment, and (*d*) characteristic odor, if any.

*Note in Plate Cultures:*

(*a*) Form.

(*b*) Size.

(*c*) Surface elevation.

(*d*) Consistency.

(*e*) Color.

*Note in Litmus Milk*:

(*a*) Whether there has been acid production.

(*b*) Whether casein is coagulated.

(*c*) Whether whey has been extruded.

(*d*) Digestion of coagulated casein (this is, peptonization).

*Note in Potato Culture:*

(*a*) Quantity of growth.

(*b*) Surface elevation.

(*c*) Consistency.

(*d*) Color, including evidence of pigment production.

In studying the cultural characteristics of the small masses of bacteria (known as colonies) it may be necessary to use a hand lens, in order that they may be studied satisfactorily.

3. *Make two smears* from (1) agar plate, (2) gelatin stab, (3) potato.

Stain one by Gram's Method, and the other with Löffler's Methylene Blue.

Make drawings in books of appearances seen in smear (film) preparations.

*In order to differentiate micro-organisms* it is necessary to know the facts in regard to their (*a*) Morphology, (*b*) Staining reaction, (*c*) Cultural characteristics on various media, (*d*) Pathogenic power, (*e*) Specific serum reaction, such as agglutination, etc.

4. *To obtain a pure culture*, in a culture tube containing many colonies of various micro-organisms.

(*a*) Take a small amount of culture material, and transfer it to a second tube.

(*b*) From the second tube transfer a small amount to a third, etc., until small individual colonies can be picked off.

5. Pure cultures can be obtained by making plates, as follows:

(*a*) Plant the culture material in a melted agar, or gelatin tube.

(*b*) Pour a plate; pick off the individual, discrete colonies that appear, and sub-culture. (Plate method is useful when agar, or gelatin, is the medium used).

Observe always:

(1) Whether the growth is better on the surface, or in the depth of the media.

(2) Whether the micro-organism shows aërobic or anaërobic tendencies.

6. *Melt two agar tubes*, and pour two plates.

(*a*) Expose to air, in the room, for 30 minutes.

(*b*) Replace the lid of the dish, and write your name on it.

(*c*) Place in the thermostat for 24 hours.

*Bacteria are classified* according to their *oxygen* requirements, as follows:

(1) The obligative aërobes—those requiring free oxygen for growth, and maintenance of activities.

(2) The obligative anaërobes—those that will not grow unless oxygen is almost rigidly excluded (certain exceptions.)

(3) The facultative anaërobes—those that grow either in the presence or absence of oxygen.

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## EXERCISE IX.

1. Conclude work of previous exercise.

2. Study cultural characteristics of the colonies appearing on the agar plate exposed to air on previous day. Note color of colonies, their form, size, consistency, etc. Note in books.

3. *Make Four Film Preparations* from the agar plate:

(*a*) Pick off four colonies, and make four separate preparations.

(*b*) Stain one by Gram's Method; one by the Acid Fast Method; one with Löffler's Methylene Blue; and one with Aqueous Gentian Violet.

(*c*) Make drawings in books of micro-organisms seen in these preparations. (Only the drawing is essential; the particular micro-organism need not be identified—this identification will come in later exercises.)

### SPORES.

4. *Method of Staining.*

(*a*) Make three films.

(*b*) "Fix" by passing through the flame 10-12 times instead of 3.

(*c*) Stain with Carbol-Fuchsin, as before.

(*d*) Wash off with water.

(*e*) Cautiously decolorize with 5% acetic acid, until pink color has nearly disappeared.

(*f*) Wash off with water.

(*g*) Counterstain with Löffler's Methylene Blue, for 1½ minutes.

(*h*) Wash off, blot, and examine.

N.B.—Spores should appear as crimson oval areas, in the blue stained bacilli.

(*i*) Make drawings of spores so stained.

*True Spores and Endospores:*

Are usually spherical, or oval.

Are non-vegetative forms, and much more resistent than the ordinary bacteria in the vegetative state.

They stain with difficulty, or not at all. This is due to the presence of a dense cellulose capsule.

They are able to withstand the action of higher temperatures, disinfectants, germicidal substances, etc.

They appear as highly refractile globules in the bacteria when unstained.

*Spore-formation.* This is interpreted as meaning that the micro-organism has assumed this state to avoid extinction in an unfavorable medium; or, it may be physiologically considered as a resting-stage in the life history of the bacterium.

It is most frequently observed in bacilli; less commonly in spirilla; and rarely in cocci.

When a spore-forming organism comes into a favorable medium, it germinates.

*Spore-bearing* is usually associated with anaërobic life-habits. Spore-bearing bacilli should be looked for in the film preparations made in this exercise.

5. If a spore-bearing bacillus was not recovered from the air, prepare another agar plate, as before. Expose to the air, and place in thermostat for 24 hours.

6. 24-hour Agar cultures of *B. Typhosus* will be given out.

(*a*) Make a sub-culture in Bouillon from one of these.

(*b*) Place in thermostat for 24 hours.

The culture so prepared will be utilized in the study of *Motility*, as seen in "Hanging Drop" preparations.

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## EXERCISE X.

1. *Study Involution Forms* (for this purpose utilize the colonies remaining on the agar plates).

(*a*) Pick off three or four colonies.

(*b*) Make film preparations.

(*c*) Stain with Löffler's Methylene Blue, in the usual way.

*Involution:* breaking down, degenerating, or old forms of micro-organisms are those showing an *atypical Morpvology*, e.g., a granular change, clubbing, irregularities in outline, or a difference in staining reaction.

Make drawings in books of the involution forms seen in the film preparations.

2. Complete work of previous exercise.

3. *Pick off colonies*, and look for spore-bearing bacilli in the agar plate.

(*a*) Stain colonies with Löffler's Methylene Blue, if this has not already been done.

(*b*) Make drawings in books.

4. *Study of Motility:* As seen in the "Hanging Drop" preparation. Utilize the bouillon-culture of B. Typhosus (planted in the previous exercise).

(*a*) Take a clean cover-glass, and on it place one or two loopfuls of the bouillon-culture.

(*b*) Smear the edge of the rubber ring on the glass slide (given out), with vaseline.

(*c*) Invert the glass slide; lower over the cover-glass, thus enclosing the drop; and with a quick, but careful movement right the slide.

(*d*) Examine with the high-dry lens. (First, however, find the edge of the drop with the low-power lens. The focussing is somewhat difficult, and must be done carefully; use a narrow diaphragm, that is, largely cut off the light. When the edge of the drop has been found, turn on the high-dry lens as directed. The bacteria are nearest the cover-glass at the edge of the drop, and, at this point, can be studied most conveniently.)

(*e*) Note the micro-organisms moving right across the field.

(*f*) Make drawings showing the movements, in a given direction, of one particular bacillus.

(*g*) Read remarks under "Motility."

(*h*) When motility has been studied in this preparation of B. Typhosus, remove the cover-glass carefully, and place it in the bichloride solution provided. (Care should be taken to place the living culture only on the cover-glass; if however, it is accidently dropped elsewhere, apply bichloride solution freely.)

## MOTILITY

To be distinguished from

(1) *Brownian Movement:* A surface-tension phenomenon; the evidence of which is an oscillating to-and-fro movement, but not motility.

(2) *Currents*: Where all the objects in the microscopic field move passively, but obviously, not as a result of their own efforts.

*Motility is the power to move independently*, and depends upon the presence of *Flagella* — long, fine, filamentous threads. The bacilli are driven by the contractile power of the Flagella. The power of motility may be lost if the micro-organism is grown in an unfavorable medium. It is essential, therefore, to choose a medium most suitable for the development of Flagella.

Micro-organisms that are motile when grown on one medium, may be non-motile when grown on others.

Flagella may be seen at one, or the other, or both poles of the bacterial cell; at both sides; or at both sides and at the poles.

The majority of motile bacteria belong either to bacilli or spirilla. Very few micro-cocci have been observed to exhibit motility.

5. *Prepare a second "Hanging Drop" Preparation* of a non-motile micro-organism (given out).

(*a*) Clean a cover-glass as before, and on it place one or two drops of distilled water.

(*b*) Add a small amount of culture material, with the platinum loop.

(*c*) Mix, and do not allow to dry.

(*d*) Examine, and make drawing.

6. *Clean all Tubes* of culture media, plates, etc., used to date:

(*a*) Sterilize in Autoclave for 20 minutes, at 15 lbs. pressure.

(*b*) Boil in saucepans for 15 minutes.

(*c*) Rinse out in clean water, and allow to drip.

(*d*) When dry, plug and re-sterilize the tubes in hot-air sterilizer, for one hour at 150° C.

(*e*) Put plates in a paper-bag, and sterilize, also, in the hot-air sterilizer for one hour.

7. *Place the Tubes* containing the living cultures of B. Typhosus, used in the day's exercise, in a saucepan of water.

(*a*) Boil for 5 minutes.

(*b*) Take a loopful of the culture, and prepare a "hanging drop."

(*c*) Look for motility.

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## EXERCISE XI.

1. Conclude work of previous exercise.

2. *Study of Milk and Water Bacteriology.*

Make Agar and Gelatine plates from a specimen of tap-water, as follows:

(*a*) Melt two agar and two gelatin tubes.

(*b*) With a sterile pipette place 1 c.c. of water in the centre of each of two plates (take care not to have any contamination from the air).

(*c*) Pour the melted agar into one plate, and the melted gelatin into the other; but do not do this until the media has cooled to about 40° C.—the temperature at which it can be held in the hand without burning.

(*d*) Mix the water and medium carefully, by tilting the plates slightly to and fro.

(*e*) When the plates have cooled, invert, and write your name on them.

(*f*) Put the agar plate in the thermostat for 24 hours at 37° C.; and the gelatin plate in the locker—that is, at room temperature (about 20° C.).

3. Given a specimen of Milk, proceed as follows:

(*a*) Melt two agar and two gelatin tubes.

(*b*) Take 0.1 c.c. of the milk in a sterile pipette; add it to a water-blank (5 c.c.) and mix thoroughly.

(*c*) Place 0.1 c.c. of this mixture (a dilution of about 1-50) in a sterile Petri dish, and pour in a tube of melted agar.

(*d*) To the remaining 1-50 dilution of milk and water, add 5 c.c. of sterile water—that is, the contents of another water-blank—and mix thoroughly.

(*e*) Place 1 c.c. of this mixture in a plate, and add the other tube of melted agar.

(*f*) Repeat this procedure for the two gelatin tubes, making the same dilutions as above, 1-100 and 1-50.

(*g*) Allow agar plates to solidify; invert; and place in the thermostat at 37° C.

Place gelatin plates in the locker at 20° C.

4. *Test for Indol.*

Add a few drops of concentrated $H_2SO_4$ to a tube of Dunham's Peptone Solution inoculated, 10 (ten) days previously, with the micro-organism, whose ability to produce Indol is to be tested.

A pink color indicates *Nitroso-Indol* (cholera-red reaction).

If the pink or deep red color does *not* appear, add 1 c.c. of Sodium Nitrite Solution (Sod. Nitrite 0.02 grams; distilled water 100 c.c.).

Now the appearance of a pink color indicates *Indol* production.

5. *Inoculate two tubes* of Dunham's Peptone Solution with a culture of B. Coli (given out).

(*a*) Put tubes, so inoculated, in the Lockers for 10 days.

(*b*) They are then to be tested for Indol.

6. *Inoculate two tubes* of Dunham's Peptone Solution with a culture of Staphylococcus (given out).

(*a*) Put tubes in Lockers for 10 days.

(*b*) They, also, are to be tested for Indol.

*Ehrlich's Method of Testing for Indol Production.*

Solution I.
- Paradimethylamide-benzaldehyde . 4 parts
- Absolute Alcohol ..... 380 "
- Hydrochloric Acid ..... 80 "

(*a*) To 10 c.c. of the bouillon-culture, suspected of containing Indol, add 5 c.c. of Solution I.

(*b*) Then add immediately 5 c.c. of Solution II. (Saturated Solution of Potassium Persulphate).

This reaction with Indol gives a sharp rose color when the mixture is shaken.

After (at the longest) 2 hours, Indol can be detected by this method; with the old, it is sometimes necessary to wait 20, and often 14 hours, before the reaction appears.

Dilutions of 1-200,000 parts are appreciable by this method, which is also specific for Indol (does not give a reaction with skatol).

## EXERCISE XII.

1. Examine the plates made yesterday; count the number of colonies that have appeared on the various plates; record the results in the books.

2. Count the number of colonies on the gelatin plates. Pick off three colonies from each.

Return plates to the locker for a second 24 hours.

3. Pick off three colonies from each remaining plate on which the colonies are numerous.

Make three film preparations.

Stain one with Löffler's Methylene Blue; one by Gram's Method; one by Acid-Fast Method.

4. Make drawings in books, showing the Morphology of the Micro-organisms found. Indicate their Staining Reaction, e.g., Gram Negatite, or Positive, etc.

*The Value of a Laboratory Examination of Water* depends greatly upon certain factors.

For example, the length of time a given specimen of water has been kept, before being examined, is very important; this may very considerably modify the bacterial count. Therefore note the time a specimen is taken; and pack it at once in ice; and so keep until the examination is made.

Water should be collected very carefully in a clean, wide-mouthed, sterile bottle with a capacity of 25-100 c.c.

(1) In the case of *city water*, always let the tap run from 8-10 minutes before collecting. Do not allow the hands to come in contact with the mouth of the bottle, or the stopper to touch anything likely to contaminate it.

(2) From *streams*, the water should be taken at a depth of 10 inches at least. Do not stir up the bed of the stream (otherwise bacteria that have sedimented will be disturbed). Note whether, or not, the stream is flowing.

(3) In the case of *wells*, note all the surroundings—the presence of out-houses, closets, sewage-pipes, cess-pools, stables, gardens, trees, etc. Also note whether the well is covered, or open to the entrance of bacteria-conveying dirt.

All such information is of extreme value in determining the probability of contamination, and in judging whether, or not, a water is potable.

*There is strong presumptive evidence* that water is contaminated—that is, that sewage micro-organisms are present—when the number of bacteria developing at 37° C, at all approximates the number developing at 20° C. *B. Coli* is generally considered the best evidence of water contamination.

*To determine the presence of B. Coli* when bacteria are found in water, the following diagnostic features are considered as sufficient evidence :—

(1) Typical Morphology—the presence of a non-spore-bearing bacillus, relatively small, and often quite thick.

(2) Gram Negative.

(3) Motile in young Agar cultures, usually. (At times this is not satisfactory as certain strains are but feebly motile.)

(4) Gas formation in dextrose bouillon (gases formed being $CO_2$ & H.

(5) Non-liquefaction of gelatin.

(6) Litmus-Milk—acidified and coagulated.

(7) Indol produced.

(8) Nitrates reduced to Nitrites.

(9) Neutral red-litmus lactose bouillon reduced, with the production of a greenish-yellow fluoresence.

## TEST FOR THE REDUCTION OF NITRATES TO NITRITES.

(*a*) Two bouillon tubes, containing nitrates, are inoculated, and, with two other inoculated tubes, are allowed to remain in the thermostat for several days.

(*b*) Then, to culture and control tubes add a small quantity of a colorless thin starch paste, containing 0.5% of KI ; also a few drops of $H_2SO_4$.

N.B.—The control tubes will remain colorless, or slowly become a pale blue ; while a dark-blue, or red-brown coloration is produced, if nitrates are present.

Normally there are a certain number of micro-organisms present in milk. It is only when these are present in excess, or are pathogenic, that the matter assumes any importance.

In planting milk or water, it should always be so suffiiciently diluted that all the bacteria present in the specimen to be examined, will be given an opportunity to develop in the media. This will not be the case if too many colonies appear on one plate. It is best, then, to so regulate the dilutions that never more than 250 colonies can appear on one plate. Even smaller numbers will facilitate counting.

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## EXERCISE XIII.

1. *Continue Bacteriology of Milk and Water.*

(*a*) Estimate the number of colonies on the gelatin plates.

(*b*) Determine the relative proportion between the number developing at 20° C. in 48 hours on gelatin, and the number developing in 24 hours at 37° C. on agar plates.

(Any approximation in relative proportions between the agar and gelatin plates—in the event of the bacterial count being high—should be regarded with suspicion as a probable evidence of pollution. *B. Coli* in 1 c.c., or less, of water in fermentation tubes, is frequently regarded as presumptive evidence of sewage contamination. Certain so-called *paracolon*, or colon-like micro-organisms, are occasionally found in normal, potable water. This may be due to a drainage of rain-fall into the water supply from regions, in the vicinity, fertilized with manure containing these micro-organisms).

(*c*) Pick off *four* colonies, and make four film preparations (48 hour) on gelatin plates.

(*d*) Stain two with Gram's Method, and two with Löffler's Methylene Blue.

## AGGLUTINATIONS.

2. *The Macroscopic Method.*

A tube with 0.1 c.c. of a serum containing agglutinins for B. Typhosus (a so-called typhoid agglutinating serum) will be given out.

(*a*) To this add 0.9 c.c. of normal salt solution, thus making a dilution in tube No. I of 1-10.

Five other small tubes will be given out.

(*b*) Number these, respectively, II, III, IV, V, and VI.

(*c*) To No. II. add 0.5 c.c. from No. I.; also 0.5 c.c. of normal salt solution; shake thoroughly. This gives a dilution of 1-20 in the second tube.

(*d*) Take 0.5 c.c. of this and place in No. III.; add 0.5 c.c. of normal salt solution; shake; and thus we have a dilution of 1-40.

(*e*) From No. III. take 0.5 c.c. and place in No. IV.; add 0.5 c.c. of salt solution; shake; and there will be a dilution of 1-80.

(*f*) From No. IV. take over 0.5 c.c. to No. V.; add 0.5 c.c. of normal salt solution; and from this a 1-160 dilution will result.

(*g*) Place 0.5 c.c. of this in No. VI.; add 0.5 c.c. of salt solution; shake thoroughly, and discard 0.5 c.c. In this tube we will have a dilution of 1-320.

Each tube now contains 0.5 c.c. of a diluted serum, dilutions ranging from 1-10 to 1-320.

(*h*) To each tube add 4 drops of a formolinized culture of B. Typhosus.

(*i*) Shake tubes again, and place in the lockers, to be read at the end of 24 hours.

3. *Microscopic Method.* Proceed as follows:

A suitable dilution, 1-10, of a typhoid serum has been prepared.

(*a*) Put a loopful of this on a glass cover-glass.

(*b*) Add a loopful of a formolinized culture of B. Typhosus.

(*c*) Mix, and invert the cover-glass over a "Hanging Drop" slide.

NOTE.—After a few minutes the bacilli are seen to run together.

When the serum contains agglutinins, this running together or clumping is evidence of agglutination of the micro-organisms.

They are seen, *macroscopically*, as small masses, or flecks at the bottom of the tubes; and *microscopically*, as many bacilli, gathered in irregular groups.

(*d*) Follow the various stages in the microscopic agglutination, and make a drawing, showing the clumped bacilli.

*Blood may be obtained from a patient's finger*, and allowed to clot in a small tube.

(*a*) Clot separated, and the serum drawn off.

(*b*) Centrifugalized to clear, and clear serum used in making dilutions.

Formolinized cultures of B. Typhosus may be used.

Twenty-four-hour-broth cultures, to which have been added 0.1% of Formolin, are allowed to stand for 48-72 hours, at the end of which time the micro-organisms are dead.

4. Make up microscopic agglutinations, and record results.

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## EXERCISE XIV.

1. *Agglutinations Continued:*

(*a*) Read the result of the tubes prepared in yesterday's exercise.

*Positive* tubes (recorded in books with a + sign) are clear, or contain small clumps of agglutinated bacilli.

*Negative* tubes (recorded with a − sign) are indicated by the persistence of a turbidity.

*Certain* tubes may be difficult to interpret, owing to an uncertainty as to the persistence of this turbidity. Such tubes are recorded with a ± sign, indicating that the tubes cannot be regarded as definitely positive, or negative.

In typhoid fever, a positive agglutination in a dilution of 1-50 of the serum may be regarded as positive.

If B. Coli and B. Paratyphosus are used as controls, and if they do not agglutinate above 1-10, then dilutions of 1-20 and 1-40 of the serum giving agglutination only with B. Typhosus, would be regarded as positive.

## AGGLUTININS

are substances produced, or increased in amount, in the blood serum of individuals infected with the micro-organism, for which the agglutinins are present.

When these substances, in the serum, are brought into contact with the micro-organism which produced them, they cause the bacilli to clump. This clumping or sedimentation phenomenon is known as *Agglutination.*

Agglutinins are relatively specific. Certain "group" agglutinins exist, however, which in lower dilutions, cause the agglutination of several of the micro-organisms of the same group, e.g., Typhoid agglutinins will agglutinate B. Coli, or B. Paratyphosus, in low dilutions.

Agglutinins can be artificially produced by injecting animals with the cultures of the micro-organisms for which we wish to produce them.

Agglutinins are antibodies, or immune bodies. They are destroyed by heating to 75° C. for 30 minutes.

It is essential that some mineral salt be present before agglutination will occur.

*Bordet's Explanation of Agglutination is as follows:*

The bacteria play only a passive role. Motility is not necessary since bacteria that have lost their motility, and

red blood cells which are inert, are both capable of being agglutinated. The agglutinins unite with the agglutinable substance in the bacteria, and lead to changes in the molecular attraction between the bacteria, and the surrounding fluid. There are two phases in the process of agglutination. In the first, the individual bacilli are affected by the agglutinins, being clumped; this partakes of the nature of a biological process. The individuality of the micro-organism affected counts only in this first phase. During the second, cells, in obedience to molecular attraction, show, in their agglutination, only such peculiarities as occur in the clumping of mineral particles.

The Agglutination phenomenon is used in the diagnosis of Typhoid Fever (where it is known as the Gruber-Widal reaction), Bacillary Dysentery, Glanders, etc.

2. A culture of *Staphylococcus Pyogenes Aureus* will be given out.

(*a*) Make subcultures in Gelatin Stab, Litmus Milk, on Agar Slant, Potato, and Glucose Agar.

(*b*) Inoculate a melted Agar tube, and pour on a plate.

(*c*) Place these cultures in the thermostat, for 24 hours, at 37° C. Put the gelatin tubes in the lockers.

3. (*a*) Make three film preparations.

(*b*) Stain one by Gram's Method, one by the Acid-Fast Method, and one with Aqueous Gentian Violet.

(*c*) Make drawings in books of the three films so prepared.

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## EXERCISE XV.

1. Conclude work of previous exercise.

2. Examine the subcultures of *Staphylococcus Pyogenes Aureus* planted the previous day.

(*a*) Note the cultural characteristics on the various media—Gelatin, Litmus Milk, Potato, Glucose Agar, Agar plate and Slant—according to the form given in an earlier exercise.

(*b*) Make two film preparations from each.

(*c*) Stain one with Aqueous Gentian Violet, and one by Gram's Method.

(*d*) Make drawings in books.

3. *Staphylococci.* One of the common groups of pus producing (pyogenic) cocci, found in boils, abscesses, carbuncles, etc., usually in the pus.

Certain varieties are pathogenic.

Of these the *Aureus* (so called because it produces a golden pigment) is the most pathogenic.

The other varieties are *Staphylococcus Pyogenes Albus* (producing a white pigment, and much less pathogenic), and *Staphylococcus Pyogenes Citreus* (producing a lemon-yellow pigment, and scarcely pathogenic at all).

*Morphology.* These cocci are usually arranged in loose, irregular masses (due to division taking place in any plane) which have been likened to clusters of grapes. This has given the name to the Staphyloceus group.

A species of the Staphylococcus (the so-called *Staphylococcus Epidermis Albus*) is almost constantly present on the skin, and in the sweat glands. These latter cocci are larger and coarser than the more pathogenic varieties, which range from $0.7^{\mu}$ to $0.9^{\mu}$ in diameter.

Staphylococci may arrange themselves in pairs (diplococcus arrangement), or in fours (tetrad arrangement).

They grow best at 28° C, but very well at 37° C. Best also under aerobic conditions (though they are facultative anaerobes), and on all the ordinary media.

They resist drying and disinfectants for some time, and are one of the hardiest non-spore-bearing micro-organisms.

They are also constantly present in the air, water, on the skin, and mucous surfaces, etc.

Pigment is produced; it is a lipochrome, and is formed most abundantly on carbohydrate media (such as potato), when an excess of free oxygen is present.

In Gelatin cultures (after 4-5 days) a gelatin, liquefying enzyme is formed.

Litmus Milk is coagulated and acidified. The casein so coagulated is not usually digested (peptonized).

Staphylococci are found in both *primary* and *secondary* infections, and in mixed infections.

They are frequently present in Osteomyelitis as a causal agent, and are found associated with suppurative processes, in any part of the body.

Staphylococci stain with all ordinary dyes, and are *Gram positive*.

Of laboratory animals, guinea pigs are relatively insusceptible. Rabbits are insusceptible to intraperitoneal infection, but pathogenicity can be determined by injecting a small amount of a 24-hour bouillon-culture, intravenously.

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## EXERCISE XVI.

1. Conclude work of previous exercise.

Complete the drawings of the various film preparations, etc.

2. Make observations on the Gelatin and Litmus Milk cultures.

*Chromogenesis, or pigment production* is a function assumed by certain organisms.

In order that a pigment be produced:

(i.) the temperature must be favorable,

(ii.) a carbohydrate medium should be used,

(iii.) a liberal supply of free oxygen must be present.

A group of micro-organisms that are but feebly pathogenic are known as the *Chromogens*, so called because of their pigment producing abilities.

Pigment production is regarded as an incidental feature in the metabolic activity of bacteria, pigments being merely by-products.

3. *Streptococcus Pyogenes.* Two films of pus containing Streptococci will be given out:

(*a*) Fix.

(*b*) Stain one with Löffler's Methylene Blue, and one by Gram's Method.

(*c*) Make drawings in books showing the cocci and pus cells.

## STREPTOCOCCUS PYOGENES.

Arranges itself in chains, as a rule, either long or short.

It is a fine coccus, and only divides in one plane, so that there is a persistence of this chain arrangement. Transitions in morphological arrangement occur between staphylococcus and streptococcus.

Is non-motile; Gram positive; stains with ordinary dyes.

Does not thrive on ordinary media. Will grow in blood agar, where it produces hemolysing colonies; on blood serum; and in bouillon containing 1% glucose. Growth on potato, scanty or absent.

Parasitic forms do not liquify; gelatin, and certain saprophytic forms, do.

Milk is sometimes coagulated by it.

The micro-organism can only be kept alive on laboratory media, with great difficulty. This can be done by the use of a Gelatin Stab, or rabbit serum broth at 8-10° C.

Virulence varies considerably as the passage through animals considerably heightens it. The strains living a parasitic existence are more virulent than the others.

May occur in *primary*, *secondary*, or *mixed* infections.

*It is found in Erysipelas*; frequently in *Puerperal Fever*; in certain skin diseases, such as *Impetigo Contagiosa*; in suppurative conditions; in ulcerative *Endocarditis*; frequently in *Otitis* media.

It is also found in the tonsils. In Angina of Scarlet Fever, and in various other anginas, it is present in the throat. In Diphtheritic sore throat the virulence of B. Diphtheriae is heightened by its presence.

It is frequently associated with B. Dysenteriae, an infectious diarrhoea in infants.

As a *secondary invader*, it is associated with B. Tuberculosis; sometimes also, in Pneumonia, it is present as a secondary infection.

Forms of streptococcus are virulent for man, and usually for rabbits.

Agglutinins may be artificially produced.

An *Immune Serum* has been prepared, which has claimed to be of value by raising the "opsonin content." Vaccines of this micro-organism are sometimes useful.

The *Micrococcus Rheumaticus* is claimed, by some, to be a strain of streptococcus.

4. Film preparations of (*a*) *Meningococcus*, (*b*) *Gonococcus* are given out.

(*a*) Stain Gonococcus film by Gram's Method.

(*b*) Make drawings of both, showing various fields.

(The Meningococcus film has already been stained).

## MENINGOCOCCUS, or M. INTRACELLULARIS, or M. MENINGITIDIS.

*Morphology.* Diplococcus, or tetrad formation; occurs characteristically in polymorphonuclear leucocytes; one micron in diameter; usually present in pairs; short chains are rarely seen, and no capsule.

Gram negative.

No change occurs in Litmus Milk.

Involution forms are common, can be grown only with difficulty on culture media.

No growth on potato; feeble growth, if any, on ordinary agar, or broth.

Fair growth on Löffler's Blood Serum sometimes.

Best Medium is { 2% glucose in 2 parts agar 1 part sheep serum } Flexner's Medium.

When attempting to cultivate it, the cerebro-spinal fluid should be quickly added to the media.

*Pathogenicity*: It is the etiologic factor in epidemic *cerebro-spinal Meningitis.*

Lesions at base of the brain extend from the optic commissure, backwards. Meninges of the entire brain are not necessarily affected; but the cord is practically always involved.

*The diagnosis* is determined by means of a lumbar puncture, when the organism is seen in smears, made from the cerebro-spinal fluid.

In 25% of cases, it is found in the blood, where blood cultures are made. (Elser.)

It is frequently found in the nasal secretions, in the early stages of the disease, and the nose is believed to be the portal of entry. The disease probably spreads from contact with infected towels, handkerchiefs, cups, etc.

Agglutinins can be artificially produced.

In the serum of patients suffering from cerebro-spinal meningitis, agglutinins are present.

Rabbits and adult guinea-pigs are insusceptible.

The serum is said to be antitoxic, to stimulate phagocytosis, and to be bactericidal.

Sometimes this micro-organism gives rise to Pneumonia, or rhinitis as a complication.

By the use of the Flexner-Jobling serum, the mortality of of cerebro-spinal meningitis has been reduced from 75% to 25%.

**GONOCOCCUS, or MICROCOCCUS GONORRHOEA.**

Morphology: very similar to the Meningococcus.

The diplococcus arrangement is seen and the cocci are arranged side by side, like coffee beans.

The Gonococcus is also found within the pus cells, or they may be free in the serum. The cocci are confined usually to the cytoplasm of the pus cell.

Gram Negative. (They are decolorized slowly, therefore expose for 10, instead of 5 minutes, to the action of alcohol.)

As a rule no growth is seen on ordinary gelatin and agar.

Blood, or blood serum, must be added to the ordinary media.

The colonies are fine, somewhat tenacious in consistency.

A temperature of about 40° C. is injurious to them.

They are sensative to drying, and will only live for about 72 hours on culture media, at room temperature. They will live longer, however, in the ice box.

Lower animals are relatively insusceptible.

*Diagnosis* depends upon

(1) The situation in which they are found.

(2) The associated clinical picture.

*Gonococcus infection* is not necessarily limited to the genito-urinary tract. In the female it may spread to the ovaries, fallopian tubes, and uterus.

(1) In the male, *Epididymitis* may be produced.

(2) *Gonorrhoeal Opthalmia* is very commonly caused by gonorrhoeal infection at birth and is accountable for 10% of all blindness. (Jordan.)

(3) Infection may be carried by the blood and lymph, and become general, finally settling down on the serous surfaces of the joints (*Gonorrhoeal Arthritis*, for treatment of which Gonococcus vaccines are useful) or of the *heart (Endocarditis)*.

(4) *Metastatic Conjunctivitis* has also been observed.

(5) Gonococci may persist in the genito-urinary tract for very long periods, even when the patient is apparently cured.

*Wertheim's Medium* consists of :—

Nutrient Agar ..... 2-3 parts
Serous Ascitic Fluid..... 1 part

*On Wertheim's Medium* the colonies of the *Gonococcus* resemble those of the *Streptococcus*.

*Gonococcus* and *Meningococcus* bear a very close relationship to one another also. They can be differentiated, however, by specific serum reactions.

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## EXERCISE XVII.

1. Conclude work of previous exercise.

2. Make four film preparations of sputum (each student to use his own) and treat as follows :

(*a*) Cover the film with glacial acetic acid for a few moments.

(*b*) Drain off and flood the preparation with Aniline Gentian Violet—apply once or twice.

(*c*) Wash in 1% NaCl solution.

(*d*) Mount in NaCl solution.

(*e*) Examine preparations, with oil-immersion lens, for encapsulated diplococci-pneumococci.

(*f*) Make drawings of the micro-organisms seen in the sputum.

*Pneumococcus* (Diplococcus lanceolatus):

The cause of

(1) 90% of the cases of lobar pneumonia.

(2) May cause pleuritis, endocarditis, meningitis, pericarditis, and occasionally rhinitis.

It is found in the sputum, in the blood stream, and at the site of the lesion in cases of lobar pneumonia.

It frequently persists in the nasal passages of those who have suffered from pneumonia. Such a "carrier" may be a grave source of danger to others.

Laboratory animals susceptible.

*Rabbits* are highly so. Intravenous injection usually kills in 36 hours, or less, causing a general septicaemia.

*Mice* are also highly susceptible.

Guinea-pigs are relatively immune.

Serum of a patient infected with this micro-organism contains agglutinins for the micro-organism in dilution of 1-40 to 1-50; it is highest about the time of the crisis.

3. Films of sputum containing pneumococcus will be given out.

(*a*) Stain with Löffler's Methylene Blue.

(*b*) Examine and make drawings.

4. *Micrococcus Catarrhalis.*

This is a diplococcus closely resembling the Meningococcus.
Gram Negative.
Found in respiratory passages, and in sputum.
Often associated with B. Influenzae.

Two points distinguish it from the Meningococcus.

(i.) It grows well on ordinary media.

(ii.) It is but slightly pathogenic.

5. *Make Drawings* of *B. Fusiformus*, and the associated *Spirochete*, seen in the film preparations (given out), stained with Aqueous Gentian Violet.

### B. FUSIFORMUS.

A thick, rather long bacillus with tapering ends; from $6^{\mu}$ to $12^{\mu}$ long; $0.6^{\mu}$ to $0.8^{\mu}$ broad; with irregular unstained areas in the cell protoplasm.

Fine spirochetes are usually seen in association with the bacillus (they will be seen as fine, wavy, spiral forms with 3 or 4 curves).

Can be grown under anaerobic conditions. The spirochete has not been cultivated.

B. Fusiformus and the spirochete are found in cases of *Vincent's Angina*—a condition characterized clinically by sore throat, and sometimes mistaken for diphtheria. Small punched out patches appear on the tonsils or soft palate; only local manifestations and often no constitutional disturbance.

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## EXERCISE XVIII.

1. Conclude work of previous exercise.

2. Make four film preparations from the pure cultures of B. Diphtheriæ provided.

(*a*) Stain with Löffler's Methylene Blue.

(*b*) Examine with oil immersion lens for B. Diphtheriæ (Klebs-Löffler).

### BACILLUS DIPHTHERIÆ.

*Morphology:* Slender rods varying from $1\text{-}6^{\mu}$ in length. The most typical morphology is seen in film preparation made from a growth 18 to 24 hours old. Several types are met with—solid, barred, and granular. Occasionally club-shaped forms are seen in film preparations made from diphtheritic membranes.

When stained with Löffler's Methylene Blue they present a *beaded*, *striated*, or *granular* appearance, due to the irregularity in the staining of the cell protoplasm, and to the presence of

metachromatic granules. In the *solid* form, the protoplasm stains uniformly throughout ; in *barred* forms it stains in irregular blocks ; and in *granular* forms only the metachromatic granules (usually one at each pole of the body of the bacillus) stain deeply. The granular type predominates in typical cases of diphtheria.

It is a non-spore bearing, Gram positive bacillus.

Grows best on Löffler's Blood Serum Mixture at a temperature of 20-37° C, in the presence of abundant oxygen, the medium being slightly alkaline. Small colonies are visible in 8-10 hours. Grows also on agar and gelatin.

No liquefaction of Gelatin. It acidifies Litmus Milk.

The micro-organism is frequently found associated with pyogenic cocci.

Virulence may or may not be modified by prolonged growth in culture media.

It usually settles down on mucous surfaces, the pharynx, larynx, etc., but occasionally in other localities. It does not become a septicaemia, or bacteremia. Grows locally, and gives off a diffusible toxin, which acts generally, but chiefly on the heart muscle, and kidney cells and nerves.

Diphtheria is a true toxaemia.

The guinea-pig is highly susceptible to the toxin.

The toxin produces all the typical lesions of the disease, except the membrane in the throat.

Diphtheria Anti-toxin was first produced by Von Behring in 1890. This anti-toxin confers an immunity which lasts for 21 days.

The danger in diphtheria is from "Carriers" (persons who have recovered from the disease but harbor the micro-organisms in their throats).

A mixed infection of Streptococcus or Staphylococcus is more serious than B. Diphteriae alone.

*Bacillus Pseudo-Diphtheriae* (Hoffman).

A short, plump, solid rod. Certain intermediate forms exist between true and Pseudo-Diphtheriae.

It does not produce acid in dextrose litmus agar, or bouillon.

It is non-virulent for guinea-pigs.

Prophylactic measures necessary in diphtheria consist in—

(*a*) Release cultures.

(*b*) The isolation of carriers.

3. Make several drawings of various types of B. Diphtheriae. Use film preparations made from several cultures where it is necessary to see the various types of the micro-organism.

4. Make one film preparation from each of the throat cultures. These are from the throats of patients suspected of having Diphtheria. The film will probably show many cocci, also other bacilli, and some of them will contain true B. Diphtheriae.

Examine each film.

Make drawings in books, and indicate the cultures in which B. Diphtheriae are found, and those in which they are absent.

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## EXERCISE XIX.

1. Conclude work of previous exercise.

2. *Water-blanks, seeded with B. Typhosus, will be given out.*

(*a*) Take two or three loopfuls of this suspension of B. Typhosus; place on a cover-glass (do not spread), dry carefully, and fix.

(*b*) Cover the film with mordant; heat carefully for five minutes; do not allow the mordant to dry on the film.

(*c*) Wash off thoroughly with water, and dry with blotter.

(*d*) Cover the film with carbol-fuchsin and steam as before for 5 minutes.

(*e*) Wash off thoroughly with water; blot; and examine film, with oil immersion lens, for flagella. (Flagella appear as fine, wavy filaments, branching off from the bacillus).

(*f*) Make drawings in books showing flagella.

3. *Inoculate with material from the culture of B. Typhosus* (given out) one tube of each of the following: Litmus Milk, Bouillon, Gelatin, Glucose Agar, Fermentation tube, and Potato.

Liquify a tube of gelatin; inoculate and pour a plate. The planted cultures are to be put in the thermostat for 24 hours at 37 C.; the gelatin is to be left at room temperature.

4. *Make two film preparations* of the culture of B. Typhosus.

(*a*) Stain one with Löffler's Methylene Blue, and one by Gram's Method.

(*b*) Make drawings in books. Indicate whether microorganism is Gram positive or negative.

5. *Inoculate with material from the culture of B. Coli* (given out) one tube of each of the following: Litmus Milk, Bouillon, Gelatin, Agar, Glucose agar, Fermentation tube, and Potato.

These cultures are also to be put in the thermostat for 24 hours at 37 C. Put the gelatin in the locker.

6. *Make one film preparation of B. Coli.*

(*a*) Stain with Gram's Method.

(*b*) Make drawings in book. Indicate whether the microorganism is Gram positive or negative.

*B. Typhosus* (Eberth).

Is a short, plump rod, 1-3$^{\mu}$ in length, and 0.5 to 0.8 in breadth.

It is actively motile.

Gram Negative.

Grows fairly well on ordinary media. There is a thin, film-like growth on potato. Colonies, on gelatin, frequently appear as bluish-white expansions.

No Indol production.

Acid is produced in dextrose agar, or dextrose bouillon, but no gas.

Litmus Milk is not coagulated.

Certain special media, the *Conradi-Drigalski, Endo and Malachite Green*, are used for the differentiation of B. Typhosus.

Agglutinins for B. Typhosus develop in the blood stream of man and animals, infected with the bacillus Typhosus. In Typhoid Fever these agglutinins usually appear on the 7-10th day of the disease. They are present in the 4th week in 90% of cases. The agglutination of the B. Typhosus by the corresponding agglutinating serum, is known as the Grüber-Widal phenomenon.

*B. Coli* is usually a short, thick rod, although considerable variation in morphology is not uncommon. It is 2-4$\mu$ in length, and 0.4 to 0.7$\mu$ in breadth. Sometimes short, oval, and coccus-like forms are seen.

The most typical members of the group are motile, but the motility is much less marked than that possessed by the B. Typhosus.

Gelatin is not liquified.

Litmus Milk is coagulated with an acid reaction in 48 hours.

Indol is produced.

Dextrose agar or bouillon are fermented with gas production ($CO_2$ & H).

It grows on all ordinary media.

It is pathogenic for *rabbits* and *guinea pigs* as a rule, when given by intra-peritoneal injection.

It is found wide spread in nature.

Practically a constant inhabitant of the intestinal tract of man and lower animals.

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## EXERCISE XX.

1. Conclude work of previous day.

2. *Study Cultural Features* of the colonies appearing on the media planted in the previous exercise.

   (*a*) Make notes in book.

   (*b*) Put gelatin tubes away to be examined at the end of 48 hours.

3. *Note whether, or not*, there has been gas production in the fermentation tubes, and in the glucose agar tubes.

4. *Make Four Film Preparations* from the cultures planted with *B. Typhosus*.

(*a*) Stain two by Löffler's Methylene Blue, and two by Gram's Method.

Also make *two film preparations* from any of the cultures of *B. Coli*.

(*b*) Stain as in the case of B. Typhosus.

(*c*) Make drawings in books of the films of both B. Typhosus and B. Coli.

5. *Study B. Tuberculosis*.

(*a*) Make three film preparations of the sputum supplied.

(*b*) Stain by Acid-Fast Method.

(*c*) Make drawings showing the acid-alcohol-fast micro-organisms.

## B. TUBERCULOSIS.

*Morphology*:—a slender rod sometimes slightly curved. About 2-4$^{\mu}$ in length, and 0.3-0.5 in breadth. The rods may be single or in clumps. Vacuoles may occur in the bacilli, causing an appearance like a row of cocci; or the unstained areas may be mistaken for a row of spores.

It is non-spore-bearing, and non-motile.

It stains poorly or not at all with ordinary dyes. By the addition of heat or a mordant, the micro-organism takes the stain and resists decolorization with acid-alcohol.

The artificial cultivation of B. Tuberculosis is difficult. It often fails to grow on favorable media, and does not grow at all on ordinary media. It grows better on subsequent subcultures, if once started. Blood serum medium, glycerin agar, glycerin bouillon or Dorset's Egg Medium are most suitable for the cultivation of B. Tuberculosis. Fine discrete colonies appear in from 10 days to three weeks. Best temperature 37-38° C. Medium should be slightly acid in reaction.

### OTHER ACID-FAST BACILLI.

(*a*) *B. Smegma*: Not acid-alcohol-fast.
Not pathogenic for guinea-pigs.

(*b*) *B. Leprae:* The disease Leprosy has a different clinical picture, and B. Lepræ cannot be grown artificially.

(*c*) *Group of Acid-Fast Branching Bacilli:* There are between 40 and 50 varieties of acid-alcohol-fast *Hay & Butter* Bacilli. These all grow well on ordinary media, and are saprophytic, as a rule.

*Tuberculins:* Are extracts of the bodies of the bacilli prepared in various ways. These are used in the diagnosis and treatment of Tuberculosis.

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## EXERCISE XXI.

1. *Conclude the study of B. Tuberculosis* from the films of sputum, stained by the Acid-Fast Method.

(*a*) Make 3 films of the sputum supplied.

(*b*) Stain by the Acid-Fast Method, and examine for B. Tuberculosis.

2. *Study B. Leprae.* Sections of stained tissue, showing B. Leprae, will be given out.

(*a*) Examine sections with oil immersion lens.

(*b*) Make drawings showing the bacillus in the tissue.

### B. LEPRAE

is a long, slender rod (resembling B. Tuberculosis) and is frequently seen in a bundle arrangement.

Has never been grown artificially on culture media.

Stains more readily than B. Tuberculosis.

Bacilli are found in the kidneys, liver, spleen, lymph-glands and in the nasal secretion.

When leprosy tissue is put into lower animals there is no multiplication of the bacilli; no leprous lesions develop; and no evidence has been adduced to show that the disease can be transmitted to lower animals.

3. *Sections of Tissue containing Treponema Pallidum* (Spirochaetae Pallida) will be given out. These sections are stained by the Silver Impregnation Method of Levaditi, and, when studied by the oil-immersion lens, the Treponema Pallidum will appear as dark, wavy, spirals on a light, brown background.

Study sections, and make drawings.

### TREPONEMA PALLIDUM.

The spirilla range from 4-20$^{\mu}$ in length, and 0.5$^{\mu}$ in thickness.

They consist of a series of fine spirals, close and regular; 3 to 12 even to 10 may be seen.

Has not yet been grown on culture media

Is the cause of syphilis; occurs constantly in the primary lesions, in secondary lesions, in the skin eruption and in the mucous patches; and also in the liver, spleen and kidneys.

It has been found in the blood stream. In cases of congenital syphilis, it is found in the tissues. In Tertiary Syphilis it is much less common.

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## EXERCISE XXII.

1. *Blood Cultures.*

These are done in cases where a *bacteremia* is suspected, or known to exist, and it is desirable to recover the microorganism producing it.

Blood cultures consist essentially in taking a small amount of the patient's blood (under strict aseptic precautions); planting it on a suitable culture media; and observing whether any growth occurs. It is most essential to always take a *small* quantity of blood to a *large* quantity of culture media—about 1 c.c. of blood to 100 c.c. of medium being the right proportions. The blood may be obtained from the ear; but it is usually desirable to puncture a vein, and either allow the blood to run freely through a needle into suitable culture media, or to withdraw a given amount of blood with a syringe, and inoculate the media. When the patient's ear or arm has been prepared,

and sterilized, and the needles have been boiled (the person doing the blood culture also having cleaned the hands) obtain the blood in one of the ways described.

It is advisable to have several kinds of media inoculated—bouillon, plain agar, glucose agar, blood-agar, and blood-agar containing glucose, all being useful.

Plates should be poured, also, and the tubes and plates incubated at 37° C. for the necessary length of time.

In making blood-cultures in *Typhoid Fever*, a small amount of blood is taken in a tube of sterile ox-bile; the tube put in the thermostat over-night at 37° C.; the next day sub-cultures are made in bouillon; and if any growth occurs, agglutinations are done.

Streptococcus, Staphylococcus, and B. Typhosus are micro-organisms that frequently set up a bacteremia.

Bacterial Vaccines may be prepared from micro-organisms, recovered by blood-culture.

2. The culture planted with blood will be put in the thermostat at 37° C, to be examined during the next exercise.

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## EXERCISE XXIII.

(1) Examine Agar plates and bouillon tubes of the blood culture made in the previous exercise.

(*a*) Note whether any growth has taken place.

(*b*) Make films of any colonies that may have appeared.

(*c*) Stain them with Löffler's Methylene Blue, and by Gram's Method.

2. *Film Preparations* of blood containing *Trypanosomes* will be given out.

Examine, and make drawings of the Trypanosomes seen.

3. *For the Study of Anaerobic Bacteria.*

Examine the film preparation of *B. Tetani* (a pathogenic, obligative anaerobe).

4. *Methods of cultivating Anaerobic Micro-organisms.*

*Media used: Agar*, containing 1.5% of either glucose, sacharose, dextrine or lactose; *Gelatin*, to which has been added 1.5% of glucose; *Bouillon*, with 2% of any one of the sugars above mentioned; and *Litmus Milk*. The media should always be freshly prepared (never more than two weeks old) and should be from 1-1.5% of normal acidity to phenolphthalein. Just before using the medium should be thoroughly boiled to drive off any oxygen that might have been absorbed; then cooled rapidly and immediately planted.

To obtain a growth of obligative anaerobic bacteria oxygen must be excluded. This may be done in any one of the following ways:

(*a*) Most simply by liquifying a tube of gelatin or of agar; driving off the free oxygen, by boiling; cooling to 40° C; inoculating; and afterwards pouring in a further quantity of liquified gelatin, or agar; and finally allowing the tube to solidify.

(*b*) In liquid media, such as bouillon, the following procedure may be adopted:

The medium is boiled at the moment of planting (as before directed) and a small quantity of liquid vaseline is added to the tube before it is quite cooled. When the tube has cooled to about 40° C, proceed to inoculate as follows:—Hold the tube inclined in the left hand, in such a way that the layer of liquid vaseline is in contact with the wall of the tube, permitting the pipette, containing the material to be inoculated, to come directly in contact with the bouillon. Blow the culture material out of the tube into the bouillon, then, by drawing it and some of the bouillon into the pipette once or twice, a more thorough admixture of the cultural material and medium is secured. By righting the tube the liquid vaseline again comes to the surface, and acts as effective barrier to the ingress of air. (Jungano and Distaso).

(*c*) A third method consists in the removal of the absorbable oxygen, from the air in contact with the cultures. This is effected by means of pyrogallic acid, and sodium hydroxide solutions (Büchner's Method). Special jars have been devised for use when one follows this method.

(*d*) Or, one may utilize the jars just mentioned in the next method, which consists in creating a vacuum; adding hydrogen to the jars; and allowing the cultures to develop in this atmosphere.

All of the above methods have been thoroughly well tested, and found to be satisfactory.

5. *Inoculate a Glucose Agar and a Gelatin Tube* with the Anaerobic Micro-organism given out.

(*a*) Make two film preparations.

(*b*) Stain one with Löffler's Methylene Blue, and one by Gram's Method.

(*c*) Make drawings in books.

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## EXERCISE XXIV.

1. Conclude work of previous exercise.

2. *Examine Cultures* of micro-organisms planted at the previous exercise.

(*a*) Note cultural features, in the books.

(*b*) Make two film preparations.

(*c*) Stain one by Gram's Method, and one with Löffler's Methylene Blue.

(*d*) Make drawings.

3. *Section of Guinea-pig Tissue* containing *B. Pestis* will be given out.

(*a*) Examine with oil immersion lens.

(*b*) Make drawings in books, showing B. Pestis.

*B. Pestis* is the casual agent in Bubonic Plague, a disease characterized by haemorrhagic septicaemia. The lymph glands, or lungs, may be involved.

A slender, oval rod, about 1.5$^{\mu}$ in length, by 0.5$^{\mu}$ in width, showing bi-polar staining.

Gram negative, and non-motile.

It grows well on ordinary media, forming, at first, fine dew-drop colonies, which soon become large, greyish and confluent.

There is no gas production in glucose or lactose bouillon.

Litmus Milk is changed to a lilac color.

In the diagnosis of Bubonic Plague a small amount of suspected material is injected into the root of the tail of a mouse, or rubbed into the skin of a guinea-pig. Death, as a rule, ensues after a short time, and characteristic lesions are seen. B. Pestis can be demonstrated in film preparations made from these lesions.

4. *Phagocytosis:* For the study of *Phagocytosis* stained film preparations will be given out.

These preparations will show many leucocytes containing bacilli. They are to be studied with the oil-immersion lens, and drawings illustrating the active stage of phagocytosis, are to be made in the books.

The mechanism of phagocytosis is complex and consists of several stages.

It is one of the most important protective agencies preventing the development of infectious diseases.