Student Guidance for Practical Work | *Midwifery*

The Biology of Microbes

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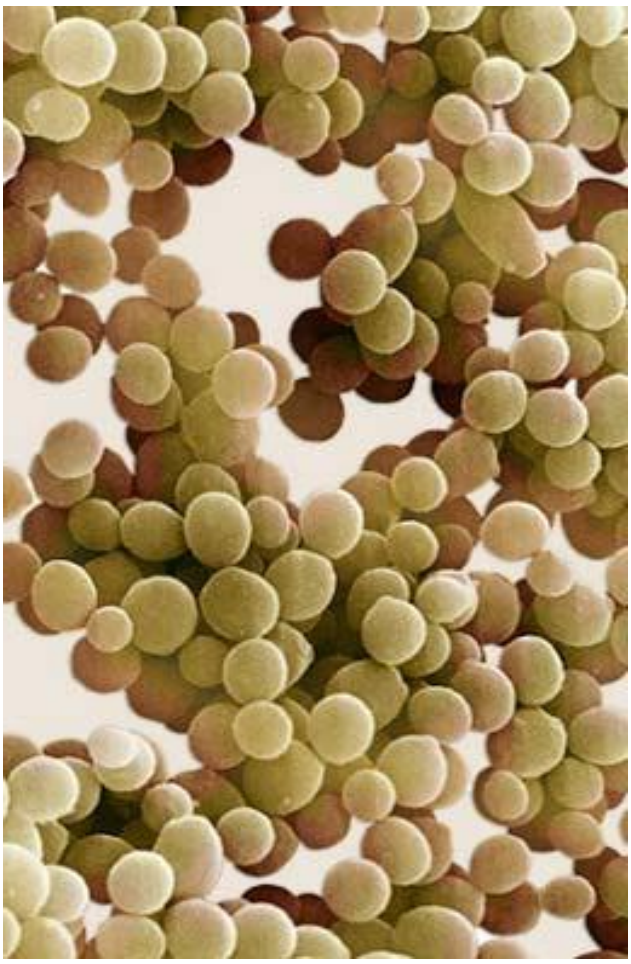

Department of Clinical Microbiology

Faculty of Medicine

Universitas Brawijaya

2019

General Rules for Students

- Respect yourself by wearing **APPROPRIATE** clothing and shoes when attending lectures and practical work. Do not wear t-shirts, jeans, nor sandals.
- **SILENCE** any handheld/gadget during lectures and practical work.
- **PREPARE** these following equipments for practical work:
  - Color pencil
  - Masker
  - Handgloves
- **READ** your text book and practical work guidance and preparation carefully before entering the laboratory.
- Each student should write **INTERIM REPORT** and **FINAL REPORT** with requirements as follow:
  - Each student must write an Interim Report for every practical work. It should be given to facilitator at the same day as the practical work day. If the practical work involves staining, each student must draw their staining result in Interim Reports. Facilitators will give back student's Interim Report on the next day. Facilitator's signature on Interim Reports also acts as evidence of attendance.
  - Final Report must be finished prior to Microbiology Final Examination and given to facilitator on March 8th, 2019. This Final Report acts as final mark of Practical Work activity together with the exam. Each student must finish his/her own report.
- Students must **BRING** their practical work guidance throughout the practical work.
- Students must arrive **FIVE MINUTES** before lectures and practical work.
- Students are **NOT** allowed to eat or drink during practical work.
- Students must wear **LABORATORY COAT** before entering the laboratory and during practical work. Your **NAME TAG** must be on your left chest.

Malang, January 14th, 2019
Head of Department of Clinical Microbiology
Faculty of Medicine, Universitas Brawijaya

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|Chapter 1 |

General Instruction

1.1 Pre-practical work preparation

- Remember that you are facing pathogenic bacteria.
- Prepare this following equipment:
  - Color pencil
  - Waterproof marker
  - Match
  - Tweezers
  - Napkin, antiseptic soap, tissue
- **Read your text book and your practical work instruction book carefully** so that you understand what you are going to do in the practical work.
- Wear your lab coat/apron when you are in the lab and during practical work. Your name tag must be on your left chest.
- Work carefully.
- If you break any of the practical work equipment, you must replace it with the new one.
- Everytime you are about to start working, you must first check the completeness of the equipment/material prepared in your desk. You should also check whether the microscope is in good condition.
- If there is something you lack of, tell the instructor.

1.2 During the practical work

- During practical work, you are not allowed to smoke, eat, or put your fingers or other things inside your mouth.
- If accident happens (even small accident), for example injury or the bacterial culture is spilled in great amount, quickly tell the instructor.
- If the bacteria spill onto the table, your skin, or your lab coat, quickly clean it with cotton moistened with Lysol solution or 70% alcohol.
- Put the used equipment (for example: pipette, object glass, swab, or other equipment) in **lysol** solution.
- Ose, tweezers, etc must be burned first before you store them.
- Bacterial culture must always be closed when not used.
- Put the waste in the waste bin/trash. Do not throw out tissue, cotton, or paper into the drainage.
- The staining process must be done in the staining rack.
- Put out the Bunsen burner when not used.

1.3 After practical work

- Clean the objective lenses (100x) with lens paper or tissue.
- Turn off the taps, fire, and lamps.
- Clean up the bench and put back all the material/equipment into its proper place.
- Wash your hand using antiseptic soap.
- You are not allowed to take the bacterial culture home.
- After every practical work, you must make a report according to the instructor's instruction.
- Use the empty pages of this instruction book to write down the result of your practical work.

|Chapter 2|

Using the Microscope for Microbiological Examination

2.1 Work place

Arrange the table and chair so that the ocular lens of the microscope is eye-level.

2.2 How to look

Try to look using both eyes opened to prevent eyestrain.

2.3 The microscope

1. Check the cleanness of the lenses, the objective lenses and the ocular lens.
2. Adjust the lens position with the light source.
3. Condenser's position:
   In the microbiology laboratory, we examine very small bacteria, so we use objective lenses with 100x magnification (using the immersion oil) whereas the focal length is short (2 mm). In order to get as powerful and as much light as possible into the objective lenses, place the condenser as high as possible. The very high position of the condenser will cause the air volume between the condenser and the object glass becomes very little, so that the entering light rays is not refracted, and the light coming into the object glass will have enough quality and quantity.
   The opposite will happen if the condenser is placed in the lower part.
4. Adjust the amount of light entering the range of visibility in order to get optimum and focused illumination by:
   - Close the condenser diaphragm.
   - Adjust the position of the mirror/lens so that the inside part of the diaphragm can be seen clearly. This means that the amount of light needed is enough.
   - Next, open the diaphragm again.
5. Place the preparation which is going to be examined on the stage.
6. Use low power objective lenses first to choose the area which is going to be examined, that is the thinner part of the preparation, where the bacteria are not clumped. Put into focus the preparation using these low power objective lenses by rotating the coarse-adjustment first, and then the fine-adjustment.
7. When the area to be examined is found, change the magnification of the objective lenses into 100x magnification. Use the immersion oil.
   The immersion oil is used to remove the refractive power of air between the preparation/object glass and the objective lenses, so that more light is entering

the objective lenses because the refractive index of immersion oil is almost the same as the refractive index of glass.
Besides immersion oil, other kinds of oil or other things that have almost the same refractive index as glass can be used, for example: Canada balsam, xylol, balsam, eupara, and glycerol.

8. After you drop the immersion oil (one drop) to the object glass, rotate the coarse-adjustment knob until unclear image is visible. Rotate the fine-adjustment knob to bring the image into focus.
9. After using the microscope, clean the objective lenses using lens paper or cotton moistened with a little xylol/benzene to remove the immersion oil.
Then, clean the remaining xylol/benzene using dry lens paper.

|Chapter 3|

Microbiological Examination

The examination in the Microbiology Laboratory is aimed to find out exactly the kind of bacteria which causes an infectious disease. This is essential to help the practitioner making diagnosis. In order to get the expected result, a laboratory technician must follow certain conditions and obey the examination procedures.

3.1 Specimen

The specimen may be: pus, blood, urine, sputum, feces, cerebrospinal fluid, pleural fluid, joint fluid, nasal swab, larynx, rectum, vagina, etc, depending on the clinical manifestation/symptoms. Sometimes the specimen is already in the form of slide or put into transport medium.

In receiving a specimen, you should consider the following things.

- Is the information on the specimen's label the same as the information in the doctor's request form?
- For example: name of patient, registration number, address, clinical diagnose, kind of specimen, etc.
- Is the specimen representative?
- For example: if the specimen needed is sputum, it is not representative to send saliva as the specimen.
- Is the collection and transportation of specimen done aseptically and properly?
- Other factors which can influence the microorganism/bacteria's life, for example: if there is disinfectant substance in the specimen's container, the bacteria will probably die before it is examined in the laboratory.

3.2 Collecting and Transporting the Specimen

Knowledge and skill in the ways of collecting and transporting the specimen is important in making a clinical diagnose of the cause of an infectious disease. For more information about this topic, especially dealing with the collection and transportation of specimen, please read the book published by the Department of Microbiology, Faculty of Medicine Brawijaya University.

3.2.1 Throat swab

Purpose:

To diagnose of throat infection, such as diphtheria, pertussis, infection by *Streptococcus sp.*, etc.

Materials:

1. Tongue spatula
2. Sterile cotton swab

3. Blood Agar Plate (BAP) medium or Chocolate Agar Plate (CAP) or Pai medium or Loffler medium.

Specimen collection:

1. Depress tongue using tongue spatula.
2. Wipe using cotton swab around larynx, especially at the inflamed part or at the spot/membrane/pseudomembrane.
3. Then the throat swab is examined directly using Gram or Neisser staining, and planted in the proper culture medium.

In transporting the specimen, it must be transported as soon as possible. The cotton swab containing the specimen should be kept in transport medium in order to prevent it from drying out.

3.2.2 Blood

Purpose:

To diagnose of several diseases, such as typhoid fever, sub acute bacterial endocarditis, septicemia, or fever of unknown origin.

Materials:

1. Cotton balls
2. 2% iodine solution
3. 70% alcohol
4. Sterile syringe and needle
5. Medium for blood culture in bottle/tube
6. Tourniquet

Specimen collection:

1. Put on the tourniquet.
2. Palpate the vena from the skin above it at the lower arm.
3. Wipe the skin with iodine (use cotton) with a little pressure circularly from the middle outward.
4. Using the same method, wipe that part of skin with 70% alcohol. Prevent it from drying.
5. Prepare the sterile syringe and needle, inject the needle into the vena.
6. After blood is aspirated into the syringe, put the tourniquet off. Take 10-20 ml of blood for adults (5 ml for children and babies). Take off the needle. Wipe the injected part with 70% alcohol (with a little pressure).
7. Move blood in the syringe aseptically into bottle/tube containing culture medium (the ratio of blood and medium used is usually 1:5). To prevent air contaminating the culture, burn the tip of the bottle on Bunsen burner in slanted position, and then carefully squirt the blood from syringe.
8. Close the bottle. If the cap is made of rubber, clean it first with alcohol to prevent contamination.
9. Incubate it in 37°C for 21 days.
10. Check the specimen every day. Identify if there is bacterial growth.
11. The culture result is said to be negative if there is no bacterial growth in 21 days.

In transporting the specimen, the blood specimen should be placed in sterile syringe with non-toxic anticoagulant substance.

3.2.3 Urine

Purpose: to find out infection in urinary tract and its cause.
Materials:

1. Urine bag (sterile bottle/tube)
2. Normal saline
3. Antiseptic substance, for example: soap, detergent, zephiran, sterile warm water

Specimen collection by clean catch midstream urine method:

1. For men:
   Clean the tip of penis/urethra with normal saline and soap by turns, the last one with sterile warm water. Throw away the first stream of urine, and collect the midstream urine.
2. For women:
   Clean vulva and labial fold the same way as for men. Urinate while standing, and collect the midstream urine.

In transporting the urine specimen, it must be kept in a sterile container and transport it to the laboratory as soon as possible. If it cannot be examined right away (in the laboratory), store it in refrigerator.

3.2.4 Pus

Purpose:to find out the cause of infection on wound.
Materials:

1. Sterile cotton swab
2. Cotton
3. 70% alcohol

Collecting method:

1. Clean the edge of wound using cotton moistened with 70% alcohol.
2. Take the pus using sterile cotton swab (be careful, do not touch the edge of the wound).
3. Take two swabs using the same method, one for direct smear examination and the other one for bacterial culture.
4. The examination should be done as soon as possible before the specimen become dry.
   In transporting the specimen, it should be kept in transport medium.

3.2.5 Vaginal Swab

Purpose: to find out the cause of infection in the vagina.

Materials:

1. Sterile cotton swab
2. Tissue
3. Vaginal speculum
4. Warm water
5. Sterile tube with medium

Collecting method:

1. Explain the sampling procedure to the patient
2. Place the patient in a supine position, support her back with a pillow, and ask politely to draw her heels toward her bottom (lithotomy position).
3. Sterilize the labia with antiseptic provided.
4. Moisten the speculum with warm water and wipe excessive mucus or fluid if necessary. Insert the speculum into the vagina to separate vaginal walls.
5. Insert the sterile cotton swab into vagina and rotate gently on to the vaginal walls.
6. Place the swab into the tube with liquid medium and place it in the incubator. Take out the speculum and clean excessive mucus or fluid.

3.3 Examination Procedure

After the specimen is received and recorded in log book, do the examination following the procedure below.

3.3.1 Direct smear and staining

Make the smear from the specimen at the object glass, then stain and observe under microscope.

- The routine staining done is Gram staining.
- Depending on the clinical diagnosis, certain staining can be carried out, for example:
  - Metachromatic/Neisser staining for diphtheria.
  - Acid fast staining for TBC and leprosy.
  - Spore staining for anthrax and tetanus.

By staining, you can identify the morphology and the characteristic of the bacteria against staining.

For certain specimens, you can make wet preparation without staining, for example when you want to see living bacteria:

- Use hanging drop method to see bacteria movement
- Use darkfield microscope to see Leptospira

3.3.2 Bacterial culture

For bacterial culture and primary isolation, use the appropriate culture medium for its growth, for example:

C. diphteriae : PAI, Loffler medium

M. tuberculosis : Lowenstein Jensen medium

N. gonorrhoe	: Thayer Martin V C N medium
Streptococcus sp.	: Blood Agar Plate medium
D. pneumoniae	: Chocolate Agar Plate medium
Enterobacteriaceae	: Mc Conkey, Eosin Methylene Blue medium

After inoculation or streaking on culture medium, incubate the medium in the incubator using optimum temperature 35-37°C for 18-24 hours.
Certain bacteria need anaerobe atmosphere for growing, so the medium must be put into anaerobic jar. Some bacteria need 5-10% CO_2 for growing; therefore, the medium must be put into candle jar.
If there is bacterial growth (colony) after incubation, bacterial identification can be carried out.
Bacterial identification is determined according to the morphology found at the direct smear examination and the form, the color/pigment of the colony at culture medium.

3.3.3 Biochemical reaction

To identify bacteria, we can do several biochemistry reactions, such as sugar fermentation test, indol production, urease production, and many other biochemical reactions.

3.3.4 Sensitivity against antibiotic/antimicrobial test

The purpose of this test is to find out whether pathogenic bacteria are sensitive towards in vitro antimicrobial. This test helps clinician in giving therapy.

3.3.5 Serological reaction

Serological reaction is essential in making diagnose, for example: Widal agglutination test for diagnosing typhoid fever.

3.3.6 Virulence/pathogenicity test

The purpose of this test is to find out whether the pathogenic bacteria are pathogen/toxigenic or not.
This test can be carried out:

- In vitro, for example coagulase test for *Staphylococcus aureus*.
- In vivo, using experimental animals (such as: rabbit, mouse, guinea pig) infected by bacteria.
  Selecting the experimental animals is based on its sensitivity towards the examined bacteria.

Another thing to be considered when carrying out microbiological examination is that every transportation or collection of bacteria from one medium to another must be in aseptic condition in order to prevent contamination.

|Chapter 4|

The Basics of Bacteriological Examination

4.1 Staining

In this practical work you will learn how to do several staining techniques which are important for clinical needs.

Making the smear for staining

- Clean the object glass with cotton or tissue and pass it above Bunsen burner flame to remove grease. Allow it to cool.
- Make one or two circles with diameter 2-3 cm on one side of object glass. Write label to each circle if necessary.
- Flip the object glass and clean the surface from fingerprints and other debris by using tissue. Pass the object glass over bunsen once to three times.
- Prepare the smear. It should be made not too thick and not too thin by:
    - ***Smear from solid culture***
      Drop one ose of sterile aquadest onto the object glass. Take some bacterial culture using an ose, and then suspense it with the aquadest on the object glass and spread evenly.
    - ***Smear from broth culture***
      Drop one ose of the broth culture onto the object glass and spread evenly. Since broth media is liquid, therefore sterile aquadest is not needed in making the smear.
- Allow the smear to air dry, then heat-fix it on the Bunsen burner for three times.

4.1.1 Simple Stain

Provided:

- Bacterial culture
- Methylene blue or safranin
- Object glass and ose
- Bunsen burner
- Sterile aquadest
- Water and bibulous paper

Procedure:

1. Make the smear on the object glass.
2. Flood it with methylene blue or safranin for ½-1 minute.
3. Wash off the stain with tap water and dry with bibulous paper.
4. Observe it under the microscope using 100x magnification objective lenses. Use immersion oil.

4.1.2 Gram Stain

Provided:

1. Bacterial culture
2. Stain for Gram stain
3. Object glass and ose
4. Bunsen burner
5. Sterile aquadest
6. Water and bibulous paper

Procedure:

1. Make the smear on the object glass.
2. Flood it with crystal violet for 1 minute. Wash off the crystal violet with tap water.
3. Flood it with lugol for 1 minute. Wash off the lugol with tap water.
4. Flood it with 96% alcohol for 5-10 seconds or until the stain is decolorized. Wash off the alcohol with tap water.
5. Flood with safranin for ½ minute. Wash off the safranin with tap water.
6. Dry with bibulous paper.
7. Observe it under the microscope using 100x magnification objective lenses.

4.1.3 Acid Fast Stain

Provided:

- Sputum from chronic cough patient
- Object glass and ose
- Bunsen burner
- Stain for acid fast staining procedure (Ziehl Neelsen method)
- Water and bibulous paper

Procedure:

1. Make the smear from the sputum specimen on the object glass. Since sputum is liquid, therefore sterile aquadest is not needed for making the smear. Allow it to air dry and then fix it.
2. Flood it with carbol fuchsin and heat it for 5 minutes, **do not let it boil and dry**. Wash off the carbol fuchsin with tap water.
3. Decolorize with acid alcohol. Wash off with tap water.
4. Flood with methylene blue for ½ minute. Wash off with tap water.
5. Dry with bibulous paper and observe under microscope using 100x magnification objective lenses.

|Chapter 5|

Fungal Examination

Objective: to study the pathogenic fungi
Provided:

- *Candida albicans* culture in Sabouraud dextrose agar (SDA)
- Filamentous fungi in SDA
- Dyes from Gram staining
- Lactophenol cotton blue (LPCB)
- 10% KOH/NaOH solution
- Specimen: skin/nail scraping, and hair

Procedure:

A. Direct microsopic examination

- Put the specimen on object glass
- Put 1 drop of KOH/NaOH on the specimen and cover it using cover glass
- Heat carefully using Bunsen burner
- Examine under microscope (objective lens 10 – 40x)
- Observe yeast/budding cells, or hyphae/mycelia

B. Staining using LPCB

- Take some culture from filamentous fungi and put on a slide
- Put 1 drop of LPCB and cover with cover glass
- Examine under microscope (objective lens 40x)
- Observe spores, hyphae (septate or non septate)

|Chapter 6|

Medical Bacteriology

6.1 *Staphylococcus sp.*

Provided:

- Staphylococcus from a patient on Nutrient Agar slant
- Empty medium: NAP and nutrient broth
- H_2O_2 3% solution
- Gram stain
- Plasma

Procedure:

Day 1:

a) Take bacteria from slant and inoculate into:
   - NAP to get isolated colony
   - Nutrient broth for catalase and coagulase test

   Incubate at 37^0C overnight (both)

b) Make smear from the colony on slant and do Gram staining; examine under microscope

Day 2:

1. Take the culture you have made the day before, and observe any pigment production by the bacteria on NAP
2. Do catalase and coagulase test using the broth culture

Procedure of coagulase test:

a) Slide coagulase test

- Prepare a clean object glass
- Make bacterial suspension on the object glass
- Add 1 drop of plasma, mix it with the bacterial suspension
- Result (+) → if there are clumping formation. If no clumping formation → confirm with tube coagulase test

b) Tube coagulase test

- Prepare a sterile test tube
- Put 0,5 ml of plasma, add 0,1 ml broth culture
- Incubate at 37^0C, and observe every 30 minutes for agglutination. If there is no reaction, incubate overnight

Procedure of catalase test:

1. Take the remaining broth culture, and put 1drop of H_2O_2 solution
2. (+): if there is bubbles formation

Demo:

1. Slide of *Staphylococcus* sp. (Gram stain)
2. Culture of *S. aureus, S. albus, and S. citreus* on NAP
3. Catalase and coagulase test (slide and tube coagulase)

6.2 *Streptococcus sp.*

Provided:

- *Streptococcus* culture on NA slant
- Blood agar plate (BAP)
- Chocolate agar plate (CAP)
- Gram stain
- H_2O_2 3%
- Brain heart infusion (BHI) broth

Procedure:

Day 1:

1. Take the bacterial culture from NA slant and inoculate into:
   a. BAP and CAP to get isolated colony and hemolytic characteristic of the bacteria
   b. BHI broth
   c. Incubate BAP, CAP (in candle jar) and BHI broth
2. Make smear from NA slant and do Gram staining

Day 2:

1. Take the culture from incubator and observe the hemolytic type on BAP
2. Make smears from BAP/CAP and BHI broth, do Gram staining and study the arrangement of the bacteria
3. Do catalase test using broth culture

Demo:

1. Gram stained *Streptococcus* slide
2. Culture of *Streptococcus pyogenes*, viridans group, and *S. fecalis* on BAP

6.3 *Mycobacterium sp.*

Provided:

1. Sputum from patients
2. Lowenstein Jensen (LJ) medium
3. Dyes for acid fast staining (Ziehl Neelsen method)
4. H_2SO_4 4% and NaOH 4% solution

Procedure:

1. Make smear of sputum, and do acid fast staining. Examine under the microscope.
2. Do concentration method to the sputum as follows:
   - Mix 1 part of sputum with 5 part of H_2SO_4 and centrifuge (3.000 rpm) for 15minutes

- Wash the sediment using normal saline by centrifuging as above, discard the supernatant

3. Inoculate LJ medium with the sediment, and incubate at 37^0C for 8 weeks (check bacterial growth every 2 weeks).

Demo:

1. LJ medium (blank)
2. *Mycobacterium sp*. culture on LJ medium.

6.4 *Enterobacteriaceae*

Provided:

- Selenite/tetrathionate broth
- McConkey agar, SS agar, EMB agar

Demo:

1. *Escherichia coli* in McConkey Agar
2. *Escherichia coli* in EMB Agar