Practical activity (8)

Investigating Microbes and Hygiene

(Teacher's Guide)

I. Objectives of the experiment

1. To make a handrub according to the WHO-recommended formulation;
2. To collect and cultivate microbial samples from your surroundings and your bare hands;
3. To differentiate bacteria into two large groups by the Gram staining technique;
4. To show the antiseptic effect of different cleaning agents; and
5. To compare the antiseptic effective of different ingredients in the handrub.

II. Expected Learning Outcomes

Upon the completion of the activities, students should be able to:

1. collect and culture microorganisms collected from the surroundings;
2. compare the antiseptic effect of different cleaning agents;
3. conduct Gram staining; and
4. make a handrub and examine its antiseptic effect.

III. Teaching notes:

1. Introduce the task by going through the "Background" with the students or have the students read through this part and the "Guiding questions about the design of the experiment" as a pre-class activity.
2. Discuss the design of the experiment with the students using the "Guiding questions about the design of the experiment".
3. Plan the laboratory work with reference to the "Time allocation for the experimental activities" in section IV according to the lesson time of your school.
4. Always remind the students about the safety precautions of each part of the experiment.
5. Go through the 'Results' and 'Discussion' with the students.

IV. Time allocation for the experimental activities:

Experimental activities		Duration	
		In class	**Out of class** (to be done by the laboratory technicians)
Lab session 1: Part (1) of the experiment			
1	Making of handrub	20 min	
Lab session 1: Part (2) of the experiment			
1	Sampling from surroundings	20 min	
2	Fingerprints on the agar plate	20 min	
3	Incubation of microbes		1–2 days
Lab session 2: Part (3) of the experiment			
1	Gram staining and microscopic observation	40 min	
Lab session 2: Part (4) of the experiment			
1	The antiseptic effect of discs soaked with different solutions	20 min	
2	Incubation of microbes		3–4 days
Lab session 3: Part (5) of the experiment			
1	Observation and analysis	30 min	
Total lesson time for the experimental activities		Lab session 1: 60 min Lab session 2: 60 min Lab session 3: 30 min	

V. Equipment, materials, and preparatory work for the experiments

A. Part (1) of the experiment: Preparation of handrub

a) Equipment (per group)

- Micropipettes (P5000, P1000, and P200) and sterile tips

b) Materials (per group)

- Sterile 15 ml centrifuge tube	× 1
- Centrifuge tube stand	× 1
- 96% ethanol (10 ml)	× 1
- 3% hydrogen peroxide (1 ml)	× 1
- 98% glycerol (0.5 ml)	× 1
- Sterile distilled water (10 ml)	× 1
- Permanent marker	× 1
- 70% ethanol in spray bottle	× 1
- Paper towel	× 1 box
- Biohazard bag	× 1
- Disposal container with 10% chlorine bleach	× 1

c) Preparatory work

Aliquots of experimental reagents (**To be done by teacher / laboratory technicians before lab session**)

1. Aliquot 10 ml 96% ethanol into sterile centrifuge tubes. Prepare one tube per group.

2. Aliquot 1 ml 3% hydrogen peroxide into sterile micro-centrifuge tubes. Prepare one tube per group.

3. Aliquot 0.5 ml 98% glycerol into sterile micro-centrifuge tubes. Prepare one tube per group.

4. Aliquot 10 ml sterile distilled water into sterile centrifuge tubes. Prepare one tube per group.

B. Part (2) of the experiment: Collection of microbiological samples from surroundings and bare hands

a) Equipment (per group)

- Mobile device × 1

b) Materials (per group)

- Sterile swab (breakable stick with a cotton bud) × 2
- Masking tape × 1
- WHO-formulated handrub from Part (1) × 1
- Hand-washing soap × 1
- Wet hand wipes × 1 pack
- Culture tube with sterile LB broth (5 ml) × 2
- Culture tube rack × 1
- LB agar plate × 2 (per student)
- Adhesive tape
- Parafilm

- Permanent marker × 1
- 70% ethanol in spray bottle × 1
- Paper towel × 1 box
- Biohazard bag × 1
- Disposal container with 10% chlorine bleach × 1

c) Preparatory work

Preparation of LB agar plates and LB broth **(To be done by teacher / laboratory technicians one day before lab session)**

1. Dissolve LB agar powder in distilled water according to the manufacturer's instructions. Autoclave the LB agar solution, and pour it into Petri dishes (15–20 ml per dish). As soon as the agar is solidified, wrap the dishes with parafilm and store them properly at 4°C until use. Prepare 2 LB agar plates per student. If there is not enough time, purchase ready-to-use LB agar plates from biotechnology companies.

2. Dissolve LB powder in distilled water according to the manufacturer's instructions. Autoclave the solution, and aliquot 5 ml into sterile culture tubes. Prepare 2 tubes per group. If there is not enough time, purchase ready-to-use LB broth from biotechnology companies.

d) Sterilisation and disposal

After the experiment, some equipment and materials to discard (solid or liquid) should be sterilised by steam under pressure (autoclave) at 121°C, 15 psi pressure for at least 30 minutes or immersed in 10% chlorine bleach for at least 2 hours.

C. Part (3) of the experiment: Gram staining of microbes collected from the surroundings

a) Equipment (per group)

- Stereomicroscope with 20X / 40X objectives × 1
- Bunsen burner × 1
- Spark lighter × 1
- Micropipette (P20) and sterile tips
- Mobile device × 1

b) Materials (per group)

- Culture tube A from Part (2) × 1
- Inoculating loop × 1
- 500 ml beaker × 1
- Microscopic slide × 1
- Coverslip × 1
- Forceps × 1 pair
- Sterile distilled water (1 ml) × 1
- Crystal violet solution (1 ml) × 1
- Gram's iodine solution (1 ml) × 1
- 96% ethanol (5 ml) × 1
- Safranin solution (1 ml) × 1
- Wooden slide holder × 1

- Permanent marker × 1
- 70% ethanol in spray bottle × 1
- Distilled water in wash bottle × 1
- Paper towel × 1 box
- Biohazard bag × 1
- Disposal container with 10% chlorine bleach × 1

c) Preparatory work

Preparation of experimental materials (**To be done by teacher / laboratory technicians before lab session**)

1. Allocate adequate amounts of dyes and staining reagents to dropper bottles. All ready-to-use dyes, staining reagents and dropper bottles can be purchased from biotechnology companies.
2. Prepare one bottle of crystal violet solution per group.
3. Prepare one bottle of Gram's iodine solution per group.
4. Prepare one bottle of 96% ethanol per group.
5. Prepare one bottle of safranin solution per group.

Notes to teacher:

The gram-positive bacteria contain a thick layer of peptidoglycan, but they lack an outer membrane. The gram-negative bacteria have a thin layer of peptidoglycan and an outer membrane that is rich in lipopolysaccharides.

Gram staining involves four major reagents: crystal violet, iodine solution, ethanol, and safranin. Crystal violet acts as the primary stain which gives the peptidoglycan layer of both types of bacteria a purple colour. Iodine solution helps fix the primary dye and is a mordant. Ethanol is then applied for decolourising the unsteady stain, such as the stain trapped by the thin layer of peptidoglycan of the gram-negative bacteria. Finally, the cells are counterstained with safranin, which gives a pink colour.

D. Part (4) of the experiment: Antiseptic study of different cleaning agents and different ingredients of the handrub

a) Equipment (per group)

- Bunsen burner × 1
- Spark lighter × 1

b) Materials (per group)

- Culture tube A from Part (2) × 1
- LB agar plate × 6
- Sterile swab (breakable stick with cotton bud) × 6
- 3 cm filter paper disc soaked with reagents × 6
- Forceps × 1 pair
- 250 ml beaker with 70% ethanol × 1
- Adhesive tape
- Parafilm

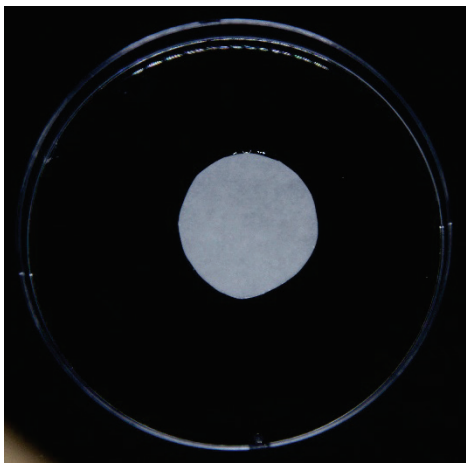
- Permanent marker × 1
- 70% ethanol in spray bottle × 1
- Paper towel × 1 box
- Biohazard bag × 1
- Disposal container with 10% chlorine bleach × 1

c) Preparatory work

Preparation of 3 cm filter paper discs soaked with reagents **(To be done by the teacher / laboratory technicians one day and 2 hours before lab session)**

1. Trim the filter papers into discs with a diameter of 3 cm. Autoclave the discs at least one day before the experiment.

2. For each filter paper disc, soak with 500 µl corresponding test solution in a petri dish (listed in the Table below) 2 hours before the start of the experiment as shown in the following figure.



Filter paper disc	1	2	3	4	5	6
Label on petri dish	96% E	80% E	H_2O_2	G	W	HR
The test solution the paper disc to be soaked with	96% Ethanol	80% Ethanol	0.125% H_2O_2	1.45 % Glycerol	Sterile distilled water	Handrub

d) Sterilisation and disposal

After the experiment, some equipment and materials to discard (solid or liquid) should be sterilised by steam under pressure (autoclave) at 121°C, 15 psi pressure for at least 30 minutes or immersed in 10% chlorine bleach for at least 2 hours.

E. Part (5) of the experiment: Observation and analysis of experimental results

a) Equipment (per group)

- Mobile device × 1

b) Materials (per group)

- LB agar plate from Part (4) × 6

- Permanent marker × 1
- Ruler × 1
- 70% ethanol in spray bottle × 1
- Paper towel × 1 box
- Biohazard bag × 1
- Disposal container with 10% chlorine bleach × 1

c) Preparatory work

No specific preparation work is required.

d) Sterilisation and disposal

After the experiment, some equipment and materials to discard (solid or liquid) should be sterilised by steam under pressure (autoclave) at 121°C, 15 psi pressure for at least 30 minutes or immersed in 10% chlorine bleach for at least 2 hours.

VI. Suggested answers to the "guiding questions about the design of the experiment".

1. Which type(s) of bacteria do you expect to find in your surroundings?

   Both gram-positive and gram-negative bacteria

2. What portable tool is suitable for collecting microbial samples from the surroundings?

   A swab: a breakable stick with cotton buds

3. What ingredients should be found in the culture medium for microbes?

   The culture medium should contain the nutrients needed to sustain the growth of a microbe, for instance, amino acids, carbohydrates, minerals… and buffering agents.

4. What is the underlying principle of Gram staining?

   Gram staining differentiates bacteria by their different components in the cell wall. Particularly, the presence of a thick layer of peptidoglycan in the gram-positive bacteria holds the crystal violet even after decolourisation by alcohol The gram-negative bacterial cells pick up the colour of the safranin solution in the counter-staining step.

5. Search for the two formulae for making handrub as suggested by the WHO.

   Formula 1: 80% ethanol (v/v), 0.125% hydrogen peroxide (v/v), 1.45% glycerol (v/v), and water.
   Formula 2: 75% Isopropyl alcohol (v/v), 0.125% hydrogen peroxide (v/v), 1.45% glycerol (v/v), and water.

   In this experiment, formula 1 was used.

6. How do we test for the antiseptic effectiveness of the handrub?

   We can do a fingerprint with bare hands before cleaning on an agar plate, and then do a fingerprint after cleaning with handrub on another agar plate. By comparing the growth of microbes on the two agar plates, the antiseptic effect can be deduced.

VII. Results

a) Part (1) of the experiment

Fill in the following table with the final concentration (in percentage) of the four ingredients required for making the WHO-recommended handrub.

	Ingredient	Amount required	Final concentrations of each ingredient in percentage (v/v)
1	96% ethanol	8333 µl (Hint: 5000 µl + 3333 µl)	*80* %
2	3% hydrogen peroxide	417 µl	*0.125* %
3	98% glycerol	145 µl	*1.42* %
4	Sterile distilled water	1105 µl	--
	Total volume	10 ml	--

b) Part (2) of the experiment

About the microbial samples collected from the surroundings

1. The choice of sampling environmental surface is

   _____, sampling area A is _____ cm^2, and

   sampling area B is _____ cm^2.

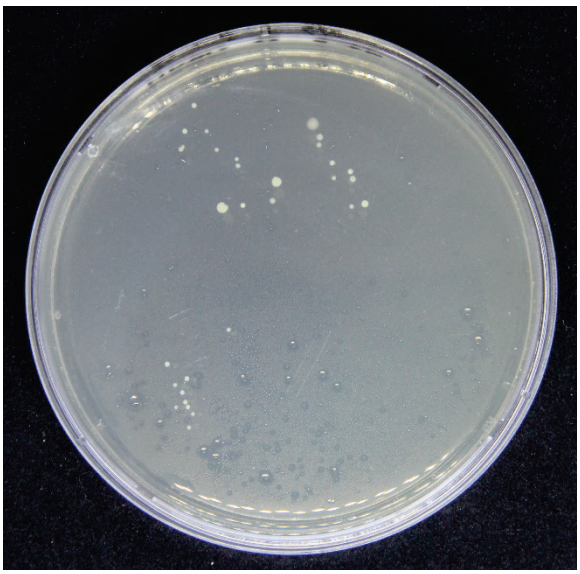
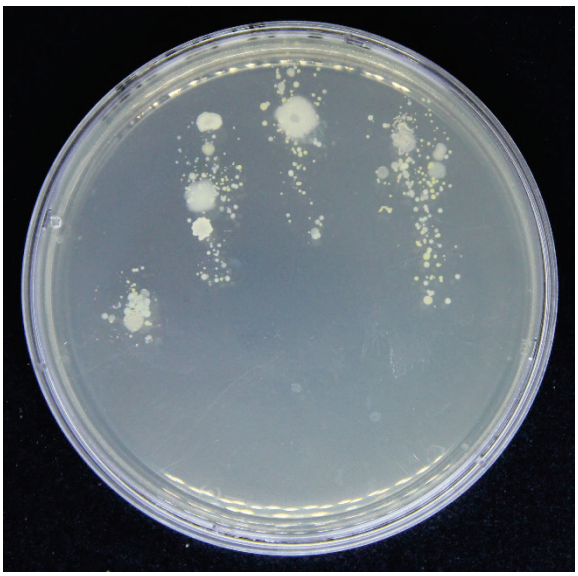
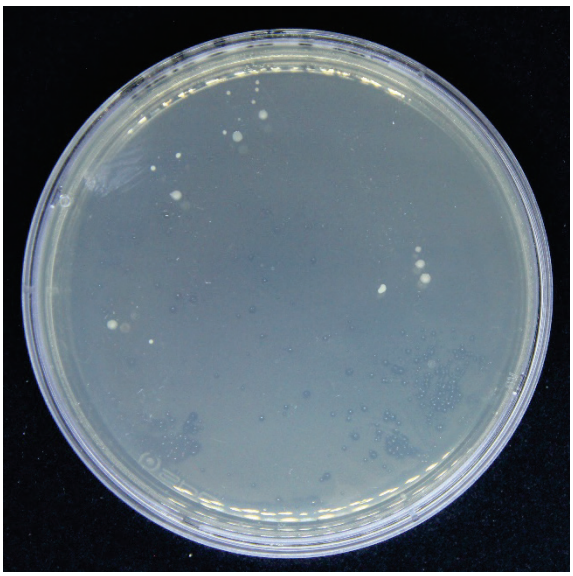
2. Which culture tube (A or B) has a cloudier broth?

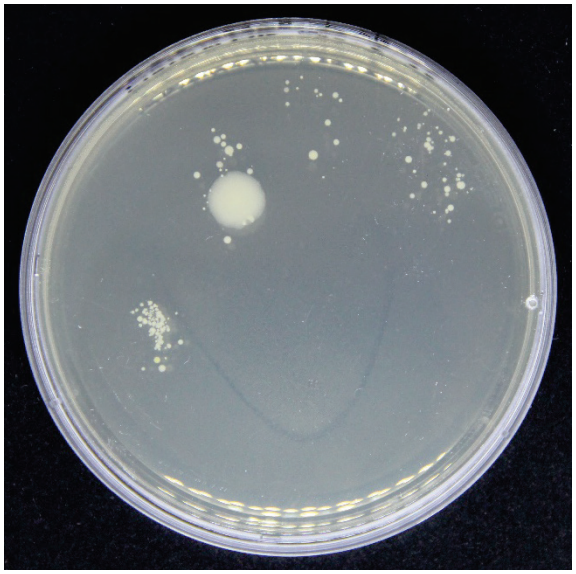
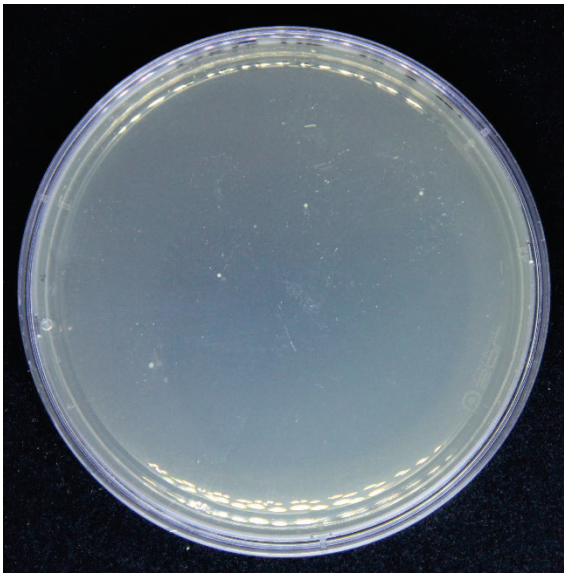
   Tube A

About the microbial samples collected from bare hands

3. Paste the photo or draw a picture of the LB agar plates after microbial incubation in the boxes below.

[For reference only]

LB agar plate X (before cleaning)	LB agar plate Y (after cleaning)
Student 1 (before cleaning) 	Student 1 (after cleaning with running water) 
Student 2 (before cleaning) 	Student 2 (after cleaning with soap) 

Student 3 (before cleaning)	Student 3 (after cleaning with handrub)
	

4. Complete the table below with your experimental data.

   [For reference only]

Group	Initial	Cleaning agent	No. of CFU on plate X	No. of CFU on plate Y
		Running water	*168*	*18*
		Soap	*TNTC*	*16*
		Handrub	*100*	*4*

   i) Which plate (plate X or plate Y) contains more bacteria?

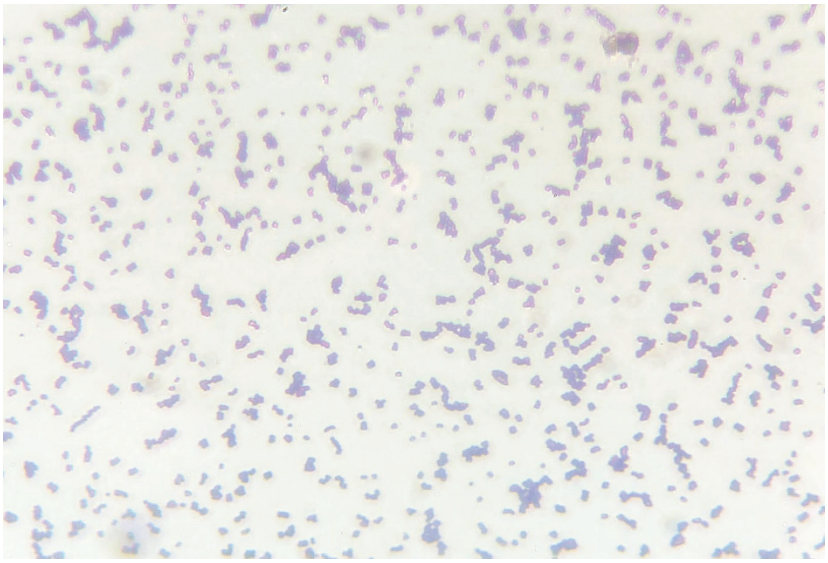
   Plate X

   ii) Does cleaning help reduce the number of bacteria?

   Yes

c) Part (3) of the experiment

Paste the photo or draw a picture of slide A after Gram staining, in the box below.



1. From the stained smear, what kind of bacteria can be found?

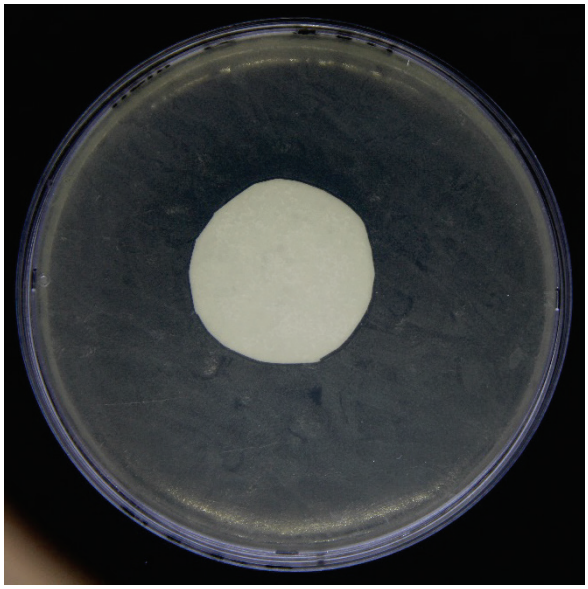
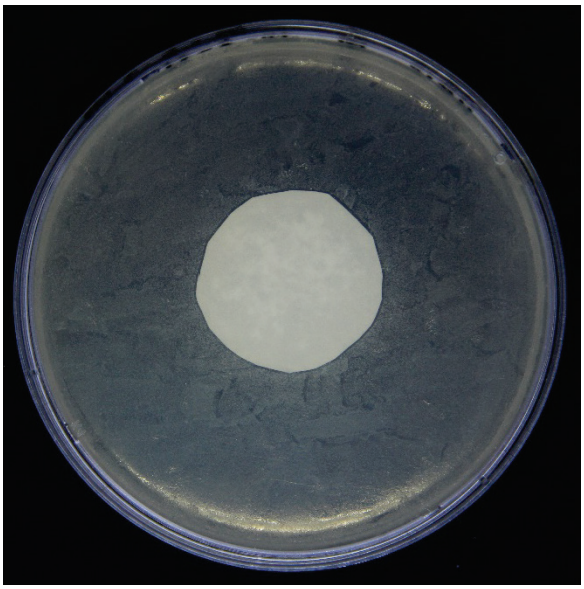
   Bacteria stained purple and bacteria stained pink are found. This means that both gram-positive bacteria and gram-negative bacteria can be found.

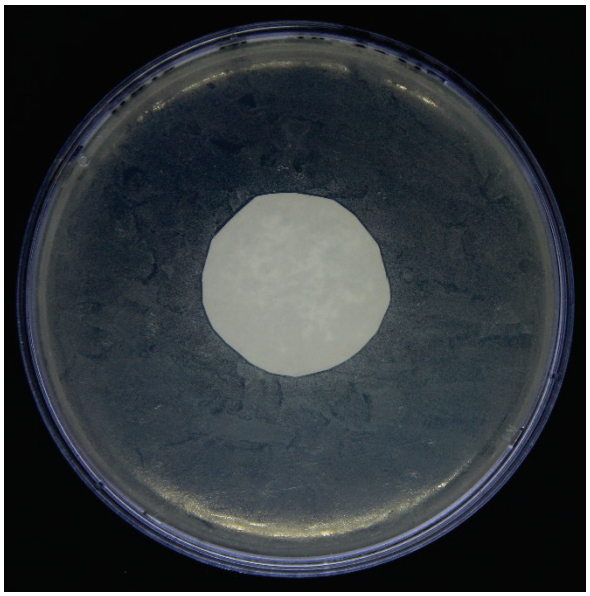
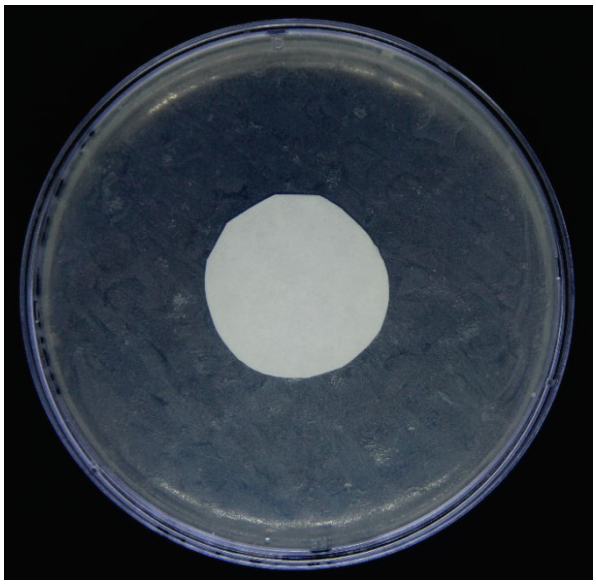
2. Which kind of bacteria is there more of in the environmental sample you collected?

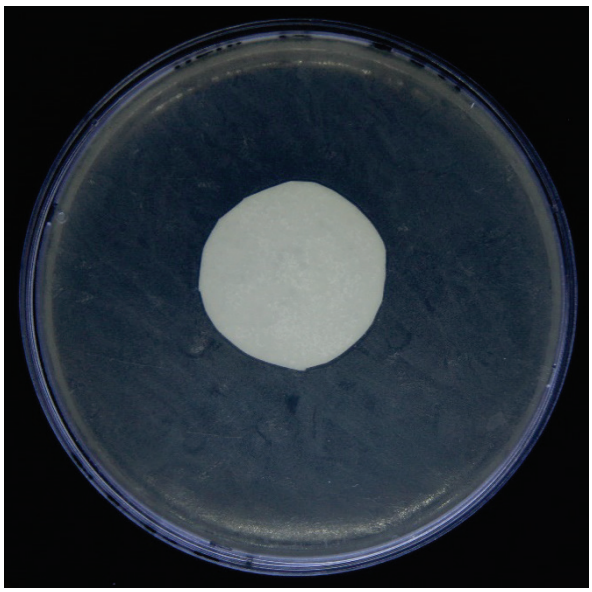
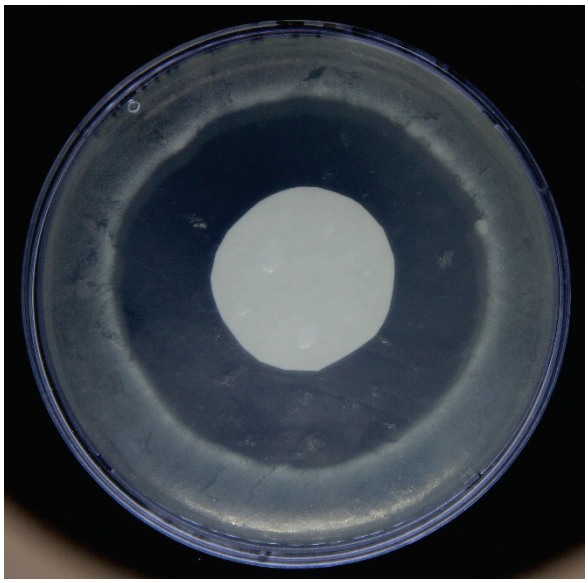
   By a rough estimation, there is no big difference in cell number between the two groups of bacteria observed.

d) Part (4) of the experiment

[For reference only]

96% ethanol 	80% ethanol 

H_2O_2 	Glycerol 

Sterilised distilled water	Handrub
	

Compare the results with those of other groups in class. Fill in the following table.

Group	Diameter of clear zone (the growth inhibition area) / mm					
	96% Ethanol	80% Ethanol	0.125% H_2O_2	1.45% Glycerol	Sterilised distilled water	Handrub
	nil	*nil*	*nil*	*Nil*	*nil*	*63*
Average diameter of clear zone						

VIII. Discussion

1. Comparing the cloudiness of tubes A and B in Part (2) of the experiment, is the disinfection with 70% ethanol effective in the inhibition of microbial growth? Explain your answer briefly.

   Yes, the disinfection with 70% ethanol is effective. The turbidity of tube A is much higher than that of tube B. This indicates that area B was much cleaner than area A after cleaning with 70% ethanol, and the growth of microbes was restricted.

2. Comparing the results of the fingerprinted LB agar plates in Part (2) of the experiment with those of other students, which cleaning agent(s) do you suggest for use in daily practice?

   Handrub is suggested to be used because it showed the strongest inhibitory effect against bacterial growth in this experiment.
   (Other answers with experimental results supported are acceptable.)

3. Search for information from microbiology books or the internet. List some common examples of gram-positive and gram-negative bacteria.

   Gram-positive:

   Staphylococcus epidermidis

   Gram-negative:

   Escherichia coli

4. Search for information from microbiology books or the internet. Suggest the function of different ingredients in the handrub.

Ingredient	Function
Ethanol	*Active substance for disinfection*
H_2O_2	*Kills spores but not the active ingredient for disinfection*
Glycerol	*Moisturising agent for skin*
Distilled water	*Solvent for other components*

5. What is the major anti-microbial component in the handrub mixture of this experiment? What is the concentration of that component? Explain its antiseptic effect.

   Ethanol is the major anti-microbial component. Its concentration in the handrub is about 80%. Ethanol can make holes in the cell wall of microorganisms. The water content in the 80% ethanol enters the cell wall via these holes and lyses the cells of the microbes. This helps kill the microorganisms faster.

6. According to the experimental results in Part (4), does the 80% ethanol provide an antiseptic effect similar to that of the handrub mixture? Explain your answer briefly.

   According to the experimental results in Part (4), 80% of ethanol did not provide an antiseptic effect similar to that of the handrub mixture even though its concentration is similar to that in the handrub. This may be due to the rapid evaporation of the 80% ethanol solution on the filter paper disc, and hence no effect can be exerted.

7. Do you think a better antiseptic effect can result by using a higher concentration of ethanol (e.g. 96% ethanol solution)? Explain your answer briefly.

   No, the antiseptic effect of absolute ethanol or high percentage ethanol (96%) should be less than that of the lower percentage of ethanol (80%). The function of ethanol is to make holes in the cell wall of microorganisms, but this may not kill them. The water content in the 80% ethanol can enter the cell wall via the holes made by the application of ethanol and lyses the cells. This helps kill the microorganisms faster.

IX. References

WHO guidelines on hand hygiene in health care: First global patient safety challenge clean care is safer care. (2019). Geneva: World Health Organization, 12. WHO-recommended handrub formulations. https://www.ncbi.nlm.nih.gov/books/NBK144054/

https://www.today.com/health/school-experiment-shows-students-importance-hand-washing-t170186

https://www.chp.gov.hk/en/healthtopics/content/460/19728.html