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Supplemental Materials

for

Do You Kiss Your Mother with That Mouth? An Authentic Large-Scale Undergraduate Research Experience in Mapping the Human Oral Microbiome

Jack T. H. Wang[1]*, Joshua N. Daly[1,2], Dana L. Willner[1,2,4], Jayee Patil[1], Roy A. Hall[1,6], Mark A. Schembri[6], Gene W. Tyson[1,2,3], and Philip Hugenholtz[1,2,3,5]
[1]School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, Queensland 4072, Australia, [2]Australian Centre for Ecogenomics, School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, Queensland 4072, Australia, [3]Advanced Water Management Centre, The University of Queensland, Brisbane, Queensland 4072, Australia, [4]Diamantina Institute, The University of Queensland, Brisbane, Queensland 4072, Australia, [5]Institute for Molecular Bioscience, The University of Queensland, Brisbane, Queensland 4072, Australia, [6]Australian Infectious Diseases Research Centre, The University of Queensland, Brisbane, Queensland 4072, Australia

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*Corresponding author. Mailing address: Room 76-426, School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, QLD, 4072, Australia. Phone: +61 7 3365 4611. Fax: +61 7 3365 4699. E-mail: t.wang1@uq.edu.au.

Appendix 1: Student instructions for human oral microbiome ALURE.

Introduction

The human oral cavity contains a smorgasbord of microorganisms. It is filled with 10^8-10^{10} colony forming units (CFU) of bacteria per gram of saliva – to put this into context, only 10^2 – 10^4 CFU of bacteria are typically found on each cm^2 of school-yard toilet seats. What this means is that your mouth contains up to a million times more bacteria than found on communal toilet seats used by thousands of children with questionable personal hygiene practices!

The microorganisms that reside in different regions of the human body comprise of complex heterogeneous populations that vary depending on a large variety of host and environmental factors. Although clinically relevant microbes at each body site are well characterised, the factors that affect the makeup of these microbial communities are still incompletely understood.

Previous studies on the microbes living in the human mouth have consisted of relatively small sample sizes. We have hundreds of students enrolled in this course, and this is a great opportunity to obtain insight into the microbial communities across a significantly larger sample population with students being the volunteers. You can volunteer to swab your own mouth and attempt to identify the microbes living within it.

You will carry out microbial identification using two different approaches

- Traditional culture-based techniques, involving Gram-staining, microscopy, colony morphology on selective/differential media, and biochemical testing
- Culture independent molecular biology techniques, including PCR, metagenomics, and next-generation DNA sequencing technology.

You will be able to compare the efficacy and accuracy of culture-dependent and independent microbial identification techniques.

SESSION 1: Core Skill-building

1.1 How do we visualize microorganisms?

Skill Building Objectives:

1. Competently use and care for a light microscope for the visualization of microorganisms using all powers of magnification, including oil immersion.
2. Competently perform the Gram stain, and be able to describe the microscopic appearance of isolates in terms of their Gram reaction and cell morphology.

Inquiry Based Objectives:

1. Can you differentiate microorganisms based on Gram-staining and Gram status?
2. Is the quality of your Gram-stain procedure comparable to laboratory standards?

Background:

The light microscope is a fundamental tool in studying all aspects of science, but especially microorganisms that we cannot see with the naked eye. The experimental information and insight scientists are able to obtain through microscopy varies depends on the type of microscope being used, one of the most important tools. When coupled with staining techniques to improve the visibility and contrast of microorganisms (E.g. the Gram-stain), microscopy is a very powerful tool in microbiology.

Materials per student:

1 light microscope, 1 set of Gram stained smears of *E. coli, S. aureus, S. pyogenes, B. subtilis*.

Tryptic Soy Agar (TSA) plates inoculated with *E. coli, S. aureus, S. pyogenes, B. subtilis*

Gram-staining kit (Bunsen burner, wire loop, slides, water, crystal violet, iodine, decolorizer, safranin)

Procedure:

1. Using Standard Operating Protocol 1, set up the light microscope to examine the Gram-stained smears of *E. coli, S. aureus, S. pyogenes, B. subtilis*, using both air and oil immersion. Noting the variation in morphology (coccus or rod-shaped?) and Gram-status (positive or negative?), draw 2 or 3 typical cells of each.
2. Using TSA plates inoculated in *E. coli, S. aureus, S. pyogenes, B. subtilis*, prepare smears and Gram stain. Refer to Standard Operating Protocol 2 for the Gram stain method.
3. Draw diagrams of each Gram stain and describe.
4. Examine the previously prepared smears and compare to your own samples.
5. Record the details of the cell walls of Gram-negative and Gram-positive bacteria.

Standard Operating Protocol 1 – Visualization of slides using light microscopy

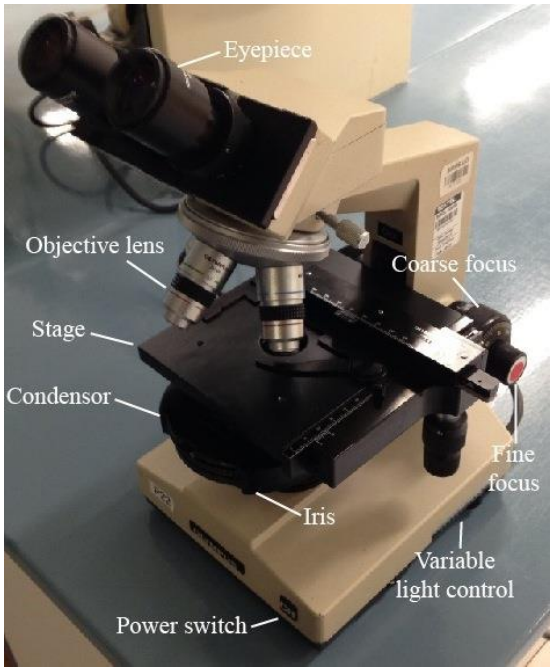

Figure A1. Schematic of a light microscope.

1. Ensure your light microscope is plugged into the power supply, and identify its different components using Figure A1.
2. Turn on the power switch and adjust the variable light control dial to high.
3. Lower the stage using the coarse focus, and swing the x10 objective into the correct position
4. Place the slide on the stage, placing the area of the smear you wish to visualize directly underneath the position of the objective. Ensure the smear is facing upward.
5. Using the coarse focus initially, focus on the slide, while ensuring that the objective does not make physical contact with the slide. Fine-tune the focal plane using fine focus until you get a clear image down the eyepiece.
6. Adjust the intensity of light coming through the eyepiece by gradually opening/closing the iris diaphragm; the variable light control can also be adjusted.
7. Without moving the stage, the position of the slide, or focal plane, bring the x40 objective lens into position. Slightly adjust the fine focus knob and the iris diaphragm again to ensure your field of view is clear and well-illuminated
8. To obtain a higher magnification, you must use the x100 objective, which is an oil immersion lens. To do this, place a drop of oil on the dried smear, and swing the x100 objective into

contact with the oil. If the sample was already focused using the x40 objective, this process should not have altered the focal plane drastically; using the fine focus knob and the iris diaphragm to locate the appropriate focal plane for your smear. This will take some practice as not only is it difficult to avoid direct contact between the oil immersion objective and the slide, the very short working distance locating the focal plane a tricky process.

9. If you are unable to see a focused image, the following are possible problems:
    a. The smear is poorly stained, or has a low concentration of cells. If this were the case, you would have been able to identify the quality of the slide using x10 or x40 objectives. This is why it is important to start with the lower magnifications initially
    b. The field of vision on the x100 objective is obstructed. This is usually due to improper care of the microscopes, and the resultant build-up of oil on the objective.
10. Once you have finished observing and drawing the specimens, always wipe off immersion oil from the objective lens after viewing, using **kimwipe tissues** which are provided for you at end of bench. Wipe the lenses in the following order: x10 first, then x40, then x100. This will ensure that the oil on the x100 objective will not cross contaminate the other non-oil immersion objectives.

Standard Operating Protocol 2: The Gram Stain

1. Prepare a smear and fix by heat (quickly pass the slide through the Bunsen flame a few times).
2. Cover the smear (not the whole slide) with **crystal violet** and leave for 1 minute.
3. Wash with water.
4. Immediately cover the smear with **iodine** and leave the iodine on for 1 minute.
5. Wash with **water**.
6. Hold the slide at a 45° angle where the smear is clearly visible. Then apply the **ethanol**, drop by drop, to the top edge of the smear until no more color runs out of the lower edge of it. The decolorisation time is usually about 10 - 15 seconds.
7. Immediately wash with **water**.
8. Counterstain with **safranin** for 1 minute.
9. Wash, blot with paper toweling, dry and examine.

RESULT: A blue color indicates Gram-positive; red: Gram-negative.

1.2 Aseptic technique

Skill-building objectives:

1. Competently apply aseptic technique towards the isolation of organisms from the environment.

Inquiry Based Objectives:

1. What are the most common mistakes made when attempting to culture microbes aseptically?
2. How can you tell if a microbial culture has been handled using proper aseptic technique?

Background:

In the handling of microorganisms, aseptic technique is another fundamental skill that is required. Whether it be sub-culturing an existing microbe from an agar plate, or handling clinical samples isolated infected wounds, you have to be certain that microbes in the surrounding environment do not contaminate the samples. Only then can laboratories maintain pure culture libraries, provide accurate clinical diagnoses, and establish a safe working environment. The Bunsen burner flame is the main mechanism through instruments used to transfer cultures can be quickly and repeatedly sterilised. Whether it is inoculating loops or Pasteur pipettes, once heated through the Bunsen burner and working within close proximity to the flame, the resulting aseptic environment will facilitate the safe handling of uncontaminated microbial samples.

Materials per student:

1 TSA plate inoculated with *E. coli*, 1x 5 mL TS broth culture of *E. coli* and *M. luteus*

1 x 10 ml sterile broth in McCartney bottle, 1 tube of sterile peptone water (PW)

1 x 10 ml sterile distilled water in McCartney bottle for moistening swabs

1 sterile plugged 150 x 13 mm test tube, 1 sterile 10 ml graduated pipette

1 sterile Pasteur pipette, 1 sterile swab

3 sterile TSA agar plates, incubation containers and racks labelled 28^0C and 37^0C

Procedure:

Using the methods as demonstrated by your supervising teaching assistant, each student carries out the following manipulations:

1. Preparation of cultures from agar plates using a sterile loop [Incubate at 37°C]:

- With a flamed inoculation loop, make the following aseptic inoculation from an isolated colony on the TSA plate inoculated with *E. coli* onto a fresh TSA plate using the 16-streak

technique for obtaining single colonies (Standard Operating Protocol 3).

2. Preparation of cultures from broth using a sterile loop [Incubate at 37°C]:
    - With a flamed inoculation loop, make the following aseptic inoculations from the mixed broth culture containing *E. coli* and *M. luteus*:
        - Onto sterile TSA agar plate, followed by 16-streaking (Standard Operating Protocol 3);
        - Into sterile tube of peptone water.
3. Transfer of sterile broth using sterile graduated pipette [Incubate at 37°C]:
    - Be careful to hold the 10 ml graduated pipette close to the top, and use a screwing action when putting on the pipette filler. If you hold the pipette further down, it is likely to break.
    - Using this sterile 10 ml pipette, transfer 5 ml of sterile broth aseptically from the McCartney bottle into the plugged test tube. Discard 10 ml pipette into cylinder of disinfectant. Label bottle and tube and set aside for incubation.
4. Environmental sampling [Incubate at 28°C]:
    - Now that you have had some practice with aseptic and pure culture techniques, you can put them to use in evaluating the microbial composition of environmental samples. You can choose a lab surface to sample (E.g. door handles, sink basins, bench tops).
    - For environmental samples, it may be easier to use either a sterile Pasteur pipette or a sterile swab to conduct the initial sampling and inoculation, followed by 16-streak dilution method (Standard Operating Protocol 3) on a TSA agar plate.
    - When using the swab, if you select a moist surface use a dry swab; with dry surfaces you need to moisten the sterile swab with sterile distilled water prior to swabbing the surface.
    - After swabbing the selected surface, use the swab to inoculate the primary area on the TSA plate, discard the swab, then use a flamed loop to continue the 16-streak procedure (Standard Operating Protocol 3).

Label all cultures (Note: plates must be labelled on the base) with a marking pen to show your name, the date, treatment/inoculum, and your bench number. These cultures will be incubated overnight and stored at 4°C until the next class. At the next class, examine each of your cultures and compare to the other students on your bench. Based on these observations, how sterile is your aseptic technique? Are there any techniques that appear to be more prone to contamination than others?

Standard Operating Protocol 3 – 16-streak dilution technique

1. Label the base of the uninoculated agar plate with your name and what you are inoculating. Place it base-up on the bench.
2. Using either a sterile swab or a wire loop sterilized by the Bunsen burner flame, sample the source of microorganisms (e.g., bacterial cultures, environmental samples). Gently rub the swab/loop over approximately one-third to one-half of the uninoculated agar plate surface. This is the primary inoculum. Close the lid of the agar plate once you have done this to minimize contamination.
3. The next step is to dilute out the primary inoculum - flame the wire loop and allow to cool. From one end of the primary inoculum, draw 4 separate streaks with the loop.
4. Flame the loop once again and allow to cool. From this point you do not enter the primary area again. From the end of the first 4 streaks, draw 4 more separate streaks.
5. Flame the loop once again. If you have trouble seeing your streaks, hold the plate obliquely to catch the light.
6. Repeat this streaking procedure using a flamed and cooled loop until you have 4 sets of 4 streaks (hence '16 streak'), flaming between each set of streaks. Ensure that each set of streaks only crosses the previous set, and be careful not to run your last set of streaks back into your original inoculum or you will pick up a lot more organisms and may not get isolated colonies. Flame the loop and return to the rack.

SESSION 2 - Sampling the Human Oral Microbiome

Skill Building Objectives:

1. Perform a mouth swab and extract bacterial DNA using pipetting and centrifugation.
2. Inoculate a variety of different media with mouth swabs while minimizing contamination.

Background:

The high-throughput metagenomic sequencing of 16S rRNA genes has provided an efficient and accurate molecular microbial diagnostic tool in identifying bacterial taxa. The 16S rRNA gene is comprised of conserved and hypervariable domains, and it is the variability within the hypervariable domains that allows the sequencing data to pinpoint the identity of the bacterial taxon (Fig. A2). In this session you will run a PCR using primers that target the V5-V8 portions of the 16S rRNA gene (Figure A2). This should have produced amplicons for all the different bacterial taxa represented within the mouth swab DNA extracts, and sequencing these amplicons will allow us to identify the bacterial taxa. At the same time, you will prepare bacterial cultures from your mouth swabs and attempt to identify the colonies using culture-dependent biochemical, immunological, and microscopy techniques.

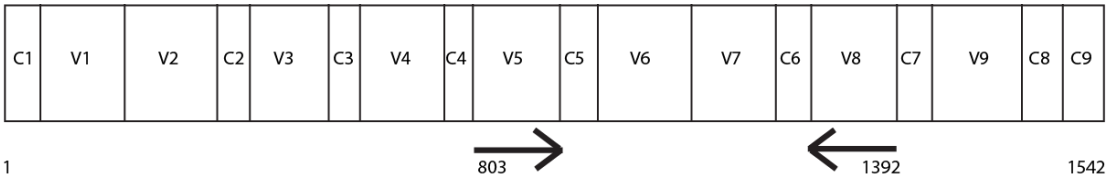

Figure A2. Schematic of 16S rRNA gene sequence. "C" and "V" represent conserved and variable domains respectively. The PCR primers used in Project 3A start and finish at positions 803 and 1392 within the gene respectively.

Materials: per student

3 Sterile swabs

1 blood agar plate, 1 Mannitol salt agar plate, 1 Mitis salivarius agar plate

Automated Pipettes: 2 μL, 20 μL, 200 μL and sterile pipette tips

Epicentre DNA extraction kit

PCR reagents (PCR tubes, 10X buffer, 25 mM $MgCl_2$, 10 mg/ml BSA, 10 mM dNTPs, 10 μM F&R primers, Taq DNA polymerase)

Vortex mixers

1 box for aerobic incubation, 1 box for anaerobic incubation

Materials: **per class**

PCR machine, heating blocks set at 65°C and 98°C

Procedure:

2.1 Collecting a Full mouth Swab and Extracting DNA

The first stage of this project involves collecting a mouth swab and extracting genomic DNA from it.

1. Each, student will be given a **unique ID code** for their samples.
2. To minimize contamination of your mouth with food and/or caffeine, it is important (where possible) to avoid drinking coffee within 2 hours of collecting the mouth swab.
3. Immediately prior to taking a mouth swab, rinse your mouth out with water twice. Make sure you take your lab-coat off and step outside of the labs to do this (no food or drink is allowed near microbial cultures in the lab!)
4. Using the DNA extraction brush (ensuring that it is sterile and not contaminated by any other part of your body), thoroughly roll the brush repeatedly over your entire mouth. This includes your tongue, along the gum lines, on either side of your cheeks, near your throat (make sure you don't gag!). Consult your teaching assistant for a proper demonstration.
5. Put the brush end into a tube containing DNA extraction solution and rotate the brush head in the solution 5-10 times to ensure all of the genetic material enters the buffer. This will extract and purify the DNA from your full mouth swabs.
6. Tightly screw on the cap on the tube, label it with your **unique ID code** and use the Vortex mixer to homogenize the sample for 10 seconds.
7. Incubate your labeled tube on the 65°C heating block for 1 minute, then Vortex mix for another 15 seconds.
8. Transfer the tube to the 98°C heating block for 2 minutes, then Vortex mix for 15 seconds.
9. Your mouth swab DNA sample is now ready to use in the PCR!

2.2 Using Mouth Swab DNA in Polymerase Chain Reactions (PCR)

The next stage involves using your mouth swab DNA in a PCR reaction with primers targeting the 16S rRNA gene of bacteria. This PCR reaction (protocol outlined in Table A1) will amplify the 16S rRNA gene of all the bacterial species found within the mouth of the volunteer, and the variability in this gene across different bacteria will allow us to use the gene sequence to identify these bacteria.

1. Locate the PCR tubes containing your **unique ID code**. There should be two of them that contain your unique ID code, each containing different suffixes. If your unique ID code is "1", then you should have two PCR tubes.
2. Using Table A2, calculate the volume of each PCR reagent to add for one 25 µL reaction. Check with your teaching assistant regarding the accuracy of these calculations (Remember $C_1xV_1 = C_2xV_2$ from introductory chemistry!).
3. Once you have done this accurately, use your calculations to prepare enough mastermix (all reagents excluding water and mouth swab DNA) for the number of PCR reactions you are running. Each student will need to prepare two PCR tubes::
    a. **1T** – This is where you add 5 µl of your mouth swab DNA, and make up to 25 µL with PCR mastermix and water.
    b. 1C – This is the control tube where you do not add any DNA. Make up to 25 µL with PCR mastermix and water
4. Once everyone on your bench has done this, place the strips of PCR tubes on your bench into the PCR machine and start the program. The PCR machine protocol is listed in Table A1.

Table A1. Stages of PCR reaction.

Stage	**PCR conditions**
Stage 1	95°C for 3:00mins
Stage 2	30-35 cycles of 95°C for 30 seconds, 55°C for 45 seconds, 72°C for 1.5 minutes
Stage 3	72°C for 10 mins
Stage 4	Remain at 10°C

Table A2. Reagents for 1 reaction in pre-prepared PCR mastermix.

Reagent stock concentration	**Required final concentration**	**Volume**
10X Buffer (Fisher Scientific)	1X	
25 mM $MgCl_2$	2 mM	
10 mg/ml BSA	0.3 mg/mL	
10 mM dNTPs	0.2 mM	
10 µM F primer	0.2 µM	
10 µM R primer	0.2 µM	
Taq DNA polymerase (12,500 units/mL)	50 units/mL	
Your mouth swab DNA	N/A	5 µl
Water	N/A	Make up to 25 µl total volume
Final Volume		**25** µl

2.3 Using Mouth Swabs to inoculate agar plates

Now that you have prepared your mouth swabs for microbial identification using PCR and next generation DNA sequencing, it is time to do things the old-school way so you can compare and contrast the different techniques. You will inoculate a number of different media with your mouth swabs and incubate these cultures at 37^0C. You will observe their growth on these plates and attempt to identify them based on Gram-staining, colony morphology, and biochemical testing.

1. Make sure that the person who is doing the mouth swab is the same student who processed their mouth swab for DNA extraction and PCR. This will allow us to directly compare the results of culture dependent and independent techniques of microbial identification
2. Using a new sterile swab thoroughly roll the swab repeatedly over your entire mouth. This includes your tongue, along the gum lines, on either side of your cheeks, near your throat
3. Inoculate swab onto primary area of blood agar plates (over a third of the plate's area) then streak out using the 16-streak method (Standard Operating protocol 3). You do not have to flame the loop between each set of 4 streaks for this, as we want to maximize the amount of bacteria isolated from the mouth.

4. Repeat steps 2 and 3 for another blood agar plate, a Mannitol salt plate and a Mitis salivarius plate. Make sure you use a new sterile swab for each plate.
5. Label each plate with your **name, date, bench, session, and unique ID code corresponding to mouth swab extracted DNA.** For your blood agar plates, clearly label which one is incubated aerobically/anaerobically.
6. Incubate one inoculated blood agar plate in the aerobic box (24 hours at 37^0C with oxygen), and the other blood agar plate in the anaerobic box (24 hours at 37^0C without oxygen). This is important as many microbes are obligate anaerobes and won't be visible unless grown in the absence of oxygen.
7. Incubate the inoculated Mannitol salt and Mitis salivarius plates in the aerobic box

SESSION 3: Culture-dependent identification of oral microbes

Skill Building Objectives:

1. Confirm the identity of oral bacterial isolates based on colony characteristics, biochemical, and immunological testing
2. Differentiate *Staphylococcus* and *Streptococcus* species via microscopy, Gram-staining, culture, biochemical, and immunological tests

Inquiry-Based Objectives:

1. What types of bacteria are most commonly found in the mouth?
2. Is there a difference in the oral bacterial distribution across different people?
3. What are the limitations of culture-based diagnostic methods?

Background

Today we will be focusing on identifying the bacteria obtained using culture-dependent identification techniques. Based on the colony characteristics on various selective and differential media, Gram-staining, microscopy, and biochemical and immunological testing, you will be able to identify the microorganisms isolated from your mouth.

The body harbors a vast number of bacterial species as normal flora, the particular organisms varying between sites of the body, due to different conditions occurring, (e.g., dry skin vs. moist mucous membranes). While *Staphylococcus epidermidis* and other organisms are common on the skin, *Staphylococcus aureus* is more frequently found in the nasal vestibule and oral cavity, with many in the population being carriers. *Streptococcus* species are Gram-positive cocci, characteristically arranged in pairs or chains. They are widely distributed in nature, some being part of the normal human flora, e.g., in the mouth. A variety of extracellular substances and enzymes are produced. An important criterion in classification of *Streptococcus* species is their ability to hemolyse red blood cells to various degrees.

We will provide **Standard Operating Protocols** (SOPs) for how to identify ***Staphylococcus*** and ***Streptococcus*** species, as they are among the most common clinically relevant microbes found on the human body. In your potential future roles as scientists, pathologists, diagnosticians and/or clinicians,

you will definitely need to know how to identify these microbes as common causative agents of disease. Once you have mastered these **SOPs**, you will be able to **select your own suite of tests** that you apply to **identify the colonies found on your own plates**.

Materials: per student

Blood, mannitol salt, and Mitis salivarius agar plates of mouth swabs inoculated in session 2

1 sterile blood agar plates, 1x10 mL sterile saline bottle

Novobiocin, bacitracin, optochin antibiotic discs

1 sterile swab, sterile forceps

Materials: per bench

Incubation box

Catalase test - 1 dropping bottle of 10 vols % H_2O_2

Slidex Staph Plus "Coagulase" test kit (Biomeriuex), BioMerieux Streptococcal typing kit

Demonstration blood and mannitol salt agar plates inoculated with *S. aureus*, *S. epidermidis* and *M. luteus*

Demonstration Mitis salivarius agar plates inoculated with various *Streptococcus* species by 16-streak method onto blood agar (incubated at 37°C for 24 h):

Streptococcus pyogenes	(Group **A**)	+ (bacitracin disc)
Streptococcus agalactiae	(Group **B)**	+ (bacitracin disc)
Streptococcus mitis	(Viridans group)	+ (optochin disc)
Enterococcus faecalis	(Group **D**)	
Streptococcus pneumonia	(pneumococcus)	+ (optochin disc)

Procedure:

1. Using your selection of the initial steps within Standard Operating Protocols 4, 5, and 6, record your presumptive identification of 2 suspected colonies on your oral swab plates.
2. Enter these results into the questionnaire using the **same unique ID code you were allocated for the mouth swab samples in session 2**. These will be collated across the class.

Standard Operating Protocol 4 – Categorization of Bacteria by Gram Status

Using the Gram-staining and microscopy skills you learnt in Session 1, and the dichotomous key and list of common bacteria presented in Tables A3 and A4, you can pick colonies from your mouth-swab inoculated plates to identify based on Gram-status and cell morphology.

Table A3. Dichotomous key for classifying major groups of bacteria from Gram-staining.

1	1A: Cells are Gram-positive	Go to 2
	1B: Cells are not Gram-positive	Go to 3
2	2A: Cells spherical in shape	Gram-positive Cocci
	2B: Cells not spherical in shape	Go to 4
3	3A: Gram-negative	Go to 5
	3B: Not Gram-negative (lack cell-wall)	*Mycoplasma*
4	4A: Cells rod-shaped	Gram-positive bacilli
	4B: Cells not rod-shaped	Go to 6
5	5A: Cells spherical in shape	Gram-negative cocci
	5B: Cells not spherical in shape	Go to 7
6	6A: Cells club-shaped	*Corynebacteria*
	6B: Cells variable in shape	*Propionibacteria*
7	7A: Cells rod-shaped	Gram-negative bacilli
	7B: Cells not rod-shaped	Go to 8
8	8A: Cells helical with several turns	*Spirochetes*
	8B: Cells comma-shaped (curved rods)	*Vibrioid*

Table A4. Common examples of bacteria categorized by Gram-status and morphology.

Gram-status and Morphology	**Bacterial Groups**
Gram-positive cocci	*Staphylococcus* (Catalase +); *Streptococcus* (Catalase -)
Gram-positive bacilli/rods	*Clostridium*; *Listeria*; *Bacillus*; *Lactobacillus*
Gram-negative cocci	*Neisseria*; *Moraxella*
Gram-negative bacilli/rods	Enterobacteria (*E. coli, Salmonella, Yersinia, Lebsiella, Serratia, Proteus*); *Pseudomonas*; *Bacteriodes* (obligate anaerobe)

Standard Operating Protocol 5 –Identification of *Staphylococcus* species

1. Typical colonies of *S. aureus* on blood agar are yellow/buff and often haemolytic. On mannitol salt agar, *S. aureus* colonies are surrounded by a bright yellow zone, while coagulase negative Staphylococci colonies are surrounded by a red or purple zone.
2. Examine demonstration cultures of the various *Staphylococcus* species and *Micrococcus luteus* on blood agar and mannitol salt plates and compare to the colonies found on your plates.
3. *Staphylococcus* species are Gram-positive cocci, usually found in grape-like clusters. They can easily be confused with *Streptococcus* species (pairs or linear chains of Gram-positive cocci) when looking down the microscope. Perform the Catalase test to separate the two: Staphylococcus should be **Catalase positive**, while Streptococcus is **Catalase negative**. Take a small part of the suspected colony and place on a glass slide, and add a few drops of hydrogen peroxide to the colony. A **positive** test is one in which gas bubbles (oxygen) are liberated when 10 volumes percent hydrogen peroxide is added to the culture.
4. When you have decided on the basis of microscopy and colony form that your swab may have contained *Staphylococcus*, perform a Coagulase test by using the Slidex Staph Plus kit test on the suspected colony (follow the manufacturer's instructions).
5. If your isolate is coagulase positive, then it is *S. aureus*. If it is coagulase negative (and you have confirmed it is *Staphylococcus* based on the above steps), then it could be either *S. epidermidis* or *S. saprophyticus.* They can be differentiated based on sensitivity to the Novobiocin antibiotic.

NB: Only carry out Steps 6-9 below if you know your colony is a Coagulase-Negative Staph (either *S. epidermidis* or *S. saprophyticus)*

6. Prepare the inoculum by suspending the suspected colony in 10 ml sterile saline and vortexing carefully with the lid firmly screwed on, to make a **faintly turbid** suspension.
7. Swab the surface of a blood agar plate with the inoculum in order to obtain an even lawn of inoculum on the agar.
8. Using the disc dispenser, place a **Novobiocin disc** onto the surface of the inoculated plate. **Using sterile forceps** ensure that the discs are fully in contact with the agar, to ensure even diffusion of the antibiotic into the agar. Incubate at 37°C overnight.
9. Check if bacterial isolate is sensitive to Novobiocin (inhibition of growth around Novobiocin disc on plate). If Novobiocin is inhibiting its growth, it is *S. epidermidis*; if not, it is *S. saprophyticus*.

Note: Antibiotic discs should only be handled with sterile forceps, as they need to be kept sterile, and some antibiotics may have toxic effects when in contact with the skin.

Standard Operating Protocol 6 –Identification of *Streptococcus* species

1. *Streptococcus* species are Gram-positive cocci, usually found in pairs or long linear chains. They can easily be confused with *Staphylococcus* species (grape-like clusters of Gram-positive cocci) when looking down the microscope. Perform the Catalase test to separate the two: *Staphylococcus* should be **Catalase positive**, while *Streptococcus* is **Catalase negative**.
2. Examine the *Streptococcus* demonstration plates for α, & ß hemolysis, which is best observed by holding the plate up to the light, with the lid still on, and looking through the agar to see changes in color. Compare the plates to Table A5 below.

- **α hemolysis** is characterized by an indistinct zone of partial destruction of erythrocytes about the colony, often accompanied by a greenish to brownish discoloration.
- **ß hemolysis** is a clear, colorless zone around the colonies, in which the erythrocytes have undergone complete discoloration.
- Non-hemolytic colonies cause no apparent clearing or discoloration of the medium.

3. Examine your blood plates for different colony types, and particularly for the presence of hemolytic streptococci. Compare your colonies to the demonstration plates and Table A5

4. Examine the Mitis salivarius plate and compare these to the demonstration plates:
    - *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus mitis*: small blue colonies about 0.2 mm diameter.
    - *S. salivarius* produces pale blue colonies 2-5 mm diameter and with a raised 'gum drop' appearance.
5. Demonstration of commercial immunological *Streptococcus* typing kit designed to serologically differentiate *Streptococcus* species by their Lancefield antigens (i.e., groups A, B, C, D, F and G). If you are sure you have isolated *Streptococcus* from your mouth swab, apply the kit by following the manufacturer's instructions to your unknown culture.

NB: Only carry out Steps 6-9 below if you know your colony is *Streptococcus*, and exhibits either α or ß hemolysis as determined above.

6. Prepare the inoculum by suspending the suspected colony in 10 ml sterile saline and vortexing carefully with the lid firmly screwed on, to make a **faintly turbid** suspension.
7. Swab the surface of a blood agar plate with the inoculum in order to obtain an even lawn of inoculum on the agar.
8. Using the disc dispenser, under the direction of your teaching assistant, place a **bacitracin disc**, and an **optochin disc** on different parts of the inoculated blood agar plate. **Using sterile forceps** ensure that the discs are fully in contact with the agar, to ensure even diffusion of the antibiotic into the agar. Incubate at 37°C overnight.
9. Determine the identity of your *Streptococcus* isolate from the freshly inoculated horse blood agar plate by using Table A5.

Table A5. Characteristics of common *Streptococcus* species.

	Organism	**Group**	**Hemolysis**	**Antibiotic sensitivity**
A	*Streptococcus pyogenes*	A	ß	Bacitracin sensitive
B	*Streptococcus agalactiae*	B	ß	Bacitracin resistant
C	*Streptococcus mitis*	Viridans	α	Optochin resistant
D	*Enterococcus faecalis*	D	None.	-
E	*Streptococcus pnuemonia*	Pneumococcus	α	Optochin sensitive

SESSION 4: Culture-independent identification of oral microbes

Skill Building Objectives:

1. Correctly interpret PCR results as visualized by gel electrophoresis.

Inquiry-Based Objectives:

1. Do you observe gel bands for the test and control reactions? Are the sizes of these bands consistent with what you expect? What does this suggest about your PCR setup?
2. Is there a difference in the oral microbial population in the class when determined by culture dependent and culture independent methods?

Background:

After collating the data on oral microbes in session 3 using culture-dependent techniques, the results from culture-independent 16S rRNA sequencing of your oral microbiome have arrived. These results are in the form of images of PCR gels resulting from the PCR you ran in session 2, as well as Operational Taxonomic Units (OTU) tables that highlight the proportion of each bacterial taxa detected with each mouth swab sample on a scale of 0 (0%) to 1 (100%) – see Table A6 below. In this session you will compare these results to the bacterial taxa identified using culture-dependent techniques, as a way to distinguish the diagnostic resolution of each of these identification techniques.

Table A6. Sample OTU table for oral microbiome analyses.

Taxon	f__Actinomycetaceae	Actinomyces	Parascardovia	Corynebacterium	Microbacterium
Sample 1	0	0.001017294	0	0.01525941	0.003051882
Sample 2	0	0	0.001	0	0
Sample 3	0	0	0	0.035211268	0
Sample 4	0	0.001003009	0.001003009	0.002006018	0.001003009

Materials: **per student**

Images of gels run on mouth-swab PCR reactions setup in session 2

OTU tables of oral microbe populations across whole course from 16S rRNA gene sequencing

Aggregate data of oral microbe populations across whole course from culture-dependent techniques

Procedure:

1. Observe the lanes on the gel corresponding to the PCR reactions you set up in session 2. Are there gel bands present in the "T" and "C" lanes?
2. Using the DNA ladder schematic, write down the size of any bands observed, as well as which lane (T or C) they appeared in. Given the PCR amplification strategy for 16S rRNA outlined in session 2, what DNA band size are you expecting to observe?
3. Compare and contrast the profiles of bacteria as identified by 16S rRNA gene sequencing in the OTU tables with culture-dependent techniques. What is the most striking difference you can observe across the two sets of results?
4. Think about the questions listed below, as they will serve as the starting point for your formal project report.

Questions:

1. Outline the different bacterial species in the oral cavity as detected by 16S rRNA gene sequencing and culture-dependent traditional microbiology. Are there any similarities amongst the groups of bacteria detected by one identification method and not the other?
2. How do culture-dependent traditional microbiology and 16s rRNA gene sequencing avoid contamination of the identification process by viral or human material?
3. **Find at least one peer-reviewed scientific research article regarding the bacteria that normally reside in the human mouth and provide references.** Describe the groups of bacteria these studies identified to be present in the human mouth.
4. How do these lists of bacteria you identified in Question 3 compare with the list we compiled through culture dependent and culture independent identification techniques? Why might our list of bacteria be different from theirs?
5. Describe 2 advantages that 16S rRNA gene sequencing has over traditional culture-dependent techniques in bacterial identification. Why is it still important to know how to do traditional culture-based bacterial identification?

Appendix 2: Faculty instructions for human oral microbiome ALURE.

SESSION 1: Core skill-building

The ALURE began with a skill-building introductory module. Given that this ALURE involved students providing their own biological material in the form of oral swabs for microbial analyses, it was important that the project protocols were given ethical clearance by the respective Institutional Review Boards. Informed consent was obtained from each student participant during the first session and prior to the commencement of laboratory activities.

Developing student competencies in microscopy was a key outcome for the first session, where students were introduced to light microscopy, which was then used to visualize a variety of microorganisms differing in size, shape, cluster, and Gram status. Utilizing appropriate aseptic technique to obtain an uncontaminated patient sample of high quality is arguably the most important determinant of diagnostic accuracy, so students were required to develop their manual dexterity through performing bacterial inoculations using a variety of instruments onto liquid broth tubes and agar plates. Teaching assistants then facilitated a student discussion regarding the expected result of each of these inoculations following incubation at 28°C or 37°C for 24 hours. These student expectations were then contrasted against the observed results to determine whether aseptic technique was performed adequately.

SESSION 2: Sampling the Human Oral Microbiome

Once the students have had a chance to develop core competencies in microscopy and aseptic technique, they were able to commence the research component of the project. Students were instructed on how to perform an oral swab using a sterile cotton swab and a MasterAmp brush (Epicentre). The entire surface of the oral cavity including the tongue, along the gum-lines, on either side of the cheeks, and near the throat was sampled; the sterile cotton swab was used to provide the primary inoculum on blood agar, Mannitol salt, and Mitis salivarius plates, while the DNA extraction brush was immersed in a buffer solution, then repeatedly heated and centrifuged according to the manufacturer's instructions. The agar plates were incubated at 37°C for 24 hours, while the extracted DNA was added to a PCR reaction utilizing primers broadly targeting the 16S rRNA gene (variable

regions V5-V8). All plates and PCR tubes were given a unique sample ID as part of the de-identification process. The amplified PCR products were sent to a sequencing facility using the Roche 454 pyrosequencing platform to phylogenetically classify the microbiota identified in the oral cavities across the entire student cohort. Utilizing the infrastructure and equipment present at the Australian Centre for Ecogenomics, UQ, the processing included additional PCR, confirmation of successful PCR amplification using gel electrophoresis, library preparation, sequencing using the 454 Roche 454 GS-FLX Titanium platform, and analysis.

Amplicons were prepared using a 2-step PCR protocol (1). Extracted DNA was first amplified using the native primers 803F and 1392wR as presented above. Each reaction (1X Buffer, 2 mM $MgCl_2$, 0.3 mg/mL BSA, 0.2 mM dNTPs, 0.2 µM primers, 0.5 units of Taq polymerase – Fisher Scientific), contained 5-10 µl of DNA and was made up to 25 µl with water. Cycling conditions were one cycle of 95°C for 3 min, followed by 30 cycles of 95°C for 30 s, 55°C for 45 s and 72°C for 90 s followed by a final extension of 72°C for 10 min. PCR products were then re-amplified in a second reaction using Roche 454 fusion primers which contained sequencing adapters and oligonucleotide barcodes in addition to 16S sequences as previously described (5). For the second step reactions, 2 µl of the PCR product from the first step was added to a 50 µl reaction. Conditions for the second step were identical to those for the first step but the number of cycles was reduced to 10. Amplicons were sequenced using the Roche 454 GS-FLX Titanium platform. Amplicon sequences were analysed as described previously (7). Briefly this involved read quality filtering and error correction using the Acacia software (2) providing output compatible with the QIIME analysis pipline (3), clustering reads into 97% identity operational taxonomic units (5), and classification of clusters against the Greengenes reference database (4) to produce community profiles.

SESSION 3: Culture-dependent identification of oral microbes

While waiting for the results of the culture-independent identification of oral microbiota through 16S rRNA gene sequencing, students had the opportunity to choose from a set of Standard Operating Protocols (SOP) for culture-dependent diagnostic tests of bacterial colony growth resulting from their oral swabs in the previous session. Colonies grown on non-selective blood agar plates were Gram-stained, and a dichotomous identification key used to classify the bacteria based on their morphology

and Gram-status. Growth on selective and differential agar media for *Staphylococcus* and *Streptococcus* species (Mannitol salt, Mitis salivarius agar plates) was also analyzed, as these two bacterial groups are among the most common clinical microorganisms typically found on the skin and in the oral cavity. The colony color, capacity to induce hemolysis, growth on selective media, and color induced by pH indicators in the media, were all part of the presumptive identification process for isolated bacterial colonies.

Students were also provided with SOPs for biochemical and immunological testing. Catalase testing differentiates *Staphylococcus* and *Streptococcus* species, and immunological detection of staphylococcal coagulase and streptococcal Lancefield antigens using latex-agglutination testing kits is able to further pinpoint the species within these respective genera. Moreover additional cultures were set up in order to determine antibiotic sensitivity to novobiocin, bacitracin, and optochin, each of which provides a differential characteristic on which to classify common *Staphylococcus* and *Streptococcus* species. Depending on their presumptive identification from the previous week, students were given the opportunity to choose which SOP they wished to employ to corroborate their presumptive identification, with the expectation that they would be able to justify the tests they have selected to their supervising teaching assistant.

In the event of plate contamination or insufficient colony growth, students will be unable to contribute their results to the collective class dataset for culture-dependent identification. However they were still able to learn the SOPs for bacterial identification by applying the protocols to *Staphylococcus* and *Streptococcus* cultures provided by the teaching team.

SESSION 4: Culture-independent identification of oral microbes

The final session of the ALURE involved aggregating the culture-dependent identification datasets across the entire student cohort, and comparing these results with the oral microbiota as detected by culture-independent 16S rRNA gene sequencing. The sequencing results were presented to students in the form of the PCR bands detected through gel electrophoresis following the initial DNA extraction, as well as an Operational Taxonomic Unit (OTU) table highlighting all of the taxa detected (6). A discussion of the disparity between the bacterial taxa identified by culture-dependent and -independent

means was led by teaching assistants, while also providing guidelines for formatting and presentation of quantitative and qualitative data consistent with scientific journal submissions. The presentation, analysis, and discussion of these results culminated in a laboratory report that all students had to submit individually as part of the course curriculum.

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