

## BM327 Microbiology Semester 2 – Lab 4 Handout

### TASK 4A: Microbial colonies on MSA plates (from TASK 3E)

You should work individually to complete this task (make independent observations of each plate), though you may wish to compare your results with your neighbours.

You will analyse the growth and colony morphology of the unknown pathogen on MSA agar (from the plates you inoculated in the previous laboratory session). You have also been provided with cultures of type strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* grown on MSA agar as controls. Record your observations in Table 1. (Refer to the handout for bacterial colony morphology descriptors.)

Table 1. Phenotypes of staphylococci grown on MSA plates

Isolate	Growth/phenotype on MSA agar?	Characteristics of colonies
<i>Staphylococcus aureus</i> (control)		
<i>Staphylococcus epidermidis</i> (control)		
Your unknown culture (# _____)		

### TASK 4B: Assaying for coagulase activity

Working as a group of two, you should assay your unknown organism and the appropriate controls for coagulase activity (Protocol 4.1).

#### Protocol 4.1: Coagulase slide test.

1. Place a drop of sterile water on a glass slide.
2. Using a flamed loop, that has been allowed to cool, remove an isolated colony from the culture plate and gently emulsify on the slide with the drop of sterile water.

3. Place a drop of plasma immediately adjacent to the bacterial suspension and mix the plasma and bacterial suspension together.
4. Gently tilt the slide back and forwards, observing for the immediate formation of a white precipitate.
5. Record your results and interpretation in Table 1.

### *Interpretation*

A positive reaction (formation of white precipitate) is typically detected 15 – 20 seconds. The test is considered negative if clumping is not observed within 2 – 3 minutes.

Table 1. Coagulase activity of *Staphylococcus* sp.

Microorganism	Coagulase test result – carefully record what you observe	Coagulase test - interpretation
Positive control ( <i>Staphylococcus aureus</i> )		
Negative control ( <i>Staphylococcus epidermidis</i> )		
Your unknown		

## TASK 4C: MLVA-typing *Staphylococcus aureus*

Working as a group of two, you should set up PCRs for MLVA typing of a *S. aureus* unknown (Protocols 4.2 and 4.3). You will use your own unknown (if it is verified to be *S. aureus* in Tasks 4A-B), or an *S. aureus* strain provided to you. You should also prepare an agarose gel (Protocol 4.4) which we will use to analyse your PCR products.

### **Top tips for setting up PCR reaction mixtures**

- Work on ice.
- Pipet carefully and accurately.
- Watch what you touch – you are covered with nucleases and your own (likely commensal) *Staphylococcus aureus*!
- Stay organized – keep track of what you have added to your reaction and what still needs to be added.
- Label your tubes – there will be 96 on the thermocycler altogether!

#### *Protocol 4.2: Set up single PCR (spa gene).*

The *spa* gene must be amplified separately as these primers do not perform well in the multiplex PCR reaction.

*Table 3. Reaction mixture for the spa single PCR*

Component	Volume for 1 reaction
5X Taq buffer	4 µl
PCR grade water	13.5 µl
F primer	1 µl
R primer	1 µl
Taq polymerase	0.5 µl
Total volume	20 µl

1. Label your PCR tube carefully with *spa* and your group number.
2. Prepare your PCR mix (Table 3) in your labelled PCR tube.
3. Use a white thin (10 µl) tip to pick one big isolated colony from the plate provided to you, and touch this to your PCR reaction mixture. Try not to add too many cells.
4. Keep the reaction mixture on ice until the whole class is ready to proceed to the amplification step (thermocycler).

#### *Protocol 4.3: Set up multiplex PCR (clfA, clfB, sdr and ssp genes).*

*Table 4. Reaction mixture for the multiplex PCR*

Component	Volume for 1 reaction
PCR grade water	7.5 µl
5X Taq buffer	4 µl
clfA-F primer	1 µl
clfA-R primer	1 µl
clfB-F primer	1 µl
clfB-R primer	1 µl
sdr-F primer	1 µl
sdr-R primer	1 µl
ssp-F primer	1 µl
ssp-R primer	1 µl
Taq polymerase	0.5 µl
Total volume	20 µl

1. Label your PCR tube carefully, including your group number.
2. Prepare your multiplex PCR mix (Table 4) in your labelled PCR tube.
5. Use a white thin (10 µl) tip to pick one big isolated colony from the plate provided to you, and touch this to your PCR reaction mixture. Try not to add too many cells.
3. Keep the reaction mixture on ice until the whole class is ready to proceed to the amplification step.

### **PCR amplification for both reactions**

Amplification of DNA fragments will be performed with an initial denaturation at 94°C for 10 minutes followed by 25 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and one minute at 72°C, with a final extension at 72°C for 5 minutes on a Bio-Rad DNA-Engine thermocycler. This PCR programme takes about 1½ hours to run.

### *Protocol 4.4: Make an agarose gel.*

1. *(Step 1 has already been done for you.) A 2% agarose solution has been made for you as follows: 2 g of agarose were added to a glass flask, and 100 ml 1X TAE buffer. The agarose solution was heated in a microwave till agarose is completely dissolved in the buffer, and then cooled down to about 50°C. Add GelRed to your cooled agarose mixture.*
2. Prepare a gel tray by taping off both ends securely and placing a comb in the desired position. Pour the agarose into the gel tray and allow this to sit undisturbed until the agarose has set.
3. When the agarose has completely set, remove the tape and place the gel into an agarose tank. [Note: ensure the gel is oriented correctly, such that the DNA samples will not run off the gel.] Fill the tank with 1X TAE buffer. Remove the comb.
4. Load the samples as follows:
  - a. Add 5 µl of *spa* PCR product to the multiplex PCR products and then add 4 µl of loading buffer to 16 µl of the combined PCR products. Load as much of these 20 µl into a well of the 2% agarose gel as possible.
  - b. Load 5 µl of the provided DNA ladder into another well.
5. When your gel is ready to run, place the cover on the tank, and check with a demonstrator to confirm that you have set it up correctly. Set the powerpack up to perform electrophoresis at 100 V for 50 minutes.
6. We will then take gel images with the “gel doc system” and post your data to the BM327 microbiology class sub-page.

## TASK 4D: Observing and recording results (from TASK 3B)

You should work individually to complete this task (make independent observations of each plate), though you may wish to compare your results with your neighbours. You will need to observe the growth of your unknown and control strains on selective/differential media and record your results and interpretation in Table 5.

Table 5. Visual observation of microbial colonies incubated on MacConkey agar

Microorganism cultured	Visual observations (colony characteristics)	Interpretation
<i>Escherichia coli</i>		
<i>Salmonella enteritidis</i>		
Your unknown		

## TASK 4E. Observing and recording results (from **Error! Reference source not found.**)

You should work individually to complete this task (make independent observations of each plate), though you may wish to compare your results with your neighbours. You will also need to observe the growth of your unknown and control strains in aerobic and anaerobic conditions and record your results in Tables 6 and 7. **Please note**, you should **not** shake/"shoogie" your fluid thioglycolate tubes.

Table 6. Visual observation (colony characteristics) of microbial growth on stiff blood agar plates incubated aerobically and anaerobically

Microorganism cultured	Observations – plates cultured <u>aerobically</u>	Observations – plates cultured <u>anaerobically</u>	Interpretation
Your unknown			
<i>Clostridium</i>			

Table 7. Visual observation of microbial growth in fluid thioglycollate medium.

Microorganism Cultured	Visual observations (turbidity/growth, colour)	Interpretation
Your unknown		
<i>Escherichia coli</i>		
<i>Clostridium</i>		

#### TASK 4F: Determining antibiotic resistance of *P. aeruginosa* isolates

Working as a group of four, you should determine whether your four *P. aeruginosa* isolates are resistant to the antibiotic gentamicin (Protocol 4.5).

##### Protocol 4.5: Kirby-Bauer Disk Diffusion Assays

1. Prepare a seed lawn for each of your four *Pseudomonas aeruginosa* isolate as follows: pipette 100 µl of the supplied culture onto the centre of a Mueller Hinton agar plate and use a sterile plastic spreader to distribute the culture evenly over the surface of the agar. The liquid should be completely absorbed into the agar before you add the antibiotic disks in step 5.
2. Obtain an empty petri dish containing twenty sterile filter discs.
3. Prepare your antibiotic dilutions as follows:
  - a. You will be provided with a 1 mg/mL stock solution of gentamicin, and you will use this to prepare four different dilutions of gentamicin. The dilutions will be added to sterile paper disks, to give four different final amounts of antibiotic.
  - b. You will be adding 20 µl of each dilution to a filter disk, but it is always a good idea to prepare a slightly larger volume to account for any pipetting errors - e.g. prepare 25 µl for each filter disk. (Since you have four strains, you should prepare enough for four filter disks.)
  - c. Prepare the dilutions so that you will end up with 4 µg, 8 µg, 12 µg and 16 µg of gentamicin when you add 20 µl of the dilution to a filter disk (e.g., you want a final concentration of 4 µg/20 µl for the first dilution).

- d. Calculate the amount of your 1 mg/mL stock solution of gentamycin, and the amount of sterile water, needed for each dilution, completing Table 8. Then prepare these gentamycin dilutions in sterile microcentrifuge tubes.

Table 8. Calculation of antibiotic dilutions

Final amount on disc	4 µg	8 µg	12 µg	16 µg
Volume stock solution				
Volume H <sub>2</sub> O				

4. Prepare your antibiotic disks and controls as follows:
  - a. For the antibiotic disks, add 20 µl of antibiotic to each disk (making sure to keep the different dilutions separated, and keep track of which disk has which dilution). Remember, you will need 4 disks for each antibiotic concentration.
  - b. For the control disks, add 20 µl of water to each disk (making sure to keep these separate from your antibiotic disks). You will need 4 control (water) disks.
  - c. All the antibiotic and control disks to dry (the liquid should soak completely into the disks before you transfer them to your agar plates).
5. Pass a forceps very briefly through a flame to sterilise them (not too hot as you do not want to overheat the metal!). Using your sterile forceps, transfer the paper disks to the seed lawns you prepared in step 1, making sure to place the disks evenly across the surface of the plate (Figure 1) as you will need to be able to measure the diameter of each zone of inhibition next week.

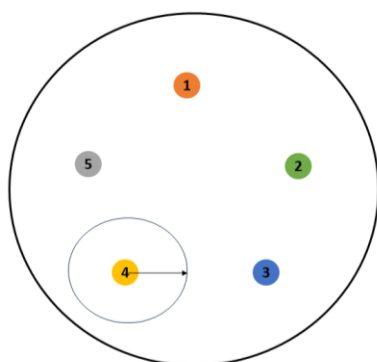


Figure 1. Placement of antibiotic disks on a Petri plate. Each coloured disk represents a different antibiotic concentration. The future zone of inhibition and its radius is shown for disk 3.

6. Be sure to press the disks firmly against the surface of the agar, so that they will not be dislodged when the plate is inverted to incubate it.
7. Your plates will be incubated at 37°C overnight for you, and you will be able to observe and record your results in the next lab session.

## Competency check: streak plate.

One of the lecturers will assess your streak plate (from Task 4A or 4D) for competency according to the following criteria:

### Streak plate competency check 3:

- ☐ Well-streaked plate, with several well-separated and distinct individual colonies
- ☐ Streaking technique needs improvement: does not make good use of the space available on the plate
- ☐ Streaking technique needs improvement: no/few single colonies obtained
- ☐ Streaking technique needs improvement: probable faults in aseptic technique
- ☐ Streaking technique needs improvement: other \_\_\_\_\_

### Labelling technique:

- ☐ Clear, legible, complete label
- ☐ Labelling is illegible/unclear
- ☐ Label placed incorrectly: \_\_\_\_\_
- ☐ Label is missing key information: \_\_\_\_\_

You should use the feedback provided by the lecturer on your streak plate to improve your technique. If you have not yet managed to pass this competency, you will be given another opportunity to streak a plate.

## Competency check: documenting and reporting on experimental results and conclusions.

Before you leave the lab, one of the lecturers will assess your work (Tables 1-2, 5-7) for competency, and give you feedback:

### Recording results check 3:

- ☐ Results and interpretation clearly and concisely recorded
- ☐ Poor sketch(es), lacking adequate detail
- ☐ Sketches/results illegible/unclear
- ☐ Lack of separation between results (raw data) and interpretation
- ☐ Failure to use accurate/precise terminology
- ☐ Results table(s) missing key information: \_\_\_\_\_
- ☐ Other: \_\_\_\_\_