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Title: Antibiotic Efficacy Testing in an Ex Vivo Model of Pseudomonas aeruginosa and Staphylococcus aureus Biofilms in the Cystic Fibrosis Lung

Authors and Affiliations:

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☐ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits. **<<If outdoor filming is possible, this will be appropriate. I have selected the second statement as this will be preferable.**

   ☒ Interviewees self-record interview statements. JoVE can provide support for this option.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 22
Number of Shots: 46

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Freya Harrison:** This method allows you to grow bacteria in an environment which cues similar cellular physiology and biofilm morphology to that observed when those bacteria infect the cystic fibrosis lungs.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Niamh Harrington:** The lungs are a waste product from the meat industry, so there are no ethical concerns. The model offers a platform to mimic human infection without a live animal model.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

OPTIONAL:

1.3. **Freya Harrison:** This technique provides a new way for researchers to screen candidate drugs for potential to enter and destroy bacterial biofilms that form in the lungs of people with cystic fibrosis.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.4. **Esther Sweeney:** With further development and validation we believe that the EPVL model could have potential use for specialized or individualized antibiotic susceptibility testing.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. Dissection and infection of ex vivo pig lung (EVPL) tissue

2.1. Obtain lungs immediately after slaughter and transport them to the laboratory in a domestic coolbox **[1]**.

2.1.1. WIDE: Establishing shot of talent taking lungs out of a coolbox.

2.2. Work on a sterilized surface and under a flame **[1]**. Place the lungs on a clean plastic chopping board covered with autoclaved aluminum foil **[2]** and check that the bronchioles remain intact. The lungs are not suitable for use if there has been any damage at the abattoir or during transport **[3]**.

2.2.1. Sterile working bench with the Bunsen burner in the shot.

2.2.2. Lungs on the chopping board.

2.2.3. Talent checking the lungs, with no damage visible.

2.3. Heat a palette knife under a flame **[1]** and very briefly touch the knife to the area of the lung surrounding the bronchiole to sterilize the surface of the tissue **[2]**. Cut away the surface tissue surrounding the bronchiole using a sterile mounted razor blade, making incisions parallel to the bronchiole to prevent any damage **[3]**.

2.3.1. Talent flaming a knife.

2.3.2. Talent touching the knife to the area surrounding the bronchiole.

2.3.3. Talent cutting away the tissue surrounding the bronchiole.

2.4. Once the bronchiole has been exposed, make a cross-sectional incision through the bronchiole at the highest point visible **[1]**.

2.4.1. Talent making the cross-sectional incision.

2.5. Using sterile forceps, lightly hold the free end of the bronchiole and cut away any remaining unwanted tissue using a sterile mounted razor blade **[1]**. Make a final cross-sectional incision across the bronchiole before any branching is visible to remove the bronchiole from the lungs **[2]**.

2.5.1. Talent cutting away any remaining tissue.

2.5.2. Talent removing the bronchiole from the lungs.

2.6. Place the bronchiole in the first DMEM-RPMI 1640 wash **[1]**. Leave it in the wash and harvest additional sections of bronchiole from the same lung to yield sufficient tissue sections for the planned experiment **[2]**.

2.6.1. Talent placing the bronchiole in the wash.

2.6.2. Talent harvesting additional tissue.

2.7. Place any additional bronchiolar sections from the same lung into the wash and leave them in the wash for at least 2 minutes **[1]**. Remove the bronchioles from the first wash and place the samples in a sterile Petri dish **[2]**.

2.7.1. Talent placing additional tissue in the wash.

2.7.2. Talent transferring the bronchioles into a Petri dish.

2.8. Lightly hold each bronchiole with sterile forceps, taking care to not damage the tissue. Remove as much remaining soft tissue as possible **[1]** and cut the tissue into 5-millimeter-wide strips using sterile dissection scissors **[2]**. *Videographer: This step is important!*

2.8.1. Talent removing soft tissue from the bronchiole.

2.8.2. Talent cutting the tissue into strips.

2.9. Place all of the bronchiolar tissue strips into the second DMEM-RPMI 1640 wash **[1]**. Leave it in the wash for at least 2 minutes, then remove the tissue strips from the second wash using sterile forceps and place them in a clean, sterile Petri dish **[2]**.

2.9.1. Talent placing the tissue into the second wash.

2.9.2. Talent transferring the tissue into a clean Petri dish.

2.10. Remove any remaining soft tissue attached to the bronchiole **[1]** and cut the strips into squares using sterile dissection scissors **[2-TXT]**. Add the third DMEM-RPMI 1640 wash into the Petri dish **[3]** and lightly mix the tissue pieces in the wash by swirling the dish **[4]**. *Videographer: This step is important!*

2.10.1. Talent removing remaining tissue from the bronchiole.

2.10.2. Talent cutting the tissue into squares. **TEXT: 5 mm x 5 mm**

2.10.3. Talent adding the third wash into the Petri dish.

2.10.4. Talent swirling the dish.

2.11. Pour the third wash out of the Petri dish without removing the tissue pieces **[1]**, then add the final SCFM wash, ensuring that all of the tissue pieces are covered **[2]**. Sterilize the tissue in SCFM under UV light for 5 minutes **[3]**.

2.11.1. Talent pouring out the third wash.

2.11.2. Talent adding the final wash to the tissue.

2.11.3. Talent putting the tissue under UV light.

2.12. Use sterile forceps to transfer each sterilized bronchiolar tissue piece into individual wells of a 24-well plate containing SCFM agarose pads **[1]**.

2.12.1. Talent transferring tissue into wells.

2.13. To infect each tissue piece with the desired bacterial strain, touch a colony grown on an agar plate with the tip of a 29-gauge needle attached to a sterile 0.5-milliliter insulin syringe **[1]**, then touch the colony onto the tissue piece, gently pricking the surface **[2]**. *Videographer: This step is important!*

2.13.1. Talent picking up bacteria from a colony.

2.13.2. Talent touching the needle to the tissue piece.

2.14. For the uninfected controls, gently prick the surface of the tissue piece with the tip of the needle **[1]**, then use a pipette to add 500 microliters of SCFM to each well **[2]**. Sterilize a breathable sealing membrane for each 24-well plate under ultraviolet light for 10 minutes **[3]**. *Videographer: This step is important!*

2.14.1. Talent pricking the surface of a control tissue with the needle.

2.14.2. Talent adding SCFM to a few wells.

2.14.3. Talent putting the breathable sealing membrane under UV light.

2.15. Remove the lid from the 24-well plate and replace it with the breathable membrane **[1]**, then incubate the plates at 37 degrees Celsius without shaking **[2]**.

2.15.1. Talent replacing the lid with the membrane.

2.15.2. Talent putting the plate in the incubator and closing the door.

3. Determination of antibiotic efficacy

3.1. Set up replicate sets of lung pieces from at least two independent lungs, using one set for a negative control and one set per each concentration of antibiotic to be tested **[1]**.

3.1.1. Talent taking a plate out of the incubator.

3.2. After 48 hours of incubation, visually inspect the uninfected tissue pieces for growth of endogenous bacteria, which would cause the medium around these sections to be turbid. If growth typical of the selected study species is observed, re-start the experiment with fresh lungs **[1]**.

3.2.1. Talent inspecting the plate for bacteria growth.

3.3. If the uninfected tissue sections show none or minimal bacterial growth, prepare one 24-well wash plate and one 24-well treatment plate. Each well of the treatment plate should contain 500 microliters of fresh SCFM without antibiotics or with the antibiotic of interest **[1]**.

3.3.1. Talent preparing plates.

3.4. Remove each infected tissue piece from the incubation plate with flame sterilized forceps **[1]**, swirl it briefly in a fresh well of the wash plate to remove any non-biofilm associated bacterial cells **[2]**, and transfer it to the appropriate well of the treatment plate **[3]**. *Videographer: This step is important!*

3.4.1. Talent removing a piece of tissue from the incubation plate.

3.4.2. Talent washing the tissue.

3.4.3. Talent putting the tissue in the treatment plate.

3.5. Seal the treatment plate with a fresh breathable membrane **[1]**, then incubate it at 37 degrees Celsius without shaking for 18 to 24 hours **[2]**.

3.5.1. Talent sealing the treatment plate.

3.5.2. Talent putting the plate in the incubator and closing the door.

3.6. Use flame sterilized forceps to remove each lung piece from the 24-well plate **[1]** and put it in a sterile 2-milliliter homogenization tube containing 1 milliliter of PBS and 1 gram of metal beads **[2]**. Bead beat for 40 seconds at 4 meters per second **[3]**.

3.6.1. Talent picking up a lung piece.

3.6.2. Talent putting the piece in a homogenization tube.

3.6.3. Talent bead beating the tissue.

3.7. Serially dilute the lung homogenate using PBS and plate it on LB agar **[1]** to determine the colony forming units in individual untreated and antibiotic-treated tissue pieces **[2]**.

3.7.1. Talent plating the homogenate.

3.7.2. Talent counting colonies.

Results

4. Results: Screening bacterial isolates for antibiotic susceptibility with the EVPL model

4.1. When grown in the ex vivo pig lung, or EVPL, biofilms of *P. aeruginosa* and *S. aureus* demonstrate increased tolerance to antibiotics **[1]** compared to susceptibility in standard, industry approved broth MIC *(spell it out 'M-I-C')* **[2]** and disc assays using standard media **[3]**.

4.1.1. LAB MEDIA: Figure 1 and 2.

4.1.2. LAB MEDIA: Figure 1 and 2. *Video Editor: Emphasize Figure 1.*

4.1.3. LAB MEDIA: Figure 1 and 2. *Video Editor: Emphasize Figure 2.*

4.2. The various effects of different antibiotics on an EVPL established biofilm are distinguishable **[1]**. For example, *P. aeruginosa* killing is achieved in EVPL with 4 to 16X MIC ciprofloxacin **[2]** but not with 4 to 8X MIC chloramphenicol **[3]**.

4.2.1. LAB MEDIA: Figure 1.

4.2.2. LAB MEDIA: Figure 1. *Video Editor: Emphasize the ciprofloxacin graph.*

4.2.3. LAB MEDIA: Figure 1. *Video Editor: Emphasize the chloramphenicol graph.*

4.3. *S. aureus* populations that are susceptible to linezolid in the disc assay are able to survive target serum concentrations and higher in EVPL **[1]**.

4.3.1. LAB MEDIA: Figure 2.

4.4. Even an optimized in vitro assay cannot accurately predict *P. aeruginosa* susceptibility to colistin in the EVPL. The amount of antibiotic required to achieve 3 $\log_{10}$ killing of EVPL-grown bacteria is often significantly higher than the MIC or the MBEC *(pronounce 'em-beck')* calculated from standard in vitro assays **[1]**.

4.4.1. LAB MEDIA: Figure 3.

4.5. In addition to distinguishing differences between antimicrobial agents, this model can highlight changes in susceptibility at different bacterial growth stages and for different antibiotic dosing intervals **[1]**. The increasing tolerance of *P. aeruginosa* biofilms to meropenem as they mature is shown here **[2]**.

4.5.1. LAB MEDIA: Figure 4.

4.5.2. LAB MEDIA: Figure 4. *Video Editor: Emphasize the increasing position of the triangles along the y-axis as the x-axis increases.*

4.6. *S. aureus* survival was measured at 4 and 24 hours post exposure to flucloxacillin, making it possible to observe differences in the reduction of bacterial cell counts across time and between isolates **[1]**.

4.6.1. LAB MEDIA: Figure 5.

4.7. Variations in bacterial load often increase with extended culture times. This can be seen in the untreated control following 48-hour biofilm development and a further 24-hour exposure to account for antibiotic dosing interval **[1]**.

4.7.1. LAB MEDIA: Figure 5. *Video Editor: Emphasize the black circles.*

Conclusion

5. Conclusion Interview Statements

5.1. **Freya Harrison:** It's crucial to maintain sterile technique while dissecting. We recommend doing the dissection in a lab, or a designated area of your lab, that is never used for microbiology work.

5.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.2.1.*

5.2. **Freya Harrison:** We have recently used the model to explore the penetration of colistin into biofilm, and it has good potential for use in research to monitor and improve drug delivery into biofilms.

5.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.