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Title: Studying Effects of Cigarette Smoke on *Pseudomonas* Infection in Lung Epithelial Cells

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **No**

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

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

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Kristen V. Fanning:** This protocol uses 3 approaches to determine if cigarette smoke extract affects Pseudomonas bacterial load in lung epithelial cells.

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

1.2. **Tiao Li:** This method could be expanded to endothelial cells or other cell types.

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

OPTIONAL:

1.3. **Chunbin Zou:** Preparation of cigarette smoke extracts, bacterial infection, and determination of bacterial load is described in detail in this video. The procedure should be easy to follow for new users.

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

Protocol

2. Cigarette Smoking Extract (CSE) Preparation

Videographer NOTE: This was a long one with a lot of long shots of pipetting. I thought it was safer to roll on these long shots for long periods just to be safe, so a preemptive apology to the editors. Also author refused to wear a lab coat.

2.1. Draw 10 milliliters of serum-free cell culture medium into a 60-milliliter syringe **[1]**. Attach the narrow end of a trimmed 1-milliliter pipette tip to the nozzle of the syringe, as an adapter to hold the cigarette **[2]**. Remove the filter from a cigarette, and attach the cigarette to the adapter **[3]**.

2.1.1. Talent draws 10 mL of medium into syringe.
2.1.2. Talent attaches pipette tip to the nozzle of the syringe.
2.1.3. Talent removes the filter from the cigarette and attaches the cigarette to the adapter.

2.2. No more than 30 minutes before performing the assay, combust the cigarette, and draw 40 milliliters of smoke-containing air into the syringe **[1-TXT]**. Mix the smoke with the medium by shaking the syringe vigorously **[2]**. Repeat the drawing process until the cigarette is completely burned out, which will require about 11 draws in approximately 7 minutes **[3]**. *Videographer: This step is important!*

2.2.1. Talent lights cigarette and pulls on syringe plunger. **TEXT: Work in a chemical hood! Cigarette smoke is toxic to humans**
2.2.2. Talent shakes the syringe vigorously.
2.2.3. Talent pulls on syringe plunger again.

2.3. To remove microorganisms and insoluble particles from the medium, filter it through a 0.22-micron filter **[1]**. Then, transfer the medium to a closed sterile tube **[2]**.

2.3.1. Talent filters medium through a 0.22-micron filter.
2.3.2. Talent transfers medium to a sterile tube.

3. *Pseudomonas* Culture

3.1. Inoculate a Tryptic Soy Broth, or TSB, agar plate with the selected *Pseudomonas* strain **[1]**. Incubate the plate overnight at 37 degrees Celsius **[2]**.

3.1.1. Talent inoculates agar plate with bacteria.
3.1.2. Talent places the plate in the incubator.

3.2. Prepare a tube containing 20 milliliters of TSB with 5 percent glycerol as the carbon source **[1]**. Collect a smear from the agar plate, and inoculate the TSB **[2]**. Incubate the *Pseudomonas* suspension in a 37-degree Celsius shaker at 200 rpm for approximately 1 hour, until the optical density at 600 nanometers is 0.6 **[3]**. *Videographer: This step is important!*

3.2.1. Talent prepares tube of medium.
3.2.2. Talent collects a bacterial smear from the agar plate, and inoculates a tube of TSB.
3.2.3. Talent places suspension in incubator.

4. Cell Culture and CSE Treatment

4.1. After culturing lung epithelial cells as described in the manuscript, add 1 milliliter of 0.25 percent trypsin to the cells **[1]**.

4.1.1. Talent adds trypsin to plate of lung epithelial cells.

4.2. After approximately 5 minutes, when the cells have completely detached from the bottom of the plate, add 10 milliliters of complete HITES *(pronounce 'hights')* medium to neutralize the trypsin **[1]**. Transfer the cells to a 15-milliliter tube **[2]**. Then, centrifuge the cells for 5 minutes, at 4 degrees Celsius and 300 times *g* **[3]**.

4.2.1. Talent adds medium to plate. NOTE: This and next shot in the same clip.
4.2.2. Talent transfers the cells to a 15-mL tube.
4.2.3. Talent centrifuges the tube.

4.3. Remove and discard the supernatant **[1]**. Then, resuspend the cells in 2 milliliters of HITES medium with 10 percent FBS **[2]**.

4.3.1. Talent removes and discards supernatant.
4.3.2. Talent resuspends cells in medium.

4.4. Pipette 10 microliters of the epithelial cell suspension onto a new plate **[1]**. Use an automated cell counter to obtain the cell concentration **[2]**.

4.4.1. Talent pipettes a small amount of cell suspension on a plate.
4.4.2. Talent inserts plate into automated cell counter.

4.5. Plate the lung epithelial cells at a concentration of 3×10^5 cells per milliliter, in a total volume of 2 milliliters of medium per well **[1 and 1a]**. Incubate the plates overnight **[2]**.

4.5.1a Added shot: medium added

4.5.1. Talent pipettes cell suspension into wells of 6-well plate.
4.5.2. Talent places plate in incubator.

Commented [AG1]: Authors: Is this pronunciation correct? If not, please correct it and send me the updated script.

4.6. When the cells reach approximately 80 percent confluency, replace the medium with HITES medium plus 1 percent FBS **[1]**. Then, add 4 percent CSE to the wells, and incubate the plates for 3 hours **[2]**.

4.6.1. Talent replaces medium in wells with new medium.
4.6.2. Talent adds CSE to the wells of the plate.

5. Bacterial Infection and Gentamycin Treatment

5.1. Add *Pseudomonas* to each well of CSE-treated lung epithelial cells **[1]**. Then, incubate the plates for 1 hour, at 37 degrees Celsius with 5 percent carbon dioxide **[2]**.

5.1.1. Talent adds Pseudomonas to the 6-well plates.
5.1.2. Talent places plates in incubator.

5.2. Next, replace the medium in the 6-well plates with 2 milliliters of fresh medium: HITES containing 4 percent CSE and 100 micrograms per milliliter gentamicin **[1]**.

5.2.1. Talent replaces medium in 6-well plates.

5.3. After incubating the plates for 1 hour at 37 degrees Celsius with 5 percent carbon dioxide, aspirate the medium from the wells **[1]**. Wash the gentamycin-treated cells twice with 2 milliliters of cold PBS **[2]**. Bacterial load in the lung epithelial cells can then be detected by the drop plate method, qRT-PCR, or flow cytometry **[3]**.

5.3.1. Talent aspirates medium from the 6-well plates.

5.3.2. Talent washes the cells with cold PBS.
5.3.3. LAB MEDIA: Figure 1.

6. Determination of Bacterial Concentration Using the Drop Plate Method

6.1. Add 1 milliliter of cell lysis buffer to each well of lung epithelial cells **[1]**.

6.1.1. Talent adds cell lysis buffer to 6-well plate.

6.2. Prepare serial dilutions of the cell lysate, ranging from 1 to 10 to 1 to 10,000 **[1].** Then, inoculate the TSB agar plate **[2]**.

6.2.1. Talent dilutes the cell lysate.
6.2.2. Talent inoculates agar plate.

6.3. After 16 hours of incubation, determine how many colony forming units were present in the lung epithelial cells **[1]** by counting the number of bacterial colonies **[2]**.
Videographer: This step is important!

6.3.1. Talent counts bacterial colonies.
6.3.2. TSB agar plate with bacterial colonies.

7. RT-qPCR Detection of Bacterial 16S rRNA

7.1. Add 0.35 milliliters of the guanidium thiocyanate lysis buffer to each well of lung epithelial cells **[1]**. Collect the cells with a cell scraper **[2]**. Pipette the lysate into a microcentrifuge tube and mix gently with the pipette **[3]**.

7.1.1. Talent adds lysis buffer to 6-well plate.
7.1.2. Talent scrapes cells from wells. NOTE: This and next shot in the same clip.
7.1.3. Talent pipettes contents of wells into microcentrifuge tubes.

7.2. Add 0.35 milliliters of freshly prepared 70 percent ethanol to the lysate, and mix well **[1]**. Transfer all samples to a spin column placed in a 2-milliliter collection tube **[2]**. Centrifuge the collection tube for 30 seconds, at 10,000 times *g* and 20 to 25 degrees Celsius **[3]**. *Videographer: This step is important!*

7.2.1. Talent adds ethanol to the tubes.
7.2.2. Talent transfers samples to a spin column in a collection tube.
7.2.3. Talent places collection tube in centrifuge.

7.3. Discard the buffer in the collection tube, and wash the column with 0.7 milliliters of wash buffer 1 **[1]**. Centrifuge the column at 10,000 times *g* for 30 seconds **[2]**.

7.3.1. Talent discards the buffer.
7.3.2. Talent washes the column with buffer 1.
7.3.3. Talent places column in centrifuge.

7.4. Wash the column twice with 0.5 milliliters of buffer **[1]**. Repeat the centrifugation at 10,000 times *g* for 2 minutes **[2]**.

7.4.1. Talent washes the column with buffer.
7.4.2. Talent places column in centrifuge.

7.5. Place the column into a new 1.5-milliliter collection tube, and add 30 to 50 microliters of RNase-free water **[1]**. After centrifuging the tube at 10,000 times *g* for 1 minute, collect the flow-through, and measure the RNA concentration **[2]**.

7.5.1. Talent places the column in a new collection tube and adds water.
7.5.2. Talent collects the flow-through from the collection tube and begins process of measuring RNA concentration.

7.6. To prepare for a 20-microliter reverse transcription reaction, mix 1 microgram of total RNA with 10 microliters of reaction buffer, 1 microliter of reverse transcriptase, and RNase-free water **[1]**. Conduct the reverse transcription reaction at 37 degrees Celsius for 1 hour and then at 95 degrees Celsius for 5 minutes, according to the manufacturer's protocol **[2]**.

7.6.1. Talent mixes RNA, buffer, reverse transcriptase, and water.

7.6.2. Talent begins reverse transcription reaction.

7.7. Then, mix 1 microliter of each cDNA template with 5 microliters of the Master Mix containing SYBR dye and 1 microliter of each 200 nanomolar specific primer **[1]**. Add water, for a total volume of 20 microliters **[2]**.

7.7.1. Talent mixes cDNA template, Master Mix, and primers.
7.7.2. Talent adds water.

7.8. Perform PCR analysis according to the manufacturer's recommendations, and then use the comparative CT method to determine the expression **[1]**.

7.8.1. Talent begins PCR process.

Results

8. Results: CSE-Treatment Increases Bacterial Load

8.1. In BEAS-2B cells, viability did not change considerably when cells were treated with 4 percent CSE for 3 hours **[1]**. One hour of *Pseudomonas* infection also did not substantially affect viability **[2]**. Finally, the use of gentamycin to kill *Pseudomonas* in the culture medium did not have a statistically significant effect on cell viability **[3]**.

8.1.1. LAB MEDIA: Figure 4.
8.1.2. LAB MEDIA: Figure 5.
8.1.3. LAB MEDIA: Figure 6.

8.2. As measured by the drop plate method, bacterial infection in BEAS-2B cells was substantially increased by treatment with CSE **[1]**. The same was true for CSE-treatment of HSAEC **[2]**.

8.2.1. LAB MEDIA: Figure 2.
8.2.2. LAB MEDIA: Figure 3.

8.3. Bacterial infection in CSE-treated BEAS-2B cells was also assessed by using RT-PCR to quantify the amount of 16S RNA present, which indicated an increase in *Pseudomonas aeruginosa* **[1]**.

8.3.1. LAB MEDIA: Figure 7.

8.4. BEAS-2B cells treated with CSE and infected with *Pseudomonas fluorescens Migula* were analyzed by flow cytometry at 509 nanometers. These results also indicated that CSE treatment increases bacterial infection **[1]**.

8.4.1. LAB MEDIA: Figure 8.

8.5. Results from fluorescent microscopy showed that GFP-labeled bacteria colocalized with BEAS-2B cells in a *Pseudomonas fluorescens Migula* infection experiment **[1]**.

8.5.1. LAB MEDIA: Figure 9.

Conclusion

9. Conclusion Interview Statements

9.1. **Kristen V. Fanning:** Cells could be exposed to cigarette smoke with robot smoking, which may mimic human behavior and make it possible to directly study the effects of cigarette smoke on lung epithelial cells.

9.1.1. INTERVIEW: Named author says the statement above in an interview-style statement while looking slightly off-camera.

9.2. **Tiao Li:** This technique could be used to explore cigarette smoke on other cell types in the context of pulmonary infection.

9.2.1. INTERVIEW: Named author says the statement above in an interview-style statement while looking slightly off-camera.

9.3. **Chunbin Zou:** The described approach could be the basis for development of effective therapies against cigarette smoke mediated lung injury and COPD exacerbation.

9.3.1. INTERVIEW: Named author says the statement above in an interview-style statement while looking slightly off-camera.