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Antibiotic efficacy testing in an ex vivo model of Pseudomonas aeruginosa and Staphylococcus aureus biofilms in the cystic fibrosis lung

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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

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KEYWORDS:

Antibiotic susceptibility testing, biofilm, cystic fibrosis, infection, MBEC, MIC

SUMMARY:

This workflow can be used to perform antibiotic susceptibility testing using an established ex vivo model of bacterial biofilm in the lungs of individuals with cystic fibrosis. Use of this model could enhance the clinical validity of MBEC (minimal biofilm eradication concentration) assays.

ABSTRACT:

The effective prescription of antibiotics for the bacterial biofilms present within the lungs of individuals with cystic fibrosis (CF) is limited by a poor correlation between antibiotic susceptibility testing (AST) results using standard diagnostic methods (e.g., broth microdilution, disk diffusion, or Etest) and clinical outcomes after antibiotic treatment. Attempts to improve AST by the use of off-the-shelf biofilm growth platforms show little improvement in results. The limited ability of in vitro biofilm systems to mimic the physicochemical environment of the CF lung and, therefore bacterial physiology and biofilm architecture, also acts as a brake on the discovery of novel therapies for CF infection. Here, we present a protocol to perform AST of CF pathogens grown as mature, in vivo-like biofilms in an ex vivo CF lung model comprised of pig bronchiolar tissue and synthetic CF sputum (ex vivo pig lung, EVPL).

Several in vitro assays exist for biofilm susceptibility testing, using either standard laboratory medium or various formulations of synthetic CF sputum in microtiter plates. Both growth medium and biofilm substrate (polystyrene plate vs. bronchiolar tissue) are likely to affect biofilm antibiotic tolerance. We show enhanced tolerance of clinical *Pseudomonas aeruginosa* and *Staphylococcus aureus* isolates in the ex vivo model; the effects of antibiotic treatment of biofilms is not correlated with the minimum inhibitory concentration (MIC) in standard microdilution assays or a sensitive/resistant classification in disk diffusion assays.

The ex vivo platform could be used for bespoke biofilm AST of patient samples and as an enhanced testing platform for potential antibiofilm agents during pharmaceutical research and development. Improving the prescription or acceleration of antibiofilm drug discovery through the use of more in vivo-like testing platforms could drastically improve health outcomes for individuals with CF, as well as reduce the costs of clinical treatment and discovery research.

INTRODUCTION:

Chronic biofilm infections affect individuals whose normal immune defenses are compromised. Groups at risk include those with the genetic condition cystic fibrosis (CF)[1]. Colonization of the abnormally thick, adhesive mucus in the respiratory tract in early infancy leads to intractable biofilm infections of the bronchioles[2,3]. The growth of bacteria as extensive matrix-encapsulated biofilms is one factor that distinguishes chronic infections of immunocompromised people from acute infections of healthy hosts and the biofilm state both protects bacteria from antibiotic exposure (due to reduced diffusion through the matrix) and decreases their antibiotic susceptibility (e.g., through induction of quiescence or upregulation of efflux pumps)[4,5]. However, disease-specific alterations in host tissue physiology and chemistry further alter bacterial physiology from that observed in acute infections or in standard laboratory growth conditions. Key examples in CF include the use of unusual carbon sources, such as fatty acids and amino acids released from lung surfactant and produced by microbial degradation of mucin, the release of micronutrients, such as iron from damaged tissues, and microaerobiosis[6-8].

The specific physicochemical conditions in a particular biofilm infection context can therefore influence responses to antibiotics. First, the structure and depth of the extracellular matrix depends on local environmental conditions, such as nutrients or shear forces. Second, environmental cues can trigger expression of specific antibiotic resistance genes. For example, the CF pathogen *Pseudomonas aeruginosa* shows increased expression of a beta-lactamase and reduced expression of porins in CF sputum versus in vitro[9], while another CF pathogen, *Burkholderia cenocepacia*, upregulates beta-lactamases and efflux pumps when grown in CF sputum[10]. Third, in-host conditions can cue a physiological or genetic switch to antibiotic-tolerant phenotypes, which are hard to recapitulate in vitro. These include small colony variants of the CF pathogen *Staphylococcus aureus*[11,12].

All of these data indicate that when diagnostic labs isolate individual clones from pathogenic biofilm and perform AST on planktonic or agar-plate grown cultures in standard laboratory

media (broth microdilution, disk diffusion or Etest), the results often do not predict which antibiotics will actually work in vivo. Even if in vitro biofilm assays are used, they may not cue an in vivo-like biofilm phenotype due to differences in the medium and attachment surface used, so assays using flow cells or high-throughput microplate platforms can over-estimate antibiotic sensitivity[13]. The same problem applies to researchers in academia and industry seeking to develop new antibiofilm agents: testing drug potential using in vitro platforms like flow cells, microtiter plates, or Center for Disease Control biofilm reactors may set the biofilm efficacy bar too low and produce false positives in the research, development pipeline.

The poor correlation between AST results and clinical outcome after antibiotic treatment in CF is well known. Many clinicians simply ignore diagnostic lab AST as there are no uniform, CF-specific guidelines for interpreting these results and instead make case-by-case decisions for prescribing. Attempts have been made to improve CF AST by using the Calgary biofilm device, which uses biofilms grown on the surface of plastic pegs set within the wells of a microplate containing standard AST medium (e.g., cation-adjusted Muller-Hinton broth)[14,15]. This assay does no better at predicting which antibiotics will work in vivo than standard planktonic AST[16]. The impact on patients with CF is stark. Despite repeated antibiotic administration (regular inhaled antibiotics and a median of 27 days/year receiving intravenous antibiotics for individuals with CF in the United Kingdom)[17], frequent and unpredictable episodes of acute pulmonary exacerbation lead to progressive lung damage and, in approximately 90% of cases, death from respiratory failure. In a recent analysis, bacterial lung infection was the strongest predictor of medication costs in CF, adding on average €3.6K/patient/year to direct healthcare costs[18,19].

For acute infections of otherwise healthy individuals, current research and policy focusing on rapid AST based on, for example, point-of-care genomic prediction is ideal[20]. But in the case of chronic CF infections, it is clear that a different approach is needed: the implementation of AST in host-mimicking models that better recapitulate the in vivo environment and pathogen metabolic state and allow for the formation of realistic biofilm structure.

We have previously developed a CF biofilm model that comprises sections of pig bronchiole incubated in synthetic CF sputum and infected with *P. aeruginosa* or *S. aureus*. Uninfected EVPL retains normal histopathology for 7 days but lab or clinical isolates of *P. aeruginosa* and *S. aureus* reproducibly form in vivo-like aggregates around the tissue, mimicking the etiology of CF infection[21-23]. We present a protocol for using this high-validity, high-throughput model as a tailored biofilm AST platform for CF and present exemplary results showing the high tolerance of pathogen biofilms to clinically-used antibiotics when grown in the model. The model could readily be incorporated into research, development pipelines for the management or prevention of biofilm formation and potentially into diagnostic AST. Most equipment used (see Table of Materials) may readily be found in a typical microbiology laboratory, although a bead beater is essential, and we have found from work with collaborators that a suitable ultraviolet germicidal cabinet may also need to be procured. As the lungs are sourced from commercial butchers or abattoirs, the model presents no ethical concerns.

PROTOCOL:

This protocol uses pig lungs sourced from a commercial abattoir that supplies meat for human consumption. Under UK legislation, using leftover tissue from animals slaughtered for meat does not require ethical approval; we advise readers to check relevant local laws and institutional guidelines before starting work.

1. Preparation of Synthetic CF Sputum Media (SCFM)

1.1. To make SCFM for use with EVPL tissue, follow the recipe outlined by Palmer et al.[24] with the modification that glucose is removed from the recipe.

NOTE: Palmer et al.'s recipe contains free amino acids, cations, anions and lactate at concentrations representative of the average concentrations found in a selection of sputum samples from CF patients. It has been shown to cue comparable carbon-usage pathways and expression of quorum sensing signals by *P. aeruginosa* PA14 to growth in medium made from lyophilised patient sputum[24]. A recipe for 1 L modified SCFM is supplied in **Table S1**.

1.2. Filter sterilize the SCFM immediately after preparation and store at 4 °C for up to 1 month.

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

2.1. Prior to dissection, prepare an agar plate/s of required bacterial strain/s for infection using whatever agar is standard in the lab for *P. aeruginosa*/*S. aureus* (e.g., lysogeny broth + 1.2% agar).

2.2. Calculate how many porcine bronchiolar tissue pieces are required for the experiment, including uninfected control tissue pieces. Multiply this number by two to repeat the experiment in two replicate lungs to confirm repeatability of results.

2.3. Multiply the total number of tissue pieces required by 0.5 to determine the volume of SCFM agarose (mL) needed to make agarose pads to make enough medium for 400 µL/tissue piece plus spare SCFM agar to account for any pipetting errors or evaporation during preparation.

2.4. Add 0.12 g of agarose to every 15 mL of SCFM required to make the desired total volume of SCFM with 0.8% weight/volume agarose.

2.5. Heat the SCFM agarose solution until the agarose is fully dissolved. A domestic microwave on low power is recommended. The time required depends on the wattage of the microwave. Allow the agarose to cool to approximately 50 °C (warm to the touch but comfortable to hold). Do not allow to cool any further.

2.6. Using a pipette, add 400 µL of the SCFM agarose to one well of a 24-well plate per tissue piece needed.

2.7. Sterilize the SCFM agarose-containing 24-well plate/s under ultraviolet light for 10 min.

2.8. Prepare three replicate washes for every intact lung being dissected using 20 mL of sterile Dulbecco's modified Eagle medium (DMEM) plus 20 mL of sterile Roswell Park Memorial Institute (RPMI) 1640 supplemented with 50 µg/mL ampicillin.

2.9. Make an aliquot of 40 mL SCFM as a final wash for every intact lung being dissected. All washes can be stored overnight at 4 °C or used immediately.

2.10. Obtain lungs from the designated source as soon as possible after slaughter, ensuring they are kept cold by transporting to the laboratory in a domestic coolbox.

NOTE: Lungs closer to the day of slaughter show less bruising from storage, but tissue kept on cold storage for up to 4 days from slaughter can also be used. As the coolbox needs to be taken into the butcher's shop or abattoir, it must be decontaminated following local lab guidelines after each use and stored outside the microbiology lab when not in use, to reduce the risk of contamination and a breach of containment.

2.11. Working on a sterilized surface and under a flame, place the lungs on a clean plastic chopping board covered with autoclaved aluminum foil. Check that the bronchioles remain intact. If there has been any damage at the abattoir or during transport the lungs are not suitable for use.

2.12. Heat a palette knife under a flame and very briefly touch the knife to the area of the lung surrounding the bronchiole to sterilize the surface of the tissue.

2.13. Cut away the surface tissue surrounding the bronchiole using a sterile mounted razor blade. Make incisions parallel to the bronchiole to prevent any damage.

2.14. Once the bronchiole has been exposed, make a cross-sectional incision through the bronchiole at the highest point visible to free the bronchiole.

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

2.16. Place the bronchiole in the first DMEM/RPMI 1640 wash. Leave the bronchiole in the wash and repeat steps 2.11-2.14 to harvest additional sections of bronchiole from the same lung as required to yield sufficient tissue sections for the planned experiment.

2.17. Place any additional bronchiolar sections from the same lung into the wash (step 2.16). Leave in the wash for at least 2 min.

2.18. Remove the bronchioles from the first DMEM/RPMI 1640 wash and place the samples in a sterile Petri dish.

2.19. Hold each bronchiole lightly using sterile forceps, making not to damage the tissue. Remove as much remaining soft tissue as possible and cut the tissue into ~5 mm wide strips using sterile dissection scissors.

2.20. Place all of the bronchiolar tissue strips into the second DMEM/RPMI 1640 wash. Leave in the wash for at least 2 min.

2.21. Remove the tissue strips from the second wash using sterile forceps, taking care not to damage the tissue. Place the tissue in a clean, sterile Petri dish.

2.22. Remove any remaining soft tissue attached to the bronchiole and cut the strips into squares (~5 mm x 5 mm) using sterile dissection scissors.

2.23. Add the third DMEM/RPMI 1640 wash into the Petri dish. Lightly mix the tissue pieces in the wash by swirling the dish.

2.24. Pour the third wash out of the Petri dish without removing the tissue pieces.

2.25. Add the final SCFM wash to the tissue-containing Petri dish, ensuring that all of the tissue pieces are covered.

2.26. Sterilize the tissue pieces in SCFM under UV light for 5 min.

2.27. Use sterile forceps to transfer each sterilized bronchiolar tissue piece into individual wells of a 24-well plate/s containing SCFM agarose pads.

2.28. To infect each tissue piece with the desired bacterial strain, touch a colony grown on an agar plate with the tip of a 29 G needle attached to a sterile 0.5 mL insulin syringe. Then touch the colony onto the tissue piece, gently pricking the tissue surface.

NOTE: Using an insulin syringe equipped with a 29 G needle allows the needle to be held accurately and comfortably while keeping fingers a safe distance from the both needle and lung tissue. It is possible to perform this step using 29 G needles that are not attached to a syringe, but this requires greater dexterity and increases the risk of a needlestick injury. Insulin syringes are readily available.

2.29. For the uninfected controls, gently prick the surface of each of the tissue piece with the tip of a 29 G needle attached to a sterile 0.5 mL insulin syringe.

2.30. Use a pipette to add 500 µL of SCFM to each well.

2.31. Sterilize a breathable sealing membrane for each 24-well plate under ultraviolet light for 10 min.

2.32. Remove the lid/s from the 24-well plate/s and replace with the breathable membrane.

2.33. Incubate the plates at 37 °C for the desired incubation (infection) time without shaking. Check that there is no visible growth of the inoculated pathogen on the uninfected control pieces (contamination control).

NOTE: If desired, ampicillin may be added to the SCFM agarose pads in step 2.5 and covering SCFM in step 2.29 to a final concentration of 20 µg/mL. This will suppress the growth of most endogenous bacteria on the lungs without affecting *P. aeruginosa* or *S. aureus* growth but, as the presence of ampicillin may affect susceptibility to other antibiotics, the reader is left to make this choice depending on the strains and antibiotics they wish to test.

3. Determination of antibiotic efficacy

NOTE: A schematic detailing the steps of this assay is provided in **Figure S1**.

3.1. To measure antibiotic tolerance of biofilms formed on EVPL, replicate sets of lung pieces, from at least two independent lungs, must be set up during the dissection and infection. One set of pieces is required for a negative control (no antibiotic treatment), and one set is required for each concentration of antibiotic to be tested.

3.2. After 48 hours of incubation, visually inspect the uninfected tissue pieces. Some growth of bacteria endogenous to the pig lung may have occurred, leading the SCFM around these sections to be turbid. If growth typical of the selected study species are observed (e.g., blue-green pigmentation diagnostic of *P. aeruginosa*), re-start the experiment with fresh lungs.

3.3. If the uninfected tissue sections show no or only minimal bacterial growth, prepare one 24-well wash plate and one 24-well treatment plate, each containing 500 µL of fresh SCFM without antibiotics or with the antibiotic of interest per well per lung tissue piece.

3.4. Remove each infected tissue piece from the incubation plate with flame sterilized forceps, swirl briefly in a fresh well of the wash plate to remove any non-biofilm associated bacterial cells, and transfer to the appropriate well of the treatment plate.

3.5. Seal the treatment plates with fresh breathable membrane.

3.6. Incubate the treatment plate/s at 37 °C without shaking for 18-24 h.

3.7. Using flame sterilized forceps, remove each lung piece from the 24-well plate and put in a sterile 2 mL homogenization tube containing 1 mL of phosphate-buffered saline (PBS) and 1 g of metal beads (**Table of Materials**).

3.8. Bead beat for 40 seconds at 4 m/s.

NOTE: Bead beating with the specific beads and homogeniser suggested in the Table of Materials does not cause significant lysis of bacteria, but each lab using the protocol should check the effects of their chosen beads and homogeniser prior to commencing AST assays.

3.9. Serially dilute the lung homogenate using PBS and plate on Lysogeny Broth (LB) agar to determine the colony forming units (CFU) in individual untreated and antibiotic-treated tissue pieces according to standard plating methods.

NOTE: Optional: Prepare duplicate plates on selective media to confirm colony identities; e.g., using mannitol salt agar for *S. aureus*.

REPRESENTATIVE RESULTS:

The EVPL model provides a high throughput assay platform, making it possible to screen a large number of bacterial isolates for antibiotic susceptibility at one time (**Figures 1** and **2**) or to screen strains against a range of antibiotic concentrations in one experiment (**Figure 3**). With practice, we have found that approximately 200 bronchiolar tissue sections can be prepared from lungs in 2 hours. The entire experiment for AST can be completed within normal working hours. Growth of *Pseudomonas aeruginosa* and *Staphylococcus aureus* isolates and the establishment of 48 h biofilm in the model is reliable and, when monitored by viable cell count, produces consistent bacterial loads (**Figures 1** and **2**). Images of tissue-associated biofilms of *Pseudomonas aeruginosa* and *Staphylococcus aureus* grown in EVPL may be found, along with protocols for preparation for light microscopy and histological staining, in our publications[21,23]. However, the reproducibility of CFU counts varies for different bacterial species. This can be quantified using standard repeatability calculations after ANOVA [25]; we have found that there is typically greater variation between CFU in replicate lung samples for *S. aureus* than for *P. aeruginosa*. We recommend that, on adoption of the model by a laboratory, repeat calculations are conducted on pilot experiments to optimize experimental techniques and to determine samples sizes to be used in final experiments (an example of this may be found in the data supplement for Sweeney et al[26]).

When grown in the EVPL, biofilms of *P. aeruginosa* and *S. aureus* demonstrate increased tolerance to antibiotics compared to susceptibility in standard, industry approved broth MIC (**Figure 1**) and disc assays using standard media (**Figure 2**). The various effects of different antibiotics on EVPL established biofilm are distinguishable, for example *P. aeruginosa* killing is achieved in EVPL with 4-16X MIC ciprofloxacin but not with 4-8X MIC chloramphenicol (**Figure 1**). A twice daily dose of 600 mg linezolid achieves a serum concentration above the MIC_{90} for susceptible pathogens (4 µg/mL)[27] and is regarded as adequate exposure without adverse side effects[28]. Data presented in **Figure 2** shows that *S. aureus* populations, susceptible to linezolid

in the disc assay, are able to survive target serum concentrations, and higher (12 µg/mL), in EVPL. There is no clear correlation between MIC and antibiotic effects on EVPL-grown biofilms for *P. aeruginosa* (**Figure 1**). Gaining a more accurate measure of in vivo antibiotic tolerance is important because sub-optimal dosing of antibiotics could increase the risk of selection for resistance in chronic infection.

It is well known that the biofilm mode of growth can significantly reduce bacterial susceptibility to antibiotics. This has led to the development of many in vitro biofilm assays and the use of minimum biofilm eradication concentration (MBEC)[14,15] instead of MIC as a more accurate predictor of susceptibility in chronic infection. The use of SCFM (in varying formulations) has also been recommended for use in MIC or MBEC testing[29]. Here we show that 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 calculated from standard in vitro assays, even when SCFM is used for these assays (**Figure 3**). This is consistent with a Cochrane review that reported that current implementations of in vitro biofilm susceptibility testing do not provide any increased predictive power for antibiotic prescribing in CF compared to standard susceptibility testing[16].

It is also simple to use the model to assess the impact of antibiotics on biofilm bacteria over time, as sufficient replica pieces of lung can be inoculated to allow destructive sampling. In addition to distinguishing differences between antimicrobial agents, the model can highlight changes in susceptibility at different bacterial growth stages or age of biofilm and for different antibiotic dosing intervals. **Figure 4** illustrates the increasing tolerance of *P. aeruginosa* biofilms to meropenem as they mature. This could be useful to determine the efficacy of novel agents, for example whether they are more effective during rapid cell division. It may also be an important consideration when setting the constraints of an experiment, as it may be necessary to standardize and validate biofilm age to avoid the age having an influence on results.

In **Figure 5**, *S. aureus* survival was measured at 4 h and 24 h post exposure to flucloxacillin and it was possible to observe differences in the reduction for bacterial cell counts across time and between isolates. This may be useful for drug development, for instance when defining pharmacokinetic and pharmacodynamic parameters or when elucidating the mode of action of a novel agent.

Variations in bacterial load often increase with extended culture times. This can be seen in the untreated control in **Figure 5** following 48 h biofilm development and a further 24 h exposure to account for antibiotic dosing interval. Variation is intrinsic to the model; each lung sample is independent from others and reflects the natural variation of lungs. It is, therefore, important to ensure that a sufficient number of replicates is included to allow for validation and an accurate interpretation of results. We refer the reader back to our recommendation to conduct repeat calculations on the data to enable the selection of robust sample sizes.

For simplicity, we have presented representative data taken from replicate tissue sections acquired from a single pair of lungs in each experiment, but in practice it is necessary to perform repeat experiments on tissue sections taken from replicate animals. This should be done in order to account for any biological variation between individual pigs, and we refer the reader to our published work for examples of how consistent the results can be between tissues taken from replicate pigs and how this variation is accounted for in statistical analysis of data using analysis of variance (ANOVA)/general linear models (GLM)[21,26].

FIGURE AND TABLE LEGENDS:

Figure 1. Total CFU of 11 CF *Pseudomonas aeruginosa* clinical isolates recovered from the EVPL model following treatment with antibiotics. Representative results of antibiotic treatment of *P. aeruginosa* in the EVPL model. Each strain was grown on EVPL tissue for 48 h then transferred to antibiotic (triangles) or PBS as a control (circles) for 18 h and the CFU/lung determined. The MIC for the appropriate antibiotic determined in standard cation-adjusted MHB is shown in brackets next to each strain (x-axis). The strains are ordered by increasing MIC values. Data were analyzed using t-tests when appropriate and Mann-Whitney U tests for non-parametric datasets. Significant differences between antibiotic treated and untreated tissues are denoted by asterisks ($P < 0.05$). **A.** Recovered viable counts from *P. aeruginosa* biofilms grown in the EVPL model and treated with 64 µg/mL chloramphenicol (highest MIC value recorded). For each isolate, the standardized mean difference in CFU between chloramphenicol-treated and untreated tissue sections was calculated using Cohen's *d.* There was no correlation between MIC value in the standard test and the decrease in viable cell numbers in the EVPL model as measured by Cohen's *d* (Spearman's rank correlation, $r_s = 0.45$, $p = 0.16$) **B.** Results of *P. aeruginosa* biofilms grown in the EVPL model and treated with 64 µg/mL ciprofloxacin (highest MIC value recorded). Values below the dashed line were below the limit of detection.

Figure 2. Total CFU of 8 *Staphylococcus aureus* CF clinical isolates recovered from the EVPL model following treatment with linezolid. Each strain was grown on EVPL tissue for 48 h then transferred to linezolid (triangles) for 24 h or were untreated as a control (circles). All strains were found to be sensitive to linezolid using the standard disk diffusion assay following EUCAST guidelines[30] (zone of inhibition > 21 mm). Data were analyzed using t-tests when appropriate and Mann-Whitney U tests for non-parametric datasets ($P < 0.05$). No significant differences between antibiotic treated and untreated were found for any of the strains. Values below the dashed line were below the limit of detection. **A.** Results of *S. aureus* biofilms in the EVPL model treated with 4 µg/mL linezolid (clinical breakpoint for sensitive/resistant according to EUCAST classification[31]). **B.** Results of *S. aureus* biofilms in the EVPL model treated with 12 µg/mL linezolid (data reproduced from Sweeney et al[23]).

Figure 3. Viable *Pseudomonas aeruginosa* cell counts of the laboratory strain PA14 and 4 CF clinical isolates recovered from the EVPL model following treatment with increasing concentrations of colistin. Each strain was grown on EVPL tissue for 48 h then exposed to colistin for 18 h. The MIC determined in standard cation-adjusted MHB medium is shown in brackets next to each strain name. The vertical lines show the MBEC value determined in MHB

(solid) and SCFM (dashed), with the exception of SED6, in which the value was the same in both media. The unfilled data points represent the lowest concentration of colistin tested that resulted in ≥ 3-$\log_{10}$ reduction in CFU/lung compared to the untreated samples (0 μg/mL colistin) (data reproduced from Sweeney et al[26]).

Figure 4. Representative viable *Pseudomonas aeruginosa* cell counts from a time course of growth on the EVPL model over 24 h, and subsequent treatment with 64 μg/mL meropenem. The laboratory strain *P. aeruginosa* PA14 and 3 CF clinical isolates were grown on EVPL tissue for the time shown on the x-axis, then transferred to meropenem (triangles) for 24 h or left untreated as a control (circles). The CFU/lung was then determined. The MIC determined in cation-adjusted MHB medium is shown in brackets next to each strain name.

Figure 5. Representative viable *Staphylococcus aureus* cell counts following growth on the EVPL model then treated with 5 μg/mL flucloxacillin over a 24 h time course. The control strain ATCC29213 and two CF clinical isolates were grown on EVPL tissue for 48 h then transferred to flucloxacillin (triangles) or left untreated as a control (circles) for 4 h and 24 h, before CFU/lung was determined (data reproduced from Sweeney et al[23]).

DISCUSSION:

The ex vivo lung model is high throughput and inexpensive and, because it uses post-consumer waste from the meat industry, it presents no ethical concerns. It is designed to mimic chronically-infected human CF airways better than currently available, in vitro AST platforms. Results presented here show that it may more accurately predict antibiotic susceptibility under these circumstances.

Critical steps in the protocol, that will ensure, reliable and reproducible results include the following.

Use consistent time and storage methods between slaughter, collection, and processing of lung samples. It is important to use lungs as soon as possible after slaughter and to keep the potential for contamination to a minimum. Differences in the ability of experimental cultures to grow in lungs if they are not as fresh as possible have been observed.

Maintain sterility in the production of SCFM and dissection of lung pieces is essential. Healthy lungs are not sterile and so the presence of commensal bacteria may reflect a ‘natural’ environment for chronic infection. Nevertheless, as previously noted, bacterial interactions within multispecies populations may alter results and susceptibility to antibiotics, so contamination should be avoided and lungs should be sterilized before use. We advocate the use of UV sterilization, as it does not appear to cause changes tin tissue integrity and, if necessary, additional antibiotic washes. However, antibiotics should be used with caution, as they may influence results by introducing selective pressures and may alter gene expression in test bacterial populations.

Use mock-infected, negative control tissue samples and cell count plates grown on a non-selective, rich medium to highlight the growth of any contaminant or commensal bacteria that have not been removed during sterilization is essential to mitigate for any impact of these bacteria on AST. It is also helpful to produce duplicate, selective agar, cell count plates specific to the organism of interest, as duplicate plates speed up colony identification and cell enumeration.

Conduct pilot experiments when first using the model and when using it with new strains or genotype of bacteria to assess biofilm CFU variations between tissue sections, allowing the selection of optimal experimental sample sizes (e.g., how many replicate tissue sections to acquire from how many replicate lungs) through the use of power calculations.

The assay uses a non-standardized inoculum, as this allows rapid inoculation after 48 hours of incubation and the formation of relatively consistent biofilms loads (especially for *P. aeruginosa*). To assay antibacterial efficacy in early biofilm growth stages, consider inoculating with a standardized CFU of colony-grown bacteria suspended in ASM. We do not recommend inoculating with planktonic bacteria: early pilot experiments showed that this leads to acute, invasive growth not reliable biofilm formation.

This protocol produces a robust prototype model for use with *P. aeruginosa*, with a great potential for development for use with *S. aureus*, but it does have some limitations that will need to be addressed for certain applications in the future. Tissue was inoculated from single colonies to allow the development of clonal populations. The results show that, for *P. aeruginosa*, this has little impact on cell numbers at 48 h. However, greater variability in bacterial load was observed for *S. aureus* and, given that different bacteria may grow differently within the model, a standardized starting inoculum and rigorous production of tissue samples of identical size and weight may be dependent on the organism of study. There may also be differences between labs due to differences in precise dissection/infection techniques or local pig breed/landrace. To assess the reproducibility of bacterial populations for individual implementations of the model, we suggest the use of repeatability calculations as part of the statistical analysis of results[25] and the use of repeatability/power calculations based on pilot experiments to calculate the optimal sample size for their use in final experiments.

One of the key advantages of EVPL over traditional plate assays is that, rather than testing for bacteria growing planktonically or on abiotic surfaces, it allows for the spatial structuring of bacterial biofilms within a host environment and with cell differentiation. This has important implications for considering the impact of physiochemical and nutrient gradients on the activity of antimicrobial agents as well as the delivery and availability of active therapies at different microenvironments within a chronic infection and cell-cell interaction between bacteria. This latter point is particularly significant, as multispecies infections are routinely observed in CF and are becoming increasingly important to infections associated with other respiratory conditions, such as asthma and chronic obstructive pulmonary disease. There is potential to develop this model for the AST for individualized patient sputum sampling in the clinical diagnostics. An analogous trial is already underway using a wound-mimicking in vitro model for growth and AST

of debrided biofilm from chronic wounds (Southwest Regional Wound Care Center in Lubbock, Texas, Dr. R. Wolcott).

Furthermore, the model uses post-mortem tissue, so the influence of the host immune response on antibiotic susceptibility is limited. Current in vitro models also do not account for host immune responses, so we do not see this as a barrier to the future use of the model in AST applications. However, the immune response is taken into consideration when pharmacokinetic and pharmacodynamic parameters and antibiotic dosing guidelines are determined. Although our studies have shown evidence of residual immune cells and responses within the tissue[23] (and S. Azimi, personal communication), this is a prime area for further optimization and development of the model if a greater match to in vivo conditions is desired.

Providing more clinically valid AST for CF will help meet a key recommendation of the UK Health, Social Care Act 2008 that "procedures should be in place to ensure prudent prescribing and antimicrobial stewardship." We believe the EVPL is an ideal candidate model to help meet this need.

ACKNOWLEDGMENTS:

We thank all of our co-authors on the original papers from which we have taken exemplary results. The work was funded by an MRC New Investigator Research Grant (grant number MR/R001898/1) awarded to FH; by PhD studentships from the BBSRC Midlands Integrative Biosciences Training Partnership (MIBTP) awarded to NEH and IA; and by the University of Warwick Undergraduate Research Support Scheme's award to FA to conduct a summer vacation research project. We thank Steve Quigley, Sons (Cubbington, Warwickshire) and John Taylor, Son (Earlsden, Coventry) for supplying lungs. We would also like to acknowledge the help of the Media Preparation Facility in the School of Life Sciences at the University of Warwick, with special thanks to Cerith Harries and Caroline Stewart, and the help of Anita Catherwood at Warwick Antimicrobial Screening Facility.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

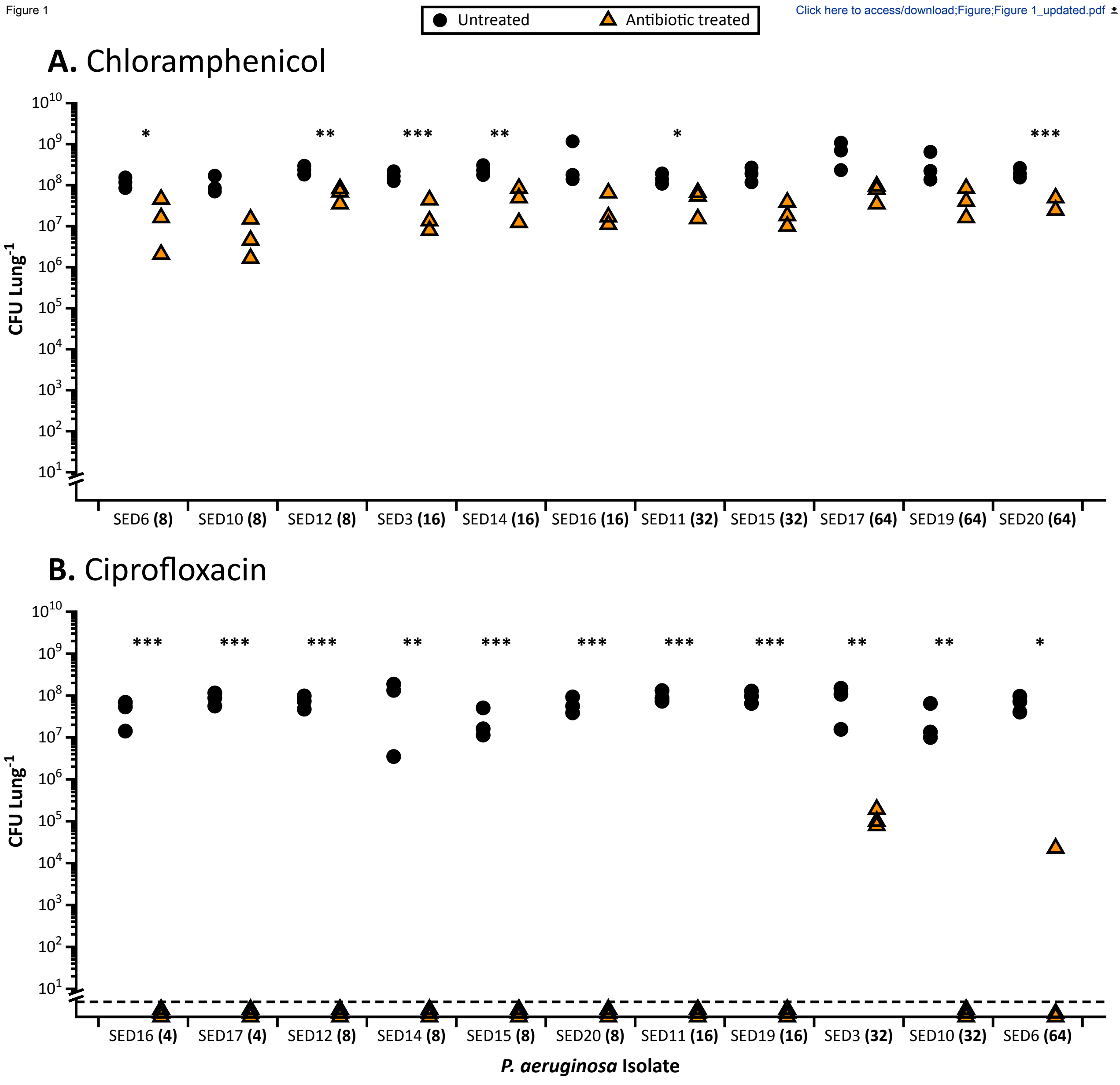


Figure 2

Click here to access/download;Figure;Figure 2_updated.pdf

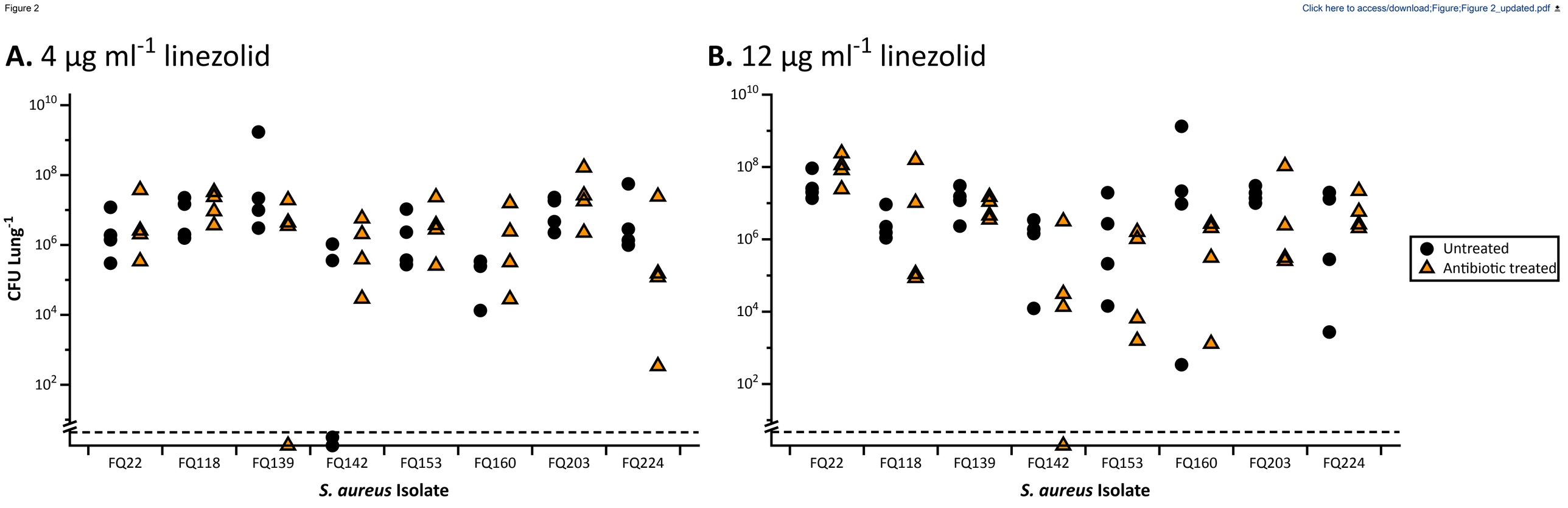


Figure 3

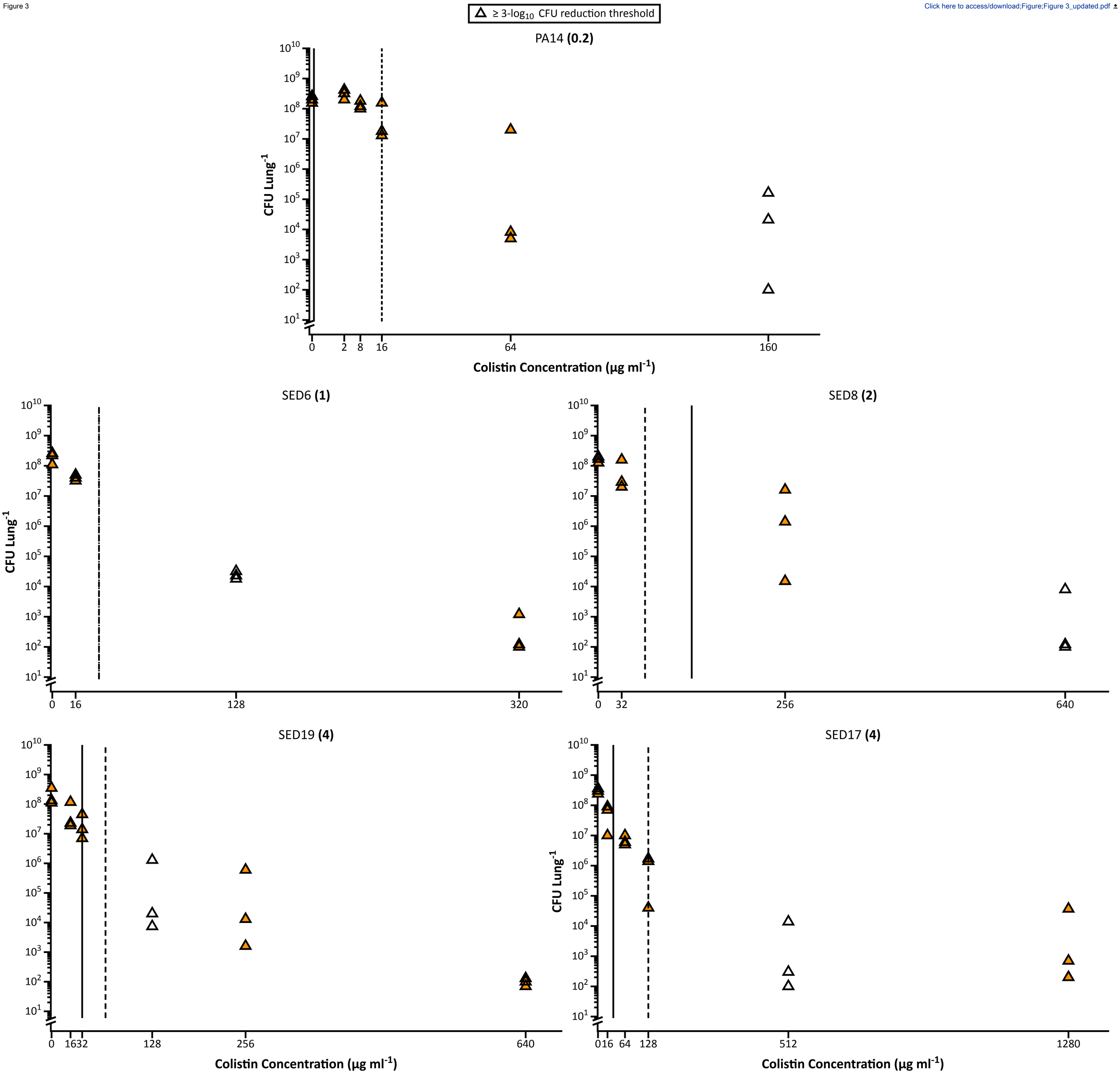


Figure 4

Click here to access/download;Figure;Figure 4.pdf

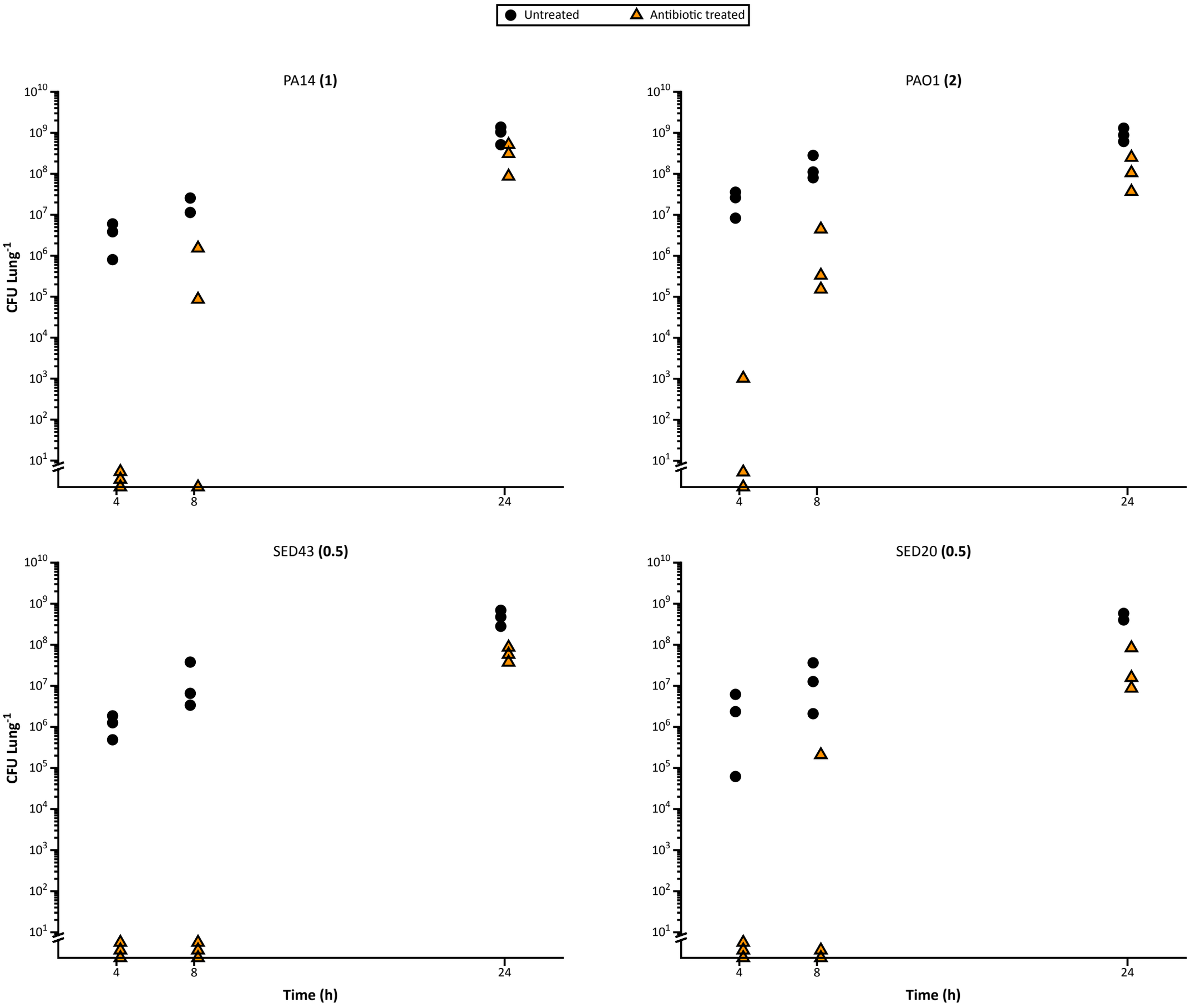
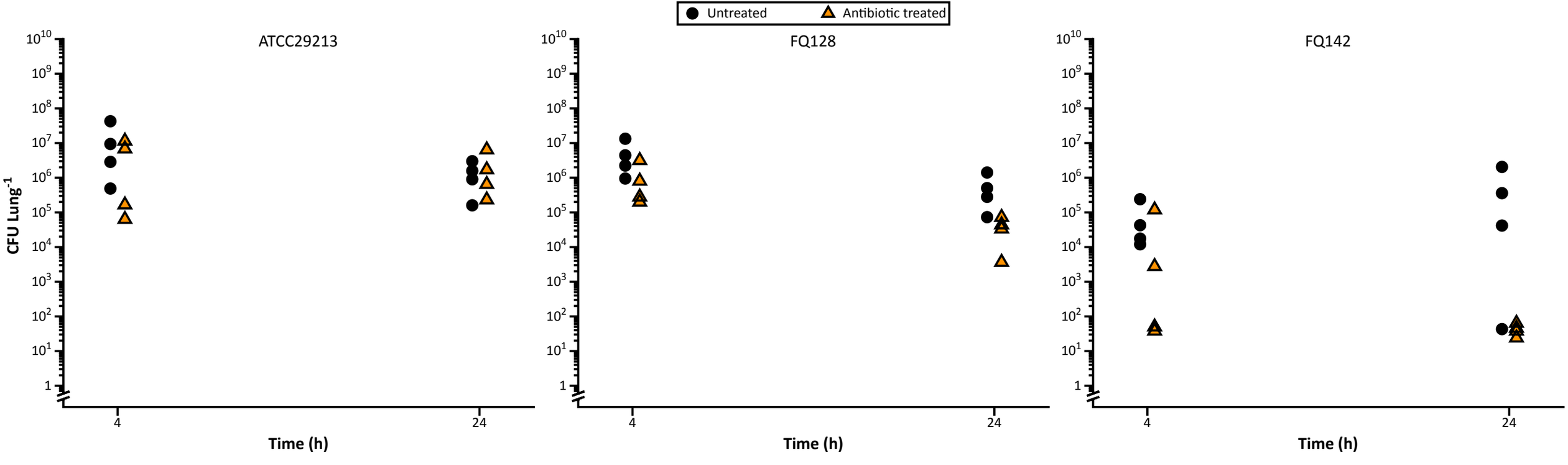


Figure 5

Click here to access/download;Figure;Figure 5.pdf



Name of Material/Equipment	Recommended supplier	Catalog Number	Comments/Description
0.5 mL insulin syringes with 29G needle attached			
24-well culture plates			
70% ethanol or similar for surface sterilizaton and flamin gof dissection equipment			
Agar plates to prepare streaks of P. aeruginosa/S. aureus (any suitable medium)			
Agarose			
Aluminum foil - pre-sterilised by autoclaving - to cover the chopping board on whcih you wil dissect lungs.			
Bead beater designed to take 2 mL tubes	MP Biomedicals	116004500	FastPrep-24 Classic bead beating grinder and lysis system
Breathe-easy or Breathe-easier sealing membrane for multiwell plates	Diversified Biotech	BEM-1 or BERM-2000	
Bunsen burner			
Chopping board - we recommend a plastic board to allow for easy decontamination with alcohol.			
Coolbox to transport lungs to lab			
Dissection scissors in different sizes			
Dulbecco's modified Eagle medium (DMEM)			
Fisherbrand 2 mL reinforced tubes	Thermo Fisher	15545809	
Fisherbrand 2.38 mm metal beads	Thermo Fisher	15505809	
Germicidal UV cabinet			
Insulin syringes - 0.5 mL with 29G needle attached.	VWR	BDAM324892	
Large pallet knife			
LB agar plates to assess CFU in lung biofilm homogenate			
Mounted razor blades			
Nalgene RapidFlow PES 75 mm x 0.1 µm x 500 ml sterile filter unit	Thermo Fisher	10474415	For filter-sterilizing SCFM
Petri dishes			
Phosphate-buffered saline			

Plastic chopping board and aluminium foil to create a sterile and cleanable dissection surface

Roswell Park Memorial Institute (RPMI) 1640 medium

SCFM ingredients as listed in Table S1

Selection of forceps (blunt tips recommended)

Selective agar plates to specifically assess P. aeruginosa / S. aureus CFU in lung biofilm homogenate, if required.

Suitable containers for disposing of contaminated sharps and pig ung tissue, according to your institution's health & safety policies.

We thank the editor and the reviewers for their comments, which have enabled us to significantly clarify the manuscript. Most notably, we have significantly edited the protocol and included a schematic of the assay. Please find a point-by-point response to comments below, along with a version of the edited manuscript with tracked changes uploaded as a supplementary file for your reference.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use. Please use American English.

Done.

2. Please shorten your title to meet the 150-character limit; consider "Antibiotic Efficacy in an Ex Vivo Model of Psueomonas aeruginosa and Staphylococcus aureus Biofilms In the Cystic Fibrosis Lung".

Title changed to "Antibiotic efficacy testing in an ex vivo model of Pseudomonas aeruginosa and Staphylococcus aureus biofilms in the cystic fibrosis lung."

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language and product recommendations from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Fisherbrand (catalog no. XYZ)

Done.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc. For example, text in lines 132-134 can be converted to a note.

Checked and changes made where necessary.

5. Although you have mentioned that you obtain the tissue for the experiments, please include an ethics statement before all of the numbered protocol steps indicating that the protocol had the approval of the appropriate review board in your institution. Alternatively, move the statement in lines 464-465 to the beginning of the protocol to explain why you do not need approval.

Ethics statement added: "This protocol uses pig lungs sourced from a commercial abattoir which supplies meat for human consumption. Under UK legislation, using leftover tissue from animals slaughtered for meat does not require any ethical approval, but we advise readers to check relevant local laws and institutional guidelines before starting work."

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We are not sure how to address this and would appreciate specific feedback on which steps are not sufficiently detailed.

7. Please consider adding all the information about stock preparation to a table that can be cited in the protocol.

Done – this is now Table S1.

8. 2.1: please cite a reference to instruct readers as to the percentage of agar to be used to prepare the agar plates. If steps 2.4 to 2.7 describe the preparation of the agar plates mentioned in 2.1, please re-number these steps as 2.1.1 etc and move them up to follow 2.1 so that the reader understands that these are the steps to be followed.

Steps 2.4-2.7 refer to agarose pads in the 24-well plates, not agar plates. Suggestion of LB+1.2% agar added to step 2.1.

9. 2.31: what is the "breathable membrane"? Please ensure that this is in the Table of Materials.

Now reads "breathable sealing membrane", exact item with catalogue number is given in the table of materials.

10. 3.2: How are you determining CFU? OD? Please specify or cite a reference.

CFU and OD are different things, this does not need further explanation or reference for microbiologists and will read very oddly if added to the paper.

11. Please highlight only up to 3 pages of protocol text for inclusion in the protocol section of the video. You can remove the highlighting from notes to stick to the 3-page limit.

Reduced.

12. Please sort the Materials Table alphabetically by the name of the material.

Done.

Reviewer #1:

Manuscript Summary:

A much needed methods paper focusing on making biofilm antibiotic susceptibility testing clinically relevant.

Major Concerns:

1. While the abstract is well written, the introduction could be reworked highlight the antibiotic tolerance aspects of biofilms more, and less on the physicochemical lung microenvironment.

We would note that this method is scheduled to be part of a special issue on biofilm methods, so we want to avoid the risk of several papers repeating similar introductory reviews of biofilm tolerance. Given the topic of the special issue, we argue that it is more useful to focus on the specific application of this model to CF.

2. Lines 99-100: 'follow their instincts for prescribing' - this can be reworded to reflect the lack of uniform guidelines and case-by-case decisions. Therefore, the need for more relevant biofilm antibiotic susceptibility testing methods.

This sounds much better, we have reworded: "… many clinicians simply ignore diagnostic lab AST as there are no uniform, CF-specific guidelines for interpreting these results, and they instead make case-by-case decisions for prescribing."

3. Lines 132-133: The Palmer, Whiteley medium is well know, but a few lines can be added on how this medium composition was devised and formulated. Why is this clinically relevant?

We have added the following: "Palmer et al's recipe contains free amino acids, cations, anions and lactate at concentrations representative of the average concentrations found in a selection of sputum samples from people with CF; it has been shown to cue comparable carbon-usage pathways and expression of quorum sensing signals by P. aeruginosa PA14 to growth in medium made from lyophilised patient sputum[24]."

4. Lines 227-228: How are the tissue bits transported? Inclusion/Exclusion criteria? What does 'ideally on same day' mean' - is there a max number of days permissible? This is important as in many settings abattoir samples may not be easily available on the day itself and transportation conditions would be important.

We have modified this step and we hope it is both clearer and a little more flexible: "Obtain lungs from source as soon as possible after slaughter, ensuring they are kept cold by transporting to the laboratory in a domestic coolbox. (Note: we have found that lungs closer to the day of slaughter show less bruising from storage, but we routinely work with tissue kept on cold storage for 4 days from slaughter. We remind the reader that as the coolbox needs to be taken into a butcher's shop or abattoir, it must be decontaminated following local lab guidelines after each use, and stored outside the microbiology lab when not in use, to reduce the risk of contamination and a breach of containment).

5. Figure 1 – Other than Figure 1A, I do not see asterisks denoting significance in Figure 1B. Not sure why this is so.

We have rectified this, and also added a dotted line to signify detection threshold (note also added to legends).

6. Figure 2 - What are the MICs using standard microdilution and disc diffusion for SA? How were concentrations for 4 and 12 ug/mL linezolid chosen?

Apologies, this wasn't clear and the legend for Fig 2 has now been edited to state "All strains were found to be sensitive to linezolid using the standard disk diffusion assay following EUCAST guidelines (zone of inhibition > 21 mm)" and that 4 and 12µg/ml linezolid are 1x and 3x the clinical breakpoint for sensitive/resistant according to EUCAST classification. Citations for EUCAST disk diffusion manual and clinical breakpoints table have been added.

7. I would also recommend a comprehensive schematic representing the assay and its steps in detail.

We think this is a great idea and we have prepared a schematic – new Figure S1 (could be used as a main figure if there is space?)

Reviewer #2:

Bacteria growing in biofilm form possess the phenotypic features usually different from their planktonic counterparts, including enhanced tolerance to antibiotics and immune defense mechanisms, which determines chronicity and recurrence of biofilm-associated infections (BAI). Thus, standard methods for susceptibility testing based on microbial suspensions are worth little in preparing the scheme of BAI treatment. In this context, the manuscript and described method are really interesting and can be a valuable tip for the scientists to assess antibiotic effect properly before the treatment of pulmonary infections in cystic fibrosis patients. However, I have some comments / questions, which need to be addressed before the acceptance of the text to be published in Journal of Visualized Experiments.

Major concerns:

1. Protocol (2): Before description the methodical steps a clear remark regarding the source of porcine tissue should be included to avoid the question about an approval from the relevant animal testing ethics committee. Complete the protocol.

We have added an ethics statement just before the protocol.

2. Protocol (2.8): Why the washes prepared for lung contain both cell culture media: DMEM and RPMI?

In all honesty, a veterinary sciences colleague suggested this and the tissue prepared this way looked healthy so we decided to stick with it and not risk changing anything.

3. Protocol (2.28): Infection step of bronchiole pieces is totally imprecise. How can you expect, that the number of bacteria will be similar after touch a colony and then tissue with syringe. Later, the bacteria will develop different in size biofilm, which will affect not only its antibiotic susceptibility, but first of all CFU number (bacteria recovered from tissue pieces). In my opinion this step of

procedure needs to be corrected to start biofilm formation from the same bacteria number. Of course the description of this step should be corrected general for the procedure (e.g. as a note), not for the results already obtained.

It is imprecise, but the only way to standardise numbers would be to suspend the cells, and inoculating them directly to the tissue surface becomes tricky. When we first started working with the model, we tried using planktonic cells standardised to particular CFU values but we found that planktonic cells produce acute / invasive pathology in the model and not a chronic biofilm state. We argue that the way to deal with this is to use repeatability and power calculations to determine optimal sample size and we have further stressed this in the Discussion, lines 765-777 now end: "To assess the reproducibility of bacterial populations for individual implementations of the model we suggest the use of repeatability calculations as part of the statistical analysis of results, and the use of repeatability / power calculations based on pilot experiments to calculate the optimal sample size for use in final experiments." We have added the following to the "critical steps…" section of the discussion as well: "The assay as presented uses a non-standardised inoculum, as this allows for rapid inoculation and after 48 hours incubation, relatively consistent biofilms loads are formed (especially for P. aeruginosa). If you wish to assay antibacterial efficacy in early biofilm growth stages, you may wish to consider inoculating with a standardized CFU of colony-grown bacteria suspended in ASM. We do not recommend inoculating with planktonic bacteria: early pilot experiments showed that this leads to acute, invasive growth not reliable biofilm."

4. Discussion (line 526-529): The use of prepared animal tissues limit an application of this method only to specialized scientific laboratories, ordinary diagnostic labs and even some research units have neither the possibility nor the time to carry out such procedures. So, the last paragraph should be more balanced (where this method can be really useful).

We respectfully disagree, we have carried out all of our work on this model in generic microbiology labs – there is no need for any equipment or facility beyond a laboratory equipped to work with Hazard Group 2 pathogens (the category to which P. aeruginosa and S. aureus belong). We have also transferred the model to our industrial partner, Perfectus Biomed Ltd., who have gained accreditation to ISO 17025 for use of the model in a microbiological testing lab. We have, however, removed the phrase "diagnostic labs" from the final paragraph of the article to avoid confusion.

Minor concerns:

1. Protocol (1.14-1.18): Are the CaCl2 and MgCl2 stocks, as well as next L-lactic and Fe(III)SO4x7H2O added to SCFM after AA stocks and pH adjustment? If YES, I propose precise: Add … to the SCFM from step 1.12

In response to the editor's suggestions, we have converted section 1 of the protocol to a table (Table S1), and we hope the order of steps is now clear.

2. Protocol (2.16): For other bronchiole steps 2.11-2.14 (not 1.11-1.14) should be repeated.
3. Protocol (2.17): Correct the number of wash step

Thanks, these have been amended.

4. Protocol (3.3): Clarify the description since this step will be used for both: uninfected tissue (control) and infected (tested material).

We have significantly re-written the protocol to aid clarity, and also provided a schematic diagram, we hope this is now clearer.

5. Line 339: "The EVPL model provides a simple … assay platform" - The method is not simple. Correct the sentence.

We have removed "simple." To give an idea of the level of tractability with practice, we have added "With practice, we have found that up to c.200 bronchiolar tissue sections can be prepared from lungs in 2 hours. The entire experiment for AST can be completed within normal working hours."

Reviewer #3:
The authors present an ex vivo lung model for antibiotic testing of bacterial isolates. Such models are highly needed, as also emphasized by the authors. I'm confident that models like this will leverage the field and redirect biofilm research towards using more clinically relevant experimental models and thereby provide more accurate antibiotic testing. The manuscript is well written and the is methodology well-presented and explained. I only have minor comments to the manuscript.

Minor Concerns:

1) L 77-85: The text is here very condensed and hard to read because of the repeated use of brackets. I suggest rephrasing to avoid the brackets. Moreover, I would prefer a clearer distinction between colony variants that are most often caused by adaptive tracking (genetic mutations) and persisters, which arise stochastically and are regulated epigenetically.

This paragraph now reads: "The specific physicochemical conditions in a particular biofilm infection context can therefore influence responses to antibiotics. First, the structure and depth of the extracellular matrix depends on local environmental conditions, such as nutrients or shear forces. Second, environmental cues can trigger expression of specific antibiotic resistance genes. For example, the CF pathogen Pseudomonas aeruginosa shows increased expression of a beta-lactamase and reduced expression of porins in CF sputum versus in vitro[9], while another CF pathogen, Burkholderia cenocepacia, upregulates beta-lactamases and efflux pumps when grown in CF sputum[10]. Third, in-host conditions can cue a physiological or genetic switch to antibiotic-tolerant phenotypes which are hard to recapitulate in vitro; examples include small colony variants of the CF pathogen Staphylococcus aureus[11,12]."

2) L 118: Many abbreviations are used throughout the text. Here EVPL is introduced without any explanation.

Thanks for spotting this, this and other omissions have been amended.

3) L 126-127: Some equipment, e.g. the UV cabinet may not be standard in all laboratories. Here you could refer to the equipment list provided to list these upfront.

We have extended the table of materials to make it more comprehensive, and the relevant sentence now reads "Most equipment used (see Table of Materials) may readily be found in a typical microbiology laboratory, although a bead beater is essential, and we have found from work with collaborators that a suitable ultraviolet germicidal cabinet may need to be procured."

4) L 220: Is this per intact lung?

Yes, this is now clarified.

5) L 281-282: It is unclear to me, why an insulin syringe should be used for inoculation and what does "touch" imply?

This step now reads: "To infect each tissue piece with the desired strain, touch a colony grown on an agar plate with the tip of a 29 G needle attached to a sterile 0.5 ml insulin syringe. Touch the colony onto the tissue piece, gently pricking the tissue surface. (Note: using an insulin syringe supplied with a 29 G needle attached allows the experimenter to hold the needle accurately and comfortably while keeping their fingers a safe distance from both needle and lung tissue. It is possible to perform this step using 29 G needles that are not attached to a syringe, but this requires greater dexterity and increases the risk of a needlestick injury. Insulin syringes are readily available)."

6) L 289: "Breathable membrane" may need specification. You could name the one you used.

Details are in the table of materials, we have been asked by the editor not to include manufacturer or brand names in the manuscript.

7) L 309-312: Did you test that the bacterial cells did not lyse upon homogenisation?

The following note has been added to the bead beating step of the protocol: "we have found that bead beating with the specific beads and homogeniser suggested in the Table of Materials does not cause significant lysis of bacteria, but we recommend that each lab using the protocol checks the effect of their chosen beads and homogeniser prior to commencing AST assays."

8) L 319-320: I assume that controls without antibiotic addition (but still containing SCFM) should also be included? This may need specification here. And maybe also specify that "500 ul antibiotics" refers to the antibiotic prepared in SCMF just above?

We have significantly re-written the protocol to aid clarity, and also provided a schematic diagram, we hope this is now clearer.

9) L 470: I would think another critical step is reproducibility across labs, countries etc. The tissue may also differ due to pig race, life history etc? Or is this only minor to other sources of variation?

We have added a point 4 to the list: "The conducting of pilot experiments by each lab when first using the model, and when using it with new strains or genotype of bacteria, in order to assess variability in biofilm CFU between tissue sections, and thus decide optimal experimental sample size (how many replica tissue sections to cut, from how many replica lungs) through the use of power calculations." And further discussion in the following paragraph: "There may also be differences between labs due to differences in precise dissection/infection technique or local pig breed/landrace." See also response to Reviewer 2's point 3.

10) L 503: How compatible is the model to microscopy analysis - incl fluorescence microscopy? This is highly relevant due to the spatial structure discussed here.

The following sentence has been added to the first paragraph of the Representative Results: "Images of tissue-associated biofilms of Pseudomonas aeruginosa and Staphylococcus aureus grown in EVPL may be found, with protocols for preparation for light microscopy and histological staining, in our publications[21,23]."

11) Figures: Personally, I like more information in the figure plots. You could e.g. specify antibiotics used in each plot etc. In my opinion, this makes it easier to instantly interpret figures, but I guess that's my subjective opinion. Figure 3 is a bit hard to read - it looks like there are also black symbols, but that may just be an effect of many overlaying orange triangles. There is no symbol specification in the plot, I recommend adding that.

We have added labels of specific antibiotic/concentration to Figures 1 & 2 where there are different ones shown. We have added colistin to the x axis labels for Figure 3 as this is the only antibiotic used here. We have also added a key to Figure 3 to say that unfilled data is the log 3 threshold.

Reviewer #4:

Manuscript Summary: The manuscript describes a new ex vivo model with the potential for assessing the effcacy of antibiotics against bioiflm formed by P.aeruginosa and S.aureus. However, I think that the aim has not been fully satisfied by the results obtained.

Major Concerns:

EVPL model is clearly inadequate for S. aureus due to the very poor repeatibility of the assay that does not allow any conclusions or deductions concerning the efficacy of the tested drug. This is clearly evident from results graphed in Figures 2 and 5: variations accounted till to 10^5 differences among replicates, also in the case of control (untreated) samples. Further, this variability could be underestimated, as it can be deduced at Lines 402-405 after considering to use lungs from different animals. Therefore, I believe that the model presented is not solid, optimised, accurate and therefore not useful, at least in the case of S. aureus. The authors should highlighted these criticism and consequently change the title and the text to highlight that EVPL could be useful in

the case of P. aeruginosa. Along with that, they should also proposed some explanation to these findings.

We have explicitly addressed the variability of CFU load for S. aureus in the first paragraph of the Representative Results, and return to it in the Discussion. This is also discussed at length in the original research article referenced for the S. aureus work, where we talk about the inherent variability in S. aureus growth as in vivo aggregates not only in our model but in elsewhere in the literature. Nonetheless we have reiterated this by modifying the first sentence of the third paragraph of the Discussion, which now reads "The protocol presented here produces a robust prototype model for use with P. aeruginosa, with great potential for development for use with S. aureus". Even a cursory read of the literature on S. aureus growth in vivo and in different infection models reveals how little is known about the in vivo pathology of this species in CF, thus it would be surprising if any model were 100% accurate.

Lines 379-387: the possibility to assess the antibiofilm activity of drugs over time is not a feature of the EPVL model; other models are useful to this.

We agree, and we have not stated that this is unique feature of the model, simply that it is possible.

The authors referred at references #26 that, however, is currently submitted and, thereore, not avalialble to this reviewer.

Ref 26 has now been published and the reference has been updated to "in press, accepted for publication in Microbiology". Hopefully we will be assigned a AOP DOI or vol/page no before we need to return proofs of the present article. Please note that the original reference included a link to the open-access preprint, therefore the article was available to the reviewers of the present manuscript.

Figure 3 caption states that MIC were obtained using standard MHB. This is not correct, since international guidelines recommend to use cation-adjusted MHB.

We have now specified that the MHB was cation-adjusted.

Minor Concerns:

Line 354: MIC values for S. aureus were measured using "disc assay"; does it stand for disk diffusion agar ? This is not clear to me, please explain. If so, justify this technical choice.

Edited to specify that this was a disk diffusion assay following EUCAST guidelines and that all isolates were classed as sensitive by having a zone of inhibition > 21 mm; reference to EUCAST manual added.

TABLE S1

1. Preparation of Synthetic CF Sputum Media (SCFM) for use with *ex vivo* pig lung model

Modified from the recipe developed by Palmer *et al*.[24] Once made, the SCFM should be filter sterilized, and may then be stored at 4°C for up to one month.

a) Main recipe

Step 1	**Chemical**	**Amount**	**Instructions**	**Final mM in 1L SCFM**
	NaCl	3.03 g	Add salts and water to clean bottle, used only for preparation of SCFM	51.85
	KCl	1.114 g		14.94
	dH2O	640 ml		N/A
Step 2	**Chemical**	**Molarity of stock made in water, filter-sterilized and stored at 4°C**	**Instructions**	**Final mM in 1L SCFM**
	Na2HPO4	0.125 M	Add 10 ml of each stock to the salts and water prepared in step 1	1.25
	NaH2PO4	0.13 M		1.30
	NH4Cl	0.228 M		2.28
	KNO3	0.0348 M		0.35
	K2SO4	0.0271 M		0.27
	MOPS	1 M		10.00
Step 3	**Chemical**		**Instructions**	**Final mM in 1L SCFM**
	19 amino acids solutions prepared according to section b)		Add 10 ml of each stock to the solution prepared in steps 1 and 2.	See section b)
Step 4	**Chemical**		**Instructions**	**Final mM in 1L SCFM**
	HCl or NaOH as required		Use to adjust pH of solution prepared in steps 1-3 to 6.8. Record volume of acid/base added.	N/A
Step 5	**Chemical**		**Instructions**	**Final mM in 1L SCFM**
	dH2O		Add to solution prepared in steps 1-4, to a final volume of 960 ml	N/A
Step 6	**Chemical**	**Molarity of stock made in water, filter-sterilized and stored at 4°C**	**Instructions**	**Final mM in 1L SCFM**
	CaCl2	0.175 M	Add 10 ml of each stock to the solution prepared in steps 1-5	1.75
	MgCl2	0.0606 M		0.61
Step 7	**Chemical**	**Molarity of stock made in water, filter-sterilized and stored at 4°C**	**Instructions**	**Final mM in 1L SCFM**
	L-Lactic acid	0.93 M	Make stock in water, pH to 7 with 5M NaOH. Add 10 ml to the solution prepared in steps 1-6.	9.30
Step 8	**Chemical**	**Molarity of working stock made in water,**	**Instructions**	**Final mM in 1L SCFM**

		immediately before adding to the recipe		
	Fe(III)SO4.7H2O	0.00036 M	Make a 0.036 M master stock, which can be filter sterilized and stored at 4°C for as long as the solution remains free of precipitate. To make the working stock, add 110 µl of the master stock to 9.890 ml dH2O and add the working stock to the solution prepared in steps 1-7.	0.0036

b) Preparation of amino acid stocks; filter sterilize before use and store at 4°C.

Amino Acid	**mM stock**	**Instructions**	**Final mM in 1 L SCFM**
Alanine	178	Dissolve in water	1.780
Arginine	30.6	Dissolve in water	0.306
Aspartate	82.7	Dissolve in 0.5 M NaOH	0.827
Cysteine	16	Dissolve in water	0.160
Glutamic Acid	154.9	Dissolve in 1 M HCl	1.549
Glycine	120.3	Dissolve in water	1.203
Histidine	51.9	Dissolve in water	0.519
Isoleucine	112.1	Dissolve in water by heating to 50°C for 30 mins on a shaker	1.121
Leucine	160.9	Dissolve in water	1.609
Lysine	212.8	Dissolve in water	2.128
Methionine	63.3	Dissolve in water	0.633
Ornithine-HCl	67.6	Dissolve in water	0.676
Phenylalanine	53	Dissolve in water	0.530
Proline	166	Dissolve in water	1.660
Serine	144.6	Dissolve in water	1.446
Threonine	107.2	Dissolve in water	1.072
Tryptophan	1.3	Dissolve in 0.2 M NaOH	0.013
Tyrosine	80.2	Dissolve in 1M NaOH	0.802
Valine	111.7	Dissolve in water	1.117

Figure S1

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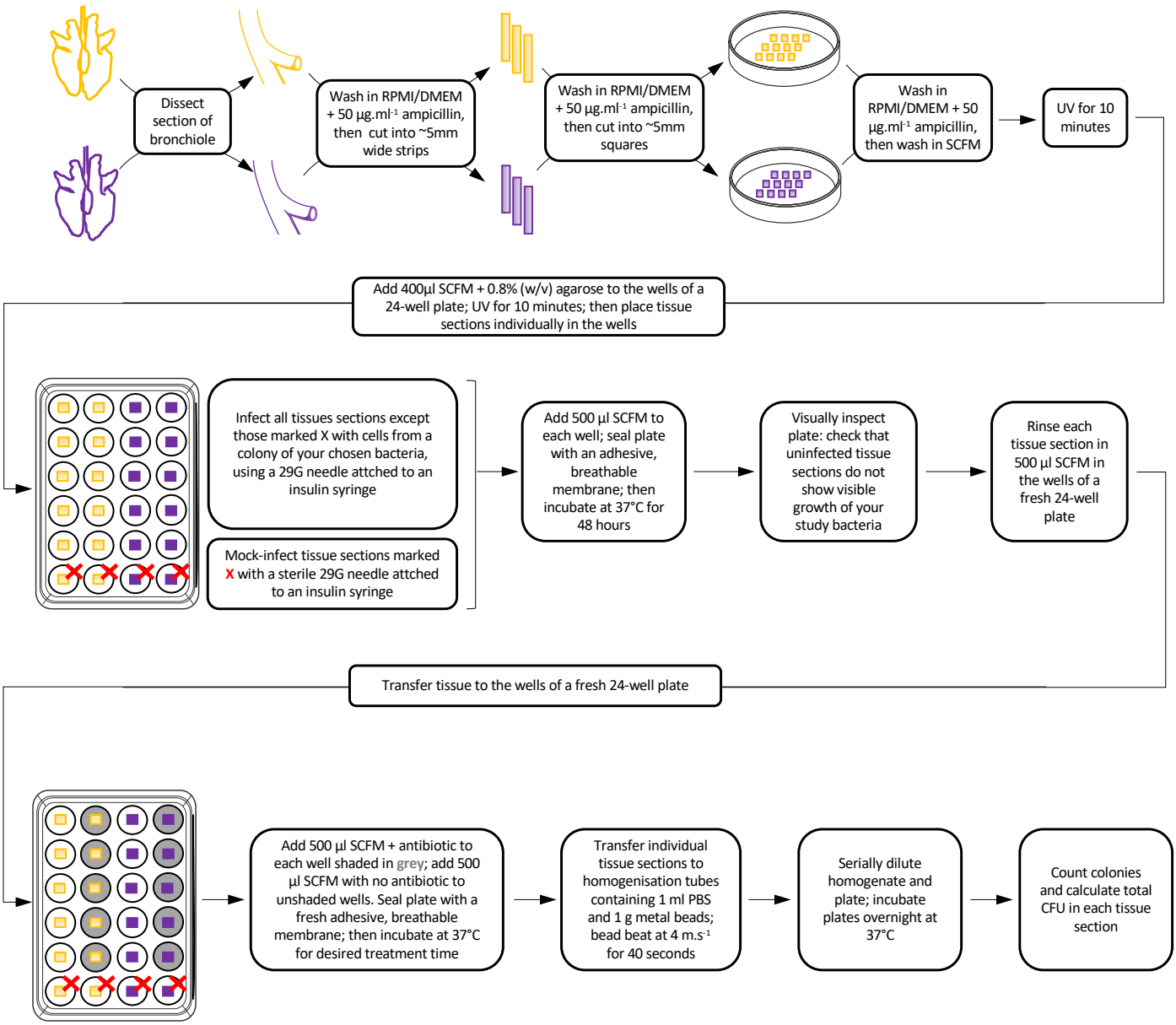

Fig. S1. Schematic of AST using the EVPL model. This shows an example experiment using tissue from two lungs (shown in orange and purple) to test the effect of one antibiotic concentration on one bacterial isolate. Each treatment (antibiotic or no antibiotic) is applied to five replica sections of tissue form each lung. Two tissue sections from each lung are used a uninfected controls, growth of the study bacterium in these sections acts as a warning of contamination during any step of the assay. Drawings of bronchioles, Petri dishes and 24-well plates are modified from Servier Medical Art under a CC-BY licence; see http://smart.servier.com.