# Methods.

1. **Sample collection.** 
   1. Collect several samples from sources where phages may be present.
   2. Two commercially available food sources (assuming target strains and associated phages will already be present).
      1. One veg sample (use a mixed salad bowl rather than a single veg, it has greater chances or use cut fruit bowls as chances of phages are higher in these).
      2. One meat sample (mince would be good)
   3. Two environmental samples (looking into environmental phage sources, with possible links to ecology and agriculture).
      1. Soil
      2. Pond water (farmland)
2. **Bacterial stock dilution. Day 1 if stocks can not be pre-prepared.** 
   1. Prepare pure culture stocks by resuspending pure culture colony in 5mL MH broth and allowing it to grow over night. Plates will need to be made up and cultured, incubated until colonies form, then an isolated colony will be inoculated into 5ml MH broth. Which in turn will be incubated overnight for use in the plaque assay.
3. **Isolation and concentration of phage stock from environmental samples.** 
   1. **Separation of phage stock. Day 1.** 
      1. In centrifuge tubes, add one parts of environmental sample and one part of 0.85% sterile buffer (PBS) 5 gram of meat, 5 gram of soil, 5 gram of salad/veg equally added to 5 mL of saline each.
      2. Mix or vortex it
      3. Centrifuge liquid samples at maximum RPM for 20mins. **(4 centrifuge tubes)**
      4. Put the supernatant in falcon tubes.
      5. Take another 5 gram of meat, 5 gram of soil, 5 gram of salad/veg samples and resuspend in 5 mL of supernatant obtained from previous steps. Repeat centrifugation to concentrate supernatant.
      6. For pond water directly use 5 mL of supernatant (concentration not required as phages do not need to be released from solid samples).
      7. Syringe filter the supernatant through a 0.22µm membrane into a sterile falcon/universal to filter out all bacteriophages. Repeat centrifugations and filtration for all the samples.
      8. Store the environmental filtrates in the fridge until needed.
4. **Double agar overlay plaque assay (day 1).** 
   1. Take 20 (falcon tubes) 50ml each and add 5 mL of molten 2x agar using pipette man. Keep these in water bath at 60oC.
   2. As negative controls, add 100ul of each bacterium and 500ul sterile saline to four respective tubes.
   3. To the 16 remaining tubes, add 100 uL of overnight bacterial culture, add 500 uL of environmental phage filtrate.
   4. Mix and plate on hard agar. Incubate overnight.
   5. So this will generate 16 plates
   6. Observe plates at regular intervals (24, 48 and 72hrs), record plaque morphology, density, and numbers.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Observation after 24 hours | | | | |
|  | *B. cereus* | *E. faecalis* | *P. aeruginosa* | *K. pneumoniae* |
| Veg |  |  |  |  |
| Meat |  |  |  |  |
| Soil |  |  |  |  |
| Pond |  |  |  |  |
| Negative control |  |  |  |  |
| Observation after 48 hours | | | | |
| Veg |  |  |  |  |
| Meat |  |  |  |  |
| Soil |  |  |  |  |
| Pond |  |  |  |  |
| Negative control |  |  |  |  |
| Observation after 72 hours | | | | |
| Veg |  |  |  |  |
| Meat |  |  |  |  |
| Soil |  |  |  |  |
| Pond |  |  |  |  |
| Negative control |  |  |  |  |

1. **Generation of phage lysates.**
   1. Plaque picking and incubation.
      1. For each plate, record the number of plaques formed and note their morphologies. Look for plates with densely packed individual plaques in a web-like pattern covering the whole plate, this indicates that several rounds of amplification (infection and lysis) have taken place. These are the plates from which plaques will be picked.
      2. Choose the best plaque (largest) for each of the bacteria from 4 sample sets and suspended in 20 mL MH broth in 50 ml Falcon tubes. Add also into these tubes 200 µL overnight culture of the corresponding bacteria.
      3. Incubate these tubes for 48 hours (over the weekend).
   2. Lysate filtration.
      1. After incubation, centrifuge each tube at maximum RPM for 20 mins.
      2. Syringe filter the supernatant through 0.22µm membrane into sterile falcon tubes.
      3. Addition of PBS to release Phages may be required prior to centrifugation.
   3. Lysate dilution (only if PFU/ml is too high)
      1. 10-5, 10-6, and 10-7 dilutions will be made up of the phage lysate and sterile MH broth.
      2. Plates will be made up for the undiluted lysate and the three dilutions, the one with the optimal plaque formation will be carried forward for use in following steps.
         1. Double agar overlay method will be repeated using 100 ul of each lysate dilution.
         2. This will be done overnight, the dilutions (or undiluted lysate) corresponding to the plates with optimum growth will be carried forward for OD readings.
2. **Optical density readings.**
3. Into a 20 ml falcon tube add 20 mL MH broth and 100 uL of one of the phage lysates, also into this tube add 200 uL overnight culture of the corresponding bacteria.
4. Repeat for all lysates and bacteria, resulting in four suspensions of MH broth, phage lysate and the corresponding bacteria.
5. Prepare 4 other 20 mL MH broth falcon tubes and add 200 µL of overnight culture.
6. These two series will be compared against each other, the phage curvettes are expected to decrease in OD while the bacteria curvettes are expected to increase in OD.
7. Measure the OD 600nm after 0, 2, 4, 6, 8hrs on day one (repeat the following day) by taking 1.5 mL culture from the tube for each reading.
8. Measure the OD 600nm by aliquoting 1.5ml of the phage culture or bacteria culture into a cuvette, measure the OD of each every two hours (9am-5pm) and repeat the next day, resulting in a total of 64 OD readings. Calibrate the spectrophotometer using a cuvette of sterile MH broth.

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Plaque suspension | OD at time point (9am – 5pm day one, 9am-5pm day two) | | | | | | | | |
| 0 hours | 2 | 4 | 6 | 8 | 24 | 26 | 28 | 30 |
| *B.cereus* |  |  |  |  |  |  |  |  |  |
| *E.faecalis* |  |  |  |  |  |  |  |  |  |
| *P.aeruginosa* |  |  |  |  |  |  |  |  |  |
| *K.pneumoniae* |  |  |  |  |  |  |  |  |  |
| Bacteria (growing cultures) | OD at time point (9am – 5pm day one, 9am-5pm day two) | | | | | | | | |
| 0 hours | 2 | 4 | 6 | 8 | 24 | 26 | 28 | 30 |
| *B.cereus* |  |  |  |  |  |  |  |  |  |
| *E.faecalis* |  |  |  |  |  |  |  |  |  |
| *P.aeruginosa* |  |  |  |  |  |  |  |  |  |
| *K.pneumoniae* |  |  |  |  |  |  |  |  |  |

1. **Minimum inhibitory concentration (MIC) determination.** 
   1. **Antibiotic stock solution preparation.** 
      1. How much carbenicillin to be dissolved in solvent is determined using the equation
         1. Where P = potency (given by manufacturer)
         2. V = volume required
         3. C = final concentration (multiplied by 1000)
         4. W = weight of antibiotic (mg) to be dissolved in solvent (ml)
      2. The potency of carbenicillin is 770µg/mg and approximately 5ml is required to for the following dilutions. Thus, is required to generate a 5ml 10,000mg/L solution.
      3. Carbenicillin will be weighed out using a microbalance.
      4. In a universal tube, dilute the full 64.935mg of carbenicillin with 5ml sterile water/saline.
      5. Vortex to mix.
      6. Filter sterilize this solution through 0.22 um syringe filters into a sterile falcon tube.
      7. For preparation of further stock solutions, from the initial 10,000 mg/L solution, prepare the following:
      8. 1 mL of 10,000 mg/L solution + 9 mL diluent (sterile water) = 1000 mg/L
      9. 100 μl of 10,000 mg/L solution + 9.9 mL diluent = 100 mg/L
   2. **Inoculum preparation.** 
      1. For each bacteria, transfer from a culture plate four morphologically similar colonies into MH broth using a sterile loop. **Alternatively, overnight cultures can be used**.
      2. Incubate cultures at 37OC in a shaking incubator until they visually reflect a 0.5 McFarland standard (15-20 mins).
      3. Prior to inoculation onto agar, gram negative bacterial suspensions (*P.aeruginosa* and *K.pneumoniae*) require dilution to 1:10 using sterile saline.
         1. Make up, in 1.5ml Eppendorf tubes, a 1ml suspension of 100ul bacteria and 900ul sterile saline.
      4. Gram positive bacterial suspensions (*B.cereus*  and *E.feacalis*) require dilution to 1:100 using sterile saline. (use Eppendorf tubes here).
         1. Make up, in 1.5ml Eppendorf tubes, a 1ml suspension of 10ul bacteria and 990ul sterile saline.
2. **Broth dilution method (macrodilution).** 
   1. **Setting up a dilution series.** 
      1. Antibiotic ranges should be prepared one step higher than the final dilution required as to account for the addition of an equal volume of inoculum. Thus, each dilution will be made up one step higher than listed in step 6.b and the final dilution will be 1024mg/L.
      2. Using a dilution range: 0 - 512 mg/L.
      3. Label 56 5ml Eppendorf tubes as follows:
      4. A - 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0 mg/L
      5. B - 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0 mg/L
      6. C - 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0 mg/L
      7. D - 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0 mg/L
      8. From the 10,000 mg/L stock, dispense the following amounts into 5ml Eppendorf tubes with a p1000 and p100 micropipette:
      9. 204.8ul into the tubes labelled 1024 A, B, C, and D.
      10. 102.4ul into tubes labelled 512 A-D
      11. 51.2 ul into tubes labelled 256 A-D
      12. 25.6 ul into tubes labelled 128 A-D
      13. 12.8 ul into tubes labelled 64 A-D
      14. 6.4 ul into tubes labelled 32 A-D
      15. 3.2 ul into tubes labelled 16 A-D
      16. From the 1000 mg/L stock, dispense the following amounts:
      17. 16.0 μl into the container labelled 8 A-D
      18. 8.0 μl into the container labelled 4 A-D
      19. 4.0 μl into the container labelled 2 A-D
      20. From the stock 100 mg/L dispense the following amounts:
      21. 20.0 μl into the bottle labelled 1 A-D
      22. 10.0 μl into the container labelled 0.5 A-D
      23. 5.0 μl into the container labelled 0.25 A-D
      24. No antibiotic is added to the tubes labelled 0 mg/L -A-D (antibiotic free growth control).
   2. **Addition of broth and inoculate.** 
      1. Into each tube add 1ml sterile MH broth using a p1000 pipette.
      2. Into each tube labelled A, add 1ml overnight culture of *B.cereus*
      3. Into each tube labelled B, add 1ml overnight culture of *E.feacalis*
      4. Into each tube labelled C, add 1ml overnight culture of *P.aeruginosa*
      5. Into each tube labelled D, add 1ml overnight culture of *K.pneumoniae.*
      6. Vortex each tube to mix.
      7. Incubate at 37oC for 18-20 hours, using a shaking incubator for aeration.
      8. After incubation, transfer suspensions into cuvettes and measure their OD.
      9. The dilutions with the closest OD reding to the control (sterile MH broth) will be considered the MIC.
3. **Antibacterial synergy assay.** 
   1. Make up 12 falcon tubes with 20ml MH broth using a pipeteman with 50ml pipettes, label them 1 A, B, C, D, 2 A-D and 3 A-D
   2. in tubes labelled A, add the MIC of carbenicillin corresponding to *B.cereus.*
   3. in tubes labelled B, add the MIC of carbenicillin corresponding to *E.feacalis.*
   4. in tubes labelled C, add the MIC of carbenicillin corresponding to *P.aeruginosa.*
   5. in tubes labelled D, add the MIC of carbenicillin corresponding to *K.pneumoniae.*
4. \*These MICs are unknown pending the completion of step 8, once known, volumes required can be determined.
   1. Into tubes A, add (100 uL) of the phage lysate generated from *B.cereus.*
5. repeat for tubes B-D, using the phage lysates generated from their corresponding bacteria.
   1. Into tubes A, add e.g. 200 µL overnight culture of *B.cereus.*
6. Repeat for the tubes B-D, using overnight cultures of their corresponding bacteria.
   1. Incubate tubes for 20 mins at room temperature.
   2. As in step 6, measure the OD 600nm at regular intervals (see table below) pipetting 1.5ml antibiotic and plaque solution into sterile cuvettes for each reading. Use a cuvette of sterile MH broth to calibrate the spectrophotometer and as negative control.
   3. Repeat steps 8a-i twice more for triplicate data.
   4. **\*Volumes used in steps 8a-g TBD after MIC determination.**

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| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Antibiotic phage synergy suspension  (run one) | OD at time point (9am – 5pm day one, 9am-5pm day two) | | | | | | | | |
| 0 hours | 2 | 4 | 6 | 8 | 24 | 26 | 28 | 30 |
| *B.cereus* |  |  |  |  |  |  |  |  |  |
| *E.faecalis* |  |  |  |  |  |  |  |  |  |
| *P.aeruginosa* |  |  |  |  |  |  |  |  |  |
| *K.pneumoniae* |  |  |  |  |  |  |  |  |  |
| Antibiotic phage synergy suspension  (run two) | OD at time point (9am – 5pm day one, 9am-5pm day two) | | | | | | | | |
| 0 hours | 2 | 4 | 6 | 8 | 24 | 26 | 28 | 30 |
| *B.cereus* |  |  |  |  |  |  |  |  |  |
| *E.faecalis* |  |  |  |  |  |  |  |  |  |
| *P.aeruginosa* |  |  |  |  |  |  |  |  |  |
| *K.pneumoniae* |  |  |  |  |  |  |  |  |  |
| Antibiotic phage synergy suspension  (run three) | OD at time point (9am – 5pm day one, 9am-5pm day two) | | | | | | | | |
| 0 hours | 2 | 4 | 6 | 8 | 24 | 26 | 28 | 30 |
| *B.cereus* |  |  |  |  |  |  |  |  |  |
| *E.faecalis* |  |  |  |  |  |  |  |  |  |
| *P.aeruginosa* |  |  |  |  |  |  |  |  |  |
| *K.pneumoniae* |  |  |  |  |  |  |  |  |  |

**Storage of samples/reagents.**

Environmental samples – store cool, do not freeze without cryoprotectant such as glycerol.

Phage stock – Can be stored cool for several months without significant loss of activity. Do not freeze without cryoprotectant.

Bacterial cultures – on agar plate or stab cultures in a fridge at 4oC.

MH media (broth and agar) – tightly closed dry container, hygroscopic and moisture sensitive.

PBS – light and moisture sensitive. Store in tightly closed dry and light resistant container. Avoid sources of ignition. Store in a cool, well-ventilated area.

**Host bacterium considerations.7**

Proper caution will be given when working with environmental samples, due to the possibility that harmful bacteria, chemicals, viruses, etc… are present.

Proper safety measures will be utilised based on the biosafety level of the bacterial host strains used in this study.

Aseptic techniques will be utilized throughout every practical step of this protocol as to prevent contamination of either the bacteria or phage.

All equipment and media components that can withstand it will be autoclaved. Media components that cannot be autoclaved will be made up with autoclaved distilled water, then the media will be passed through a 0.22µm membrane.

**Safety considerations7**

All solid waste should be autoclaved prior to disposal.

Use of surrogate strains may be used if pathogenic strains exceed the biosafety level of the laboratory, be aware that data derived from use of surrogate strains may not extrapolate to pathogenic strains.

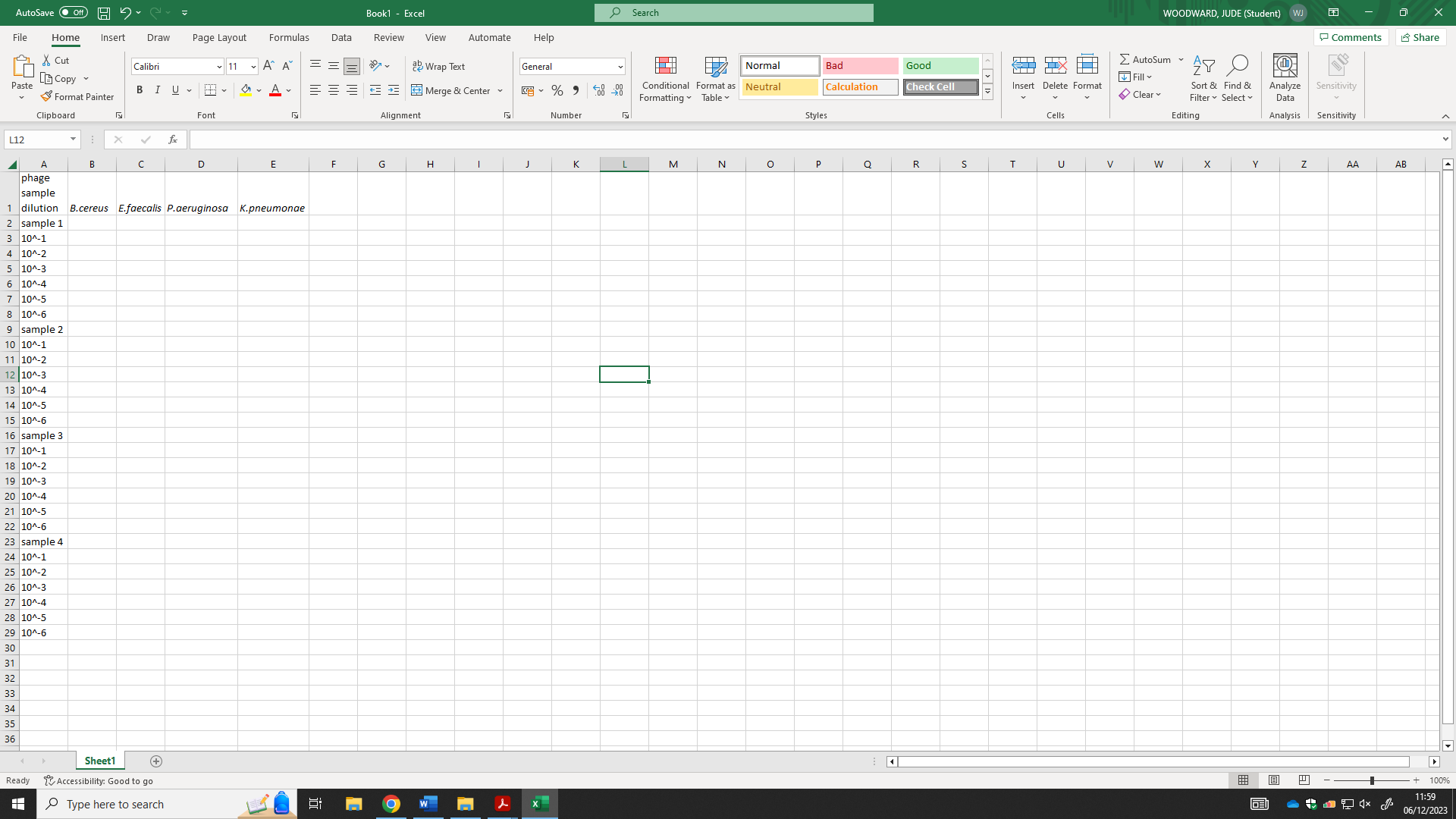
Some reagents used may be dangerous, read their associated MSDS and implement appropriate safety controls.

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**Appendix.**

**1. Example tick box table for marking off every combination of bacterial host strain and phage sample dilution.**



2. serial dilution of phage stock

a. combine 100µL environmental sample and 900µL phosphate buffered saline (PBS) in a sterile tube.

b. combine 100µL of the previous solution with 900µL PBS in a sterile tube.

c. repeat step b to produce dilutions of e.g.; 10-5, 10-6, and 10-7. Further dilutions may be made up based on further consideration into the optimum dilution.

d. these dilutions can be utilised in step 2 of the protocol (overlay titre method) to yield plaques.