Final Report for Conquer CF Innovation Grant 2021
Designing Optimal Phage Cocktails for Kids with Cystic Fibrosis: DOCK-CF

Lay summary of the project
Phages (viruses that selectively infect and kill bacteria) that demonstrate potent killing activity against the most common and problematic bacteria that infect children with cystic fibrosis will be identified. These bacteria and phages will have their full genetic material sequenced and this data will be combined with the results of laboratory experiments on phage-bacteria interactions to predict phage combinations that can be tested in future clinical trials in children with cystic fibrosis.

Project aims / objectives:
Overall hypothesis: Well-defined bacterial subtypes are predictable early colonisers of cystic fibrosis (CF) patients, and these can be effectively targeted for eradication by lytic phages and phage cocktails.

Aim 1: Characterise (genotypic and phenotypic) phages with lytic ('killing') activity against dominant subtypes of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in children with CF (cohort 1).

Aim 2: To design maximally effective 'phage cocktails' against the majority (~80%) of clinical isolates of *S. aureus* and *P. aeruginosa* from cohort 1; confirm their in vitro efficacy and antibiotic synergy against bacterial isolates from a separate cohort of children with CF (cohort 2); and validate safety and efficacy of phage and phage-antibiotic combinations in a mouse lung infection model.

Through prior seed funding from the Australasian Society for Infectious Diseases, systematic biobanking of a representative sample of key pathogens from CF patients at The Children's Hospital at Westmead (CHW, cohort 1) was undertaken between June 2020 and December 2021. A total of 57 *Pseudomonas aeruginosa* and >230 *Staphylococcus aureus* isolates were collected over this time.

Funding from the RACP (Research Establishment Fellowship) and from Cystic Fibrosis Australia (Conquer CF Innovation grant) allowed for work on the *P. aeruginosa* isolates to be undertaken towards answering part of both aims of the project between 2021 and 2022 (inclusive 15 months maternity leave).

Results

Bacterial isolates

57 *P. aeruginosa* isolates were collected from children with cystic fibrosis at The Children's Hospital at Westmead. DNA was extracted and isolates were sequenced and analyzed using in house whole genome sequencing workflow.

As shown in **Figure 1.**, the *P. aeruginosa* population is quite diverse with no sequence type (ST) dominating. We detected 31 different STs. Eight isolates were of a mucoid phenotype, and all isolates had at least one intact efflux pump.

Bacteriophages and phage susceptibility profiles

All bacterial isolates were screened against a panel of 17 lytic phages and at least one lytic phage has been identified with activity against each bacterial target. Seven lytic phages were selected for further characterization and to test efficacy individually by spot test and liquid kinetics for the 3 phages with the broadest host range (**Figure 2**).

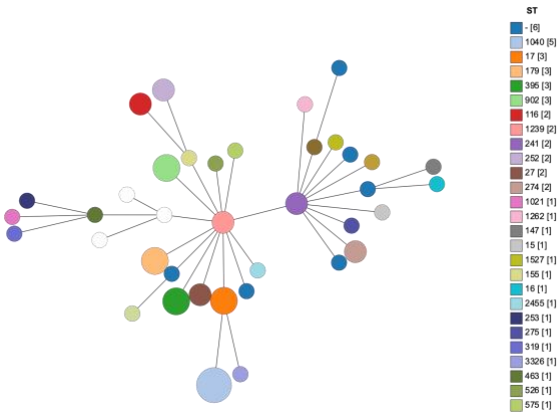


Figure 1. MLST minimum spanning tree based on seven housekeeping genes (*acsA*, *aroE*, guaA, *mutL*, *nuoD*, *ppsA*, *trpE*).

Manual annotation of important phage genetic features and comparison with publicly available phage genomes are currently ongoing. We have confirmed that the phages selected for this study do not carry antibiotic resistance genes, virulence genes and no genetic elements related to a temperate lifestyle.

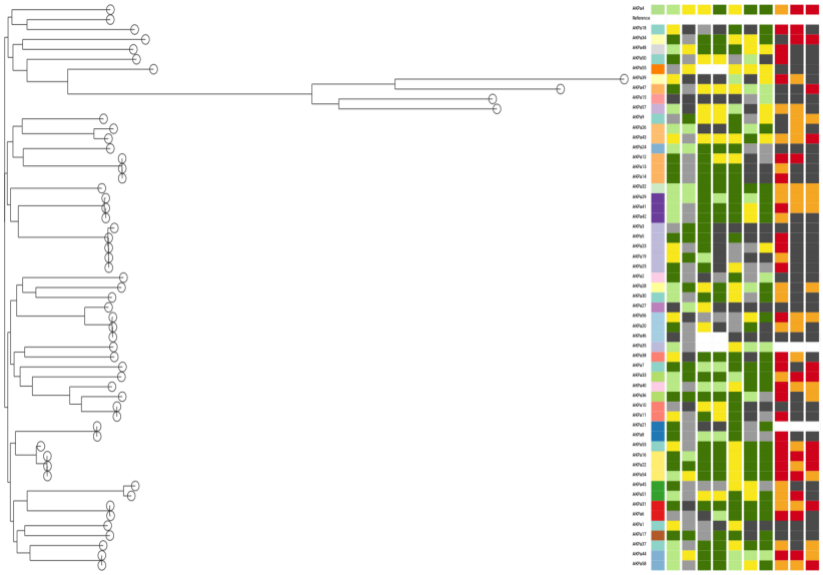


Figure 2. Phage susceptibility profile measured by spot test. Column 1: ST profile. Columns 2 to 8: phage spot test results. Dark green: Full activity=clear spot, light green: Full+=some colonies in spot. Yellow: Partial activity=opaque spot +/- clear halo. Light grey: Poor activity=very opaque spot. Dark grey: No activity=no clearing. Columns 9 to 11 phage liquid kinetics results. Red: more than 15 h bacterial growth inhibition. Orange: between 10 to 15 h bacterial growth inhibition. Dark grey: less than 10 h growth inhibition.

The 3 best phages (broadest host ranges) were selected for screening by liquid growth kinetics in combination (as a cocktail). The combination (phage cocktail) was noted to have a positive (growth inhibition) or negative (absence of growth inhibition) impact depending on different isolates tested. We also observed that the positive behavior of one phage used individually would not guarantee the success of the combination. This work is ongoing.

Phage stability

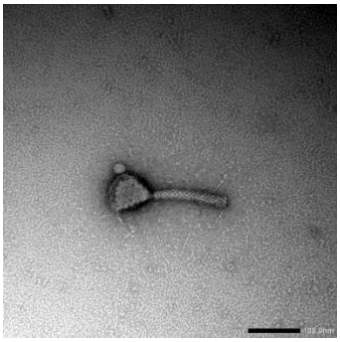
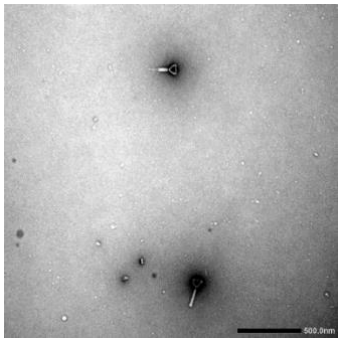
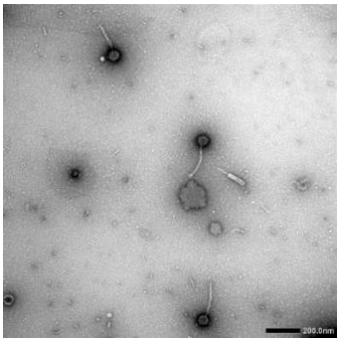
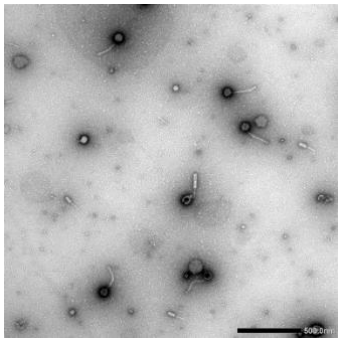
Phage titers have been tested under different conditions:

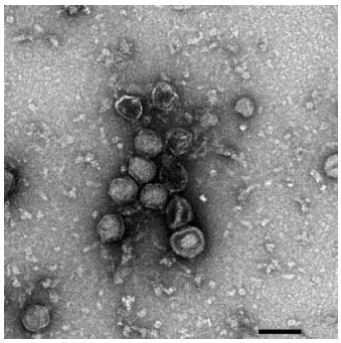
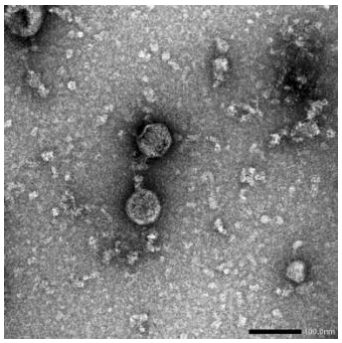
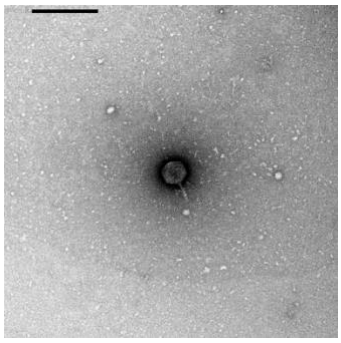
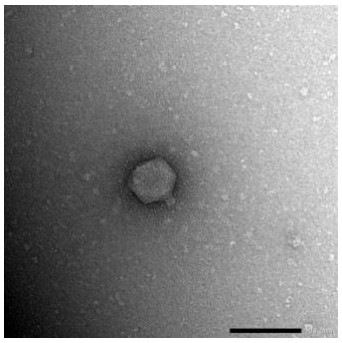
- Temperature (room temperature [21^{o}C] for 24h and 1 week, and 37^{o}C for 24h)
- pH: Room temperature for 4h at pH 3, 6, 7, 8
- Glycerol 50%, -80 ^{o}c for 1 month

All the phages seem highly stable under the tested conditions. The titers remain high (10^{11} pfu/mL-10^{12}pfu/mL) at all ranges of temperature and pH tested. The chloroform condition has not been tested yet.

Virion morphology by transmission electron microscopy (TEM)

The selected phages were subjected to TEM analysis for their morphological examination. TEM was conducted at the Westmead Electron Microscopy Facility (Westmead, Australia). TEM for Pa9F, Pa9R and Pa135 are currently ongoing.

Pae7 Belongs to the Myoviridae family. Characteristic features include an icosahedral head, a long tail and 6 short spikes.		
Pae8 Belongs to the Siphoviridae family. Characteristic features include an icosahedral head, long flexible tail and 4 fibers.		

Pa133 Belongs to the Myoviridae family. Characteristic features include an icosahedral head and a short tail.		
Pa134 Belongs to the Myoviridae family. Characteristic features include an icosahedral head and a short tail.		

Minimum Inhibitory Concentration Assay / Checkboard assays

The minimal inhibitory concentrations (MICs) for selected antibiotics were determined using the broth microdilution method. Five bacterial isolates were selected based on their antibiotic susceptibility profile and their phenotype: mucoid (MPA) or non-mucoid (NMPA). Phages (Pae7, Pa133 and Pa135) were tested individually and in combination with 3 antibiotics individually: Ciprofloxacin (fluoroquinolone), Meropenem (Carbapenem), Amikacin (Aminoglycosides).

Overall, [1] the addition of phages that inhibit the growth for at least 10h to an antibiotic contribute to a significant MIC drop; [2] no antagonistic effects have been observed, [3] inconsistency in MIC values when mucoid *P. aeruginosa* isolates are tested, [4] the combination of phages and antibiotics result in appearance of bacterial resistant subpopulations in some cases.

Immediate next steps:
Further funding will be sought to complete outstanding experiments to allow these results to be published. Additional funding will also be sought to undertake work on the *S. aureus* isolates that have been collected to date.

We thank Conquer CF and the Australian Cystic Fibrosis Research Trust (ACFRT) for their support of our research.

Dr Ameneh Khatami, on behalf of the DOCK-CF research team