

Supplementary Materials for

Mixed strain pathogen populations accelerate the evolution of antibiotic resistance in patients

Supplementary Table 1. Comparison of *P. aeruginosa* positivity in first enrolment sample between single and mixed strain patients.

| Patient | Mixed or single strain? | First enrolment sample <i>P. aeruginosa</i> positive? |
|---------|-------------------------|---|
| 1 | Single strain | Negative |
| 3 | Single strain | Positive |
| 4 | Single strain | Positive |
| 5 | Single strain | Positive |
| 6 | Single strain | Positive |
| 7 | Single strain | Positive |
| 12 | Single strain | Positive |
| 13 | Single strain | Negative |
| 19 | Single strain | Negative |
| 21 | Single strain | Negative |
| 22 | Single strain | Negative |
| 23 | Single strain | Negative |
| 24 | Single strain | Negative |
| 25 | Single strain | Negative |
| 26 | Single strain | Negative |
| 27 | Single strain | Positive |
| 29 | Single strain | Positive |
| 30 | Single strain | Positive |
| 31 | Single strain | Negative |
| 32 | Single strain | Positive |
| 33 | Single strain | Positive |
| 34 | Single strain | Negative |
| 35 | Single strain | Positive |
| 2 | Mixed strain | Negative |
| 8 | Mixed strain | Positive |
| 9 | Mixed strain | Negative |
| 10 | Mixed strain | Positive |
| 11 | Mixed strain | Negative |
| 14 | Mixed strain | Negative |
| 15 | Mixed strain | Negative |
| 16 | Mixed strain | Positive |
| 17 | Mixed strain | Negative |
| 18 | Mixed strain | Positive |
| 20 | Mixed strain | Positive |
| 28 | Mixed strain | Positive |

The first enrolment was *P. aeruginosa* positive in a total of 12/23 (52.17%) single strain patients and 6/12 (50.00%) mixed strain patients. Whether first enrolment sample was *P. aeruginosa* positive did not differ significantly between single strain and mixed strain patients (unpaired two tailed t-test; p=0.9073).

Supplementary Table 2: Time (days) between first and last sample for antibiotic-treated single strain and mixed strain patients.

| Patient number | Mixed or single strain? | Time (days) between first and last sample |
|----------------|-------------------------|---|
| 8 | Mixed strain | 4 |
| 15 | Mixed strain | 7 |
| 17 | Mixed strain | 4 |
| 10 | Mixed strain | 7 |
| 16 | Mixed strain | 20 |
| 18 | Mixed strain | 16 |
| 29 | Single strain | 7 |
| 32 | Single strain | 4 |
| 34 | Single strain | 4 |
| 6 | Single strain | 21 |
| 35 | Single strain | 22 |
| 5 | Single strain | 30 |
| 4 | Single strain | 14 |

Time (days) between first and last sample did not differ significantly between antibiotic-treated single and mixed strain patients (unpaired two tailed t-test; $p=0.3211$).

Supplementary Table 3: ANOVA testing responses to antibiotic treatment.

Response variable: $P_{R(F),x} - P_{R(I),x}$ where $P_{R(F),x}$ is the proportion of isolates resistant from patient y to antibiotic x at the final time point and $P_{R(I),x}$ is the proportion of isolates from patient y resistant to antibiotic x at the initial time point

Pathogen diversity: A categorical variable reflecting patients that were colonized by single strain and mixed strain populations.

Patient: We nested patient within pathogen diversity as patients were colonized by either a single strain or mixed strain population of *Pseudomonas*.

Antibiotic: Resistance was measured to six different antibiotics (meropenem, ceftazidime, gentamicin, ciprofloxacin, aztreonam, tazobactam)

Initial Resistance: $P_{R(I),x}$ is the proportion of isolates from patient y resistant to antibiotic x at the initial time point

Response type: A binary variable reflecting whether or not antibiotics were used to treat a given patient during the sampling interval (ie direct responses) or not (collateral responses)

A) Full model

| Term | DF | SS | F | P |
|---------------------------------------|----|-------|-------|--------|
| Pathogen diversity | 1 | 8456 | 15.03 | .0003 |
| Patient [Pathogen diversity] | 11 | 24244 | 3.91 | .0004 |
| Antibiotic | 5 | 2391 | .85 | .52 |
| Initial resistance | 1 | 17391 | 30.9 | <.0001 |
| Response type | 1 | 16.13 | .029 | .866 |
| Initial resistance*pathogen diversity | 1 | 9.98 | .0177 | .894 |
| Initial resistance*Antibiotic | 5 | 2515 | .894 | .49 |
| Error | 52 | 29253 | | |

B) Reduced model – constructed by sequentially removing non-significant terms, beginning with interaction terms.

| Term | DF | SS | F | P |
|------------------------------|----|-------|-------|--------|
| Pathogen diversity | 1 | 8393 | 15.98 | .0002 |
| Patient [Pathogen diversity] | 11 | 28919 | 5.00 | <.0001 |
| Initial resistance | 1 | 17180 | 32.70 | <.0001 |
| Error | 64 | 33626 | | |

C) Full model excluding the data from patient 8

| Term | DF | SS | F | P |
|---------------------------------------|----|-------|-------|--------|
| Pathogen diversity | 1 | 6505 | 12.17 | .0011 |
| Patient [Pathogen diversity] | 10 | 24365 | 4.55 | .0002 |
| Antibiotic | 5 | 1727 | .64 | .66 |
| Initial resistance | 1 | 16910 | 31.6 | <.0001 |
| Response type | 1 | 24.47 | .045 | .83 |
| Initial resistance*pathogen diversity | 1 | 24.76 | .046 | .83 |
| Initial resistance*Antibiotic | 5 | 4202 | 1.57 | .18 |
| Error | 47 | 25121 | | |

Supplementary Table 4: Summary of negative binomial regression models for the number of *de novo* variants detected. Coefficients and p-values (two-sided Wald test) are given for resistance variants (a) and other variants (b), including rerunning analysis with an outlier which did not change the overall conclusion (c, d).

| (a) glm.nb(resistance.variants ~ infection.type*isolate.count) | Estimate | Std. Error | z value | Pr(> z) |
|---|----------------|----------------|----------------|----------------|
| (Intercept) | 2.87155 01 | 0.95946 467 | 2.99286 69 | 0.00276 37 |
| Infection.type (mixed vs. single) | 2.26900 02 | 2.28450 297 | 0.99321 39 | 0.32060 572 |
| Isolate.count (no. of isolates) | 0.13180 623 | 0.05133 518 | 2.56756 165 | 0.01024 166 |
| Interaction between infection type and no. of isolates | 0.04884 186 | 0.10804 483 | 0.45205 175 | 0.65123 17 |

| (b) glm.nb(nonresistance.variants ~ infection.type*isolate.count) | Estimate | Std. Error | z value | Pr(> z) |
|--|----------------|----------------|----------------|----------------|
| (Intercept) | 0.42021 8 | 0.44753 492 | 0.93896 13 | 0.34775 063 |
| Infection.type (mixed vs. single) | 0.52385 72 | 0.57749 448 | 0.90712 06 | 0.36434 3 |
| Isolate.count (no. of isolates) | 0.12915 218 | 0.03094 087 | 4.17416 147 | 2.99E- 05 |
| Interaction between infection type and no. of isolates | 0.00666 | 0.04064 429 | 0.16386 | 0.86984 136 |

| (c) With 25-0925 outlier: glm.nb(resistance.variants ~ infection.type*isolate.count) | Estimate | Std. Error | z value | Pr(> z) |
|---|---------------|----------------|----------------|----------------|
| (Intercept) | 3.36122 27 | 1.02884 311 | 3.26699 25 | 0.00108 697 |
| Infection.type (mixed vs. single) | 1.78746 35 | 2.33750 836 | 0.76468 76 | 0.44445 762 |
| Infection.type (mixed vs. single) | 0.18347 49 | 0.05340 47 | 3.43555 715 | 0.00059 134 |
| Interaction between infection type and no. of isolates | 0.00168 54 | 0.11209 265 | 0.01503 59 | 0.98800 352 |

| (d) With 25-0925 outlier: glm.nb(nonresistance.variants ~ infection.type*isolate.count) | Estimate | Std. Error | z value | Pr(> z) |
|--|----------------|----------------|----------------|----------------|
| (Intercept) | 0.28289 04 | 0.45670 829 | 0.61941 17 | 0.53564 52 |
| Infection.type (mixed vs. single) | 0.68521 73 | 0.59202 829 | 1.15740 63 | 0.24710 641 |
| Isolate.count (no. of isolates) | 0.11354 428 | 0.03077 403 | 3.68961 384 | 0.00022 459 |
| Interaction between infection type and no. of isolates | 0.01143 137 | 0.04144 573 | 0.27581 529 | 0.78268 994 |

Supplementary Table 5: ANOVA testing for a trade-off between growth rate and resistance.

Response variable: Growth rate, the mean growth rate of 8-10 (check) replicate cultures of each isolate in culture medium

Pathogen diversity: A categorical variable reflecting isolates from patients that were colonized by single strain or mixed strain populations.

Resistance phenotype: A categorical variable reflecting isolates that were MDR (resistant to 3 or more antibiotics) or non-MDR (resistant to 0-2 antibiotics)

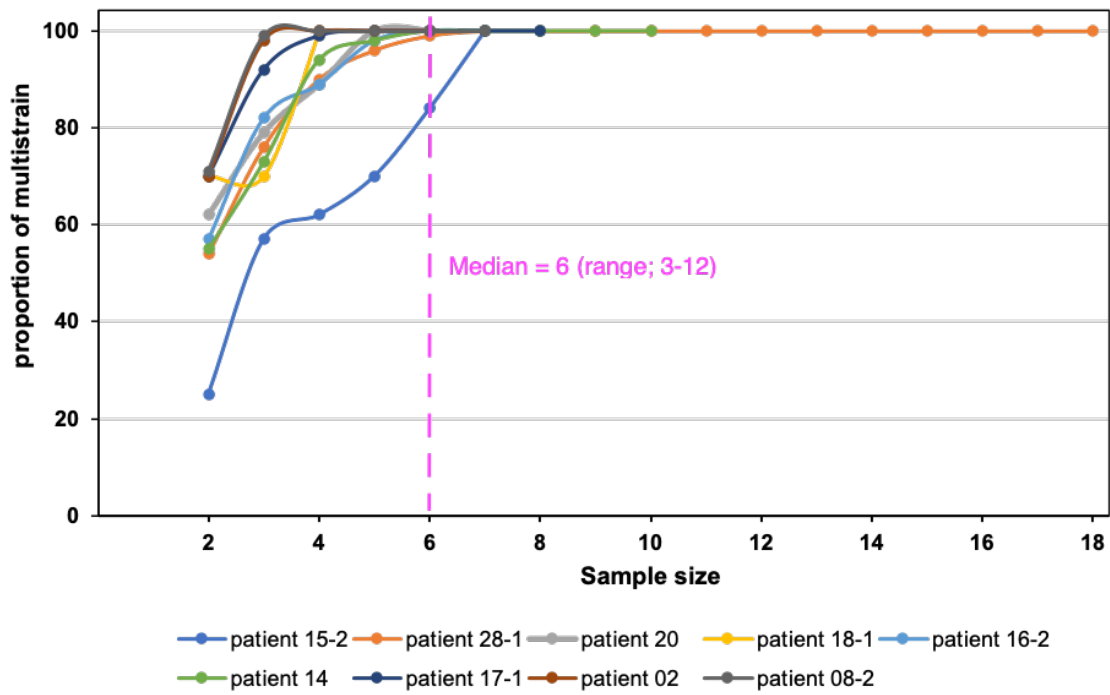
Patient: We nested patient within pathogen diversity as patients were colonized by either a single strain or mixed strain population of Pseudomonas.

| Term | DF | SS | F | P |
|---|-----------|-----------|----------|----------|
| Pathogen diversity | 1 | .062 | .126 | .72 |
| Resistance phenotype | 1 | 2.66 | 5.42 | .021 |
| Pathogen diversity*Resistance phenotype | 1 | 5.52 | 11.24 | .0010 |
| Patient [Pathogen diversity] | 8 | 14.4 | 3.66 | .0006 |
| Error | 167 | 82 | | |

Supplementary Table 6: Characteristics of study cohort.

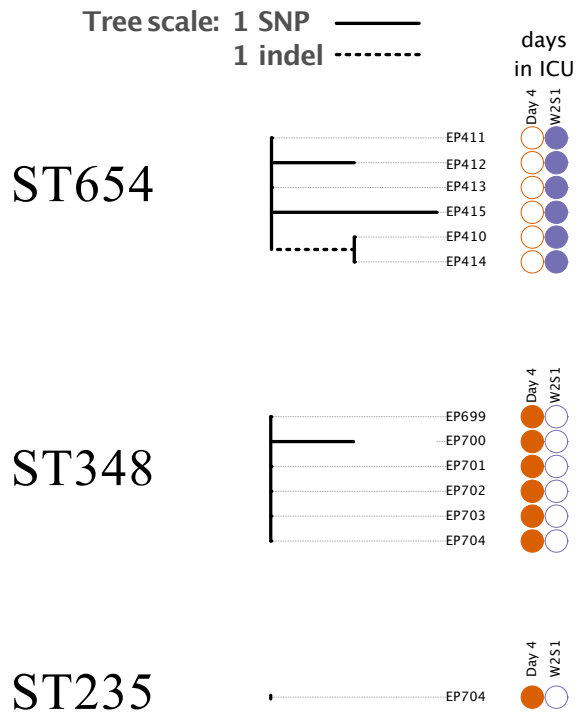
| | | Included, n (%) |
|-------------------------------------|----------------|-----------------|
| | | (n = 35) |
| Female sex | | 13 (36.1) |
| Age | (median, IQR) | 63 (49-72) |
| APACHE IV score at ICU admission | (median, IQR) | 45 (34-68) |
| Body Mass Index, BMI | (median, IQR) | 23 (26-31) |
| ICU admission specialty | Medical | 15 (42.9) |
| | Surgery/trauma | 20 (57.1) |
| Origin prior to ICU | Home/community | 16 (45.7) |
| | Healthcare | 19 (54.3) |
| Prior antibiotic use | | 10 (27.8) |
| Diabetes | | 6 (17.1) |
| European region | North | 3 (8.6) |
| | South | 24 (68.6) |
| | East | 3 (8.6) |
| | West | 5 (14.3) |

Supplementary Figure 1: Rarefaction analysis of mixed strain patient samples, showing the proportion of subsamples that contain at least 2 strains as sample size increase.



The median sample size of the single strain patient samples was 6 isolates, with a range between 3 and 12.

Supplementary Figure 2: Genetic diversity of isolates recovered from patient 15.



Phylogenetic reconstruction of ST654, ST348 and ST235 isolates from patient 15 indicating SNPs and indels.