Changes in colony morphology and antibiotic resistance in response to *in vitro* exposure of *Klebsiella pneumoniae* to the antibiotic cephalothin.

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Abstract

Klebsiella pneumoniae is a gram-negative nosocomial pathogen and causative agent of many hospital acquired infections. *K. pneumoniae* infections have become increasingly of interest due to the rise of hypervirulent variants and multidrug resistant strains. Modeling how antibiotic resistance evolves in *K. pneumoniae* will allow us to better understand exactly how the bacterium acquires resistance to various antibiotics. A previous experiment in our lab exposed a strain of *K. pneumoniae* to low but increasing concentrations of the antibiotic cephalothin. As a result, the strain evolved to be mucoid with elongated cellular morphology and resistant to multiple antibiotics. This study aimed to repeat the same experimental approach with multiple cultures, to determine if different genomic mutations could result in the same endpoint of antibiotic resistance. Five cultures of *K. pneumoniae* 43816 were exposed to increasing amounts of the antibiotic cephalothin over a 14-day period. After the 14 day experiment, cultures were assayed for changes in antibiotic susceptibility, colony, and cellular-level morphology. Preliminary results indicate evolved resistance to cephalothin and tetracycline, but not kanamycin. Further, alterations in the colony morphology have been noted with a mix of small and large colony phenotypes. This variation of colony morphology in the adapted population may indicate different genetic mutations that correspond to these large and small colony variants. Current work is determining the relationship between colony morphology and antibiotic resistance.

Objectives

- Identify evolutionary characteristics that *K. pneumoniae* develops in response to increasing antibiotic resistance *in vitro*.
- Characterize various morphology changes in *K. pneumoniae* in response to *in vitro* antibiotic resistance development.
- Determine how the *in vitro* development of cephalothin resistance in *K. pneumoniae* affects susceptibility to other antibiotics and biofilm formation.

Methods

Klebsiella pneumoniae (ATCC 43816) was grown in low but increasing concentrations of cephalothin over a period of 14 days to induce the development of antibiotic resistance. Five replicates (labeled A, B, C, D, E) of this strain were independently exposed to the antibiotic and a sixth isolate was left untreated for the 14 days (labeled U). A sample of each replicate was frozen at each 12-hour time-point.

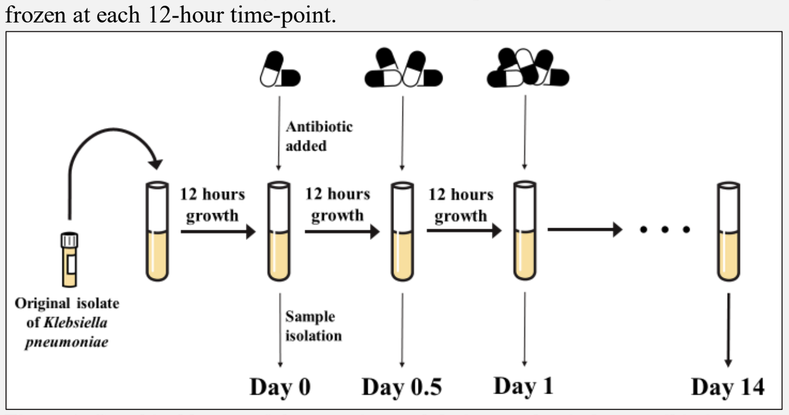

Figure 1. Graphical layout of the experimental design used to induce antibiotic resistance. *K. pneumoniae* was grown in low but increasing concentrations of the antibiotic cephalothin over a period of 14 days.

Minimum inhibitory concentrations (MICs) for susceptible and adapted isolates were then examined. Additionally, colony morphology changes were noted and their effects on MICs were investigated. To assess biofilm formation, *K. pneumoniae* was grown in a 96-well plate and planktonic bacteria were removed. The remaining adherent bacteria enveloped within biofilm were stained with crystal violet and quantified.

Results

Development of Antibiotic Resistance Leads to Phenotypic Changes

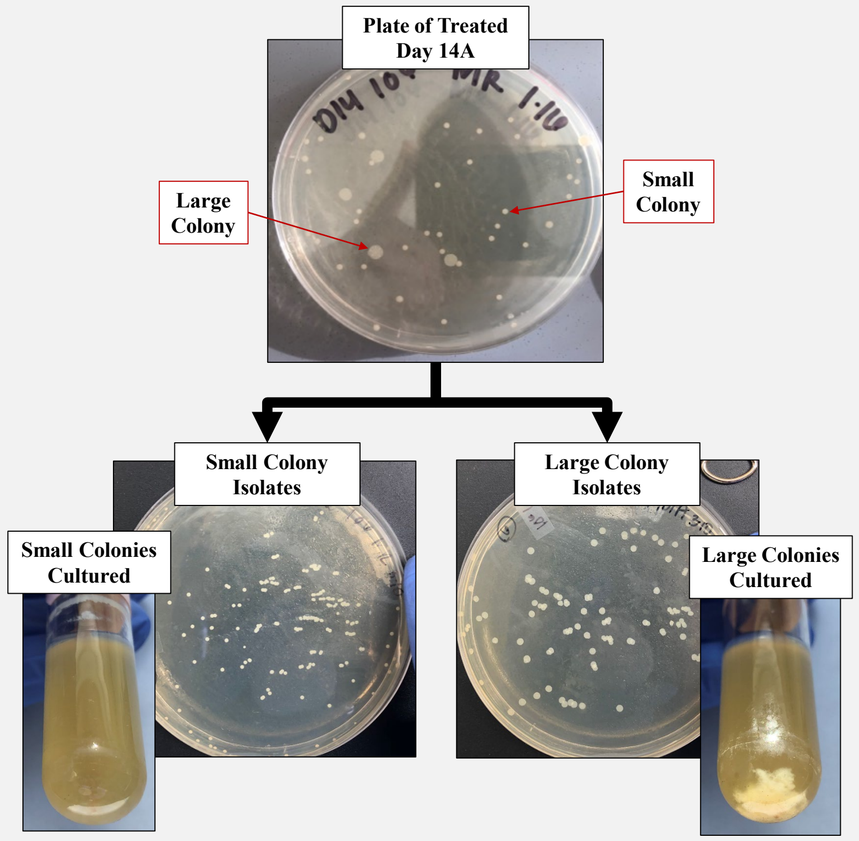

Figure 2. Phenotypic morphology changes. Colonies of one treated replicate after 14 days of growth in cephalothin (14A) displayed large and small variants. Isolation of individual colonies gave rise to their respective phenotypes. Culturing individual colonies in media showcased further phenotypical differences in that large colony variants appeared to have large aggregates in culture whereas small colony variants in culture did not.

Development of Antibiotic Resistance Leads to Changes in Biofilm Formation

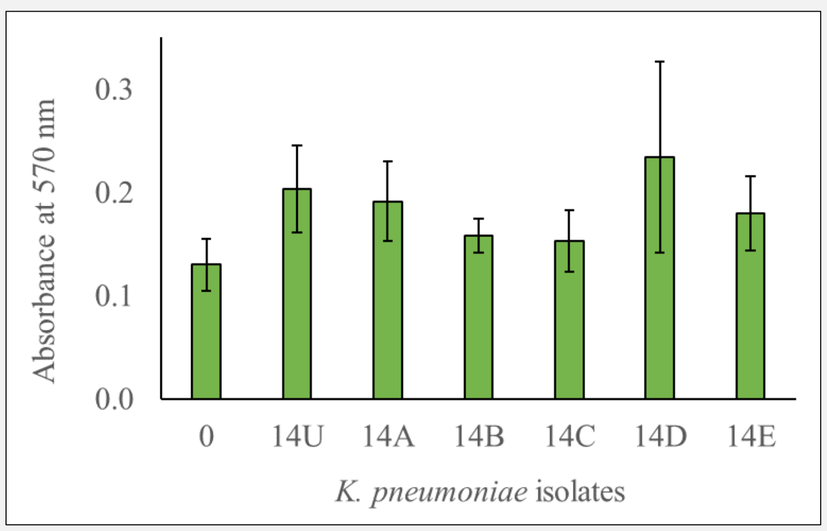

Figure 3. Biofilm-forming abilities of K. pneumoniae isolates. Cultures were grown and stained with crystal violet following the removal of planktonic bacteria to examine biofilm formation. Isolate numbers represent the day of isolation during the experiment, where A-E represent treated replicates and U represents the untreated condition. Error bars represent SD of the mean (n = 11).

Results (cont.)

Minimum Inhibitory Concentrations

Table 1. Breakpoints in µg/mL for starting and ending isolates. Day 0 was susceptible to cephalothin. All values represent concentrations of antibiotic (in µg/mL) needed to inhibit the growth of *K. pneumoniae*, where 14U represents the untreated control condition and A-E represent replicate conditions that were treated with cephalothin.

Breakpoints in µg/mL	Day 0	Day 14U	Day 14A	Day 14B	Day 14C	Day 14D	Day 14E
Cephalothin	8	4	31	62.5-125	31-62.5	31-62.5	31-62.5
Kanamycin	8	8-16	4	4	4	4	4
Tetracycline	4	8	8-16	8-31	16	16	16
Amikacin	31	31	16	16	8	4	62.5

Table 2. Minimum inhibitory concentrations of cephalothin for isolated and recombined 14A small and large colony variants. All values represent concentrations of antibiotic (in µg/mL) needed to inhibit the growth of *K. pneumoniae*, where 14U represents the untreated control condition and A-E represent replicate conditions that

Cephalothin MICs in µg/mL	MIC 50	Breakpoint
Small	8	16
Large	8	16
Recombined	31	62.5

Increased from original breakpoint of 31 µg/mL

Conclusions

- Induced cephalothin-resistant *K. pneumoniae* strains showed increased resistance to cephalothin, mild resistance to tetracycline and amikacin, and no change in resistance to kanamycin.
- A phenotypic change in colony morphology (large and small colonies) was observed in one treated replicate. For Day 14A, plates contained both small and large colonies. Individual colonies were isolated and showed the same but lower MIC 50s and breakpoints for cephalothin. However, once colonies were recombined, resistance was amplified.
- All isolates were able to produce biofilm. Day 0 was the weakest biofilm producer, and all Day 14 isolates (including the untreated condition) saw increases in biofilm production.

Future Directions

- Whole-genome sequencing will be used to identify the genetic mutations responsible for:
  - the evolution of antibiotic resistance
  - differences between small and large colony variants that affect their apparent synergetic relationship.
- Assessment of other replicates for potential changes in biofilm formation.
- Examination of a potential relationship between colony morphology and biofilm formation.
- Investigation of any relationship between biofilm formation and antibiotic resistance.

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