The acquisition of a flocculent phenotype in response to *in vitro* exposure of *Klebsiella pneumoniae* to the antibiotic cephalothin.

Sean M. Dorenkott, and Terri N. Ellis
Biology Department, University of North Florida

Abstract

Klebsiella pneumoniae is a gram-negative bacterial pathogen that is notorious for being the 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 bacterial populations acquire resistance to various antibiotics. Presently, it is the aim of our laboratory to determine if different genomic mutations in *K. pneumoniae* acquired in response to antibiotic treatment could result in the same endpoint of antibiotic resistance. In our current experiment, five cultures of *K. pneumoniae* ATCC 43816 were exposed to low but increasing amounts of the antibiotic cephalothin over a 15-day period. After the 15-day experiment, alterations in the morphology of bacterial colonies have been noted. All tested strains have demonstrated a flocculent phenotype, which is a rarely-seen and understudied characteristic of *K. pneumoniae*. The flocculent phenotype is a type of protective biofilm that, rather than being attached to a surface (bodily or otherwise), is free-floating. This allows these bacterial aggregates (often called "flocs") to be carried throughout the body by the blood or other fluids in the form of large, difficult-to-treat structures. Here, a novel method of quantifying antibiotic resistance of bacterial cells trapped in flocs is presented. Preliminary data from the current study indicates that flocculent structures provide *K. pneumoniae* with substantially increased protection from antibiotics.

Objectives

- Identify evolutionary characteristics that *K. pneumoniae* develops in response to antibiotic treatment *in vitro*.
- Develop a novel method for quantifying antibiotic resistance of cells trapped in flocculent structures
- Elucidate how the appearance of the flocculent phenotype is linked to the evolution of antibiotic resistance in *K. pneumoniae*.

Methods

Klebsiella pneumoniae (ATCC 43816) was grown in low but increasing concentrations of cephalothin over a period of 15 days to induce the development of antibiotic resistance. Five replicates (labelled A, B, C, D, E) of this strain were independently exposed to the antibiotic for the entire period.

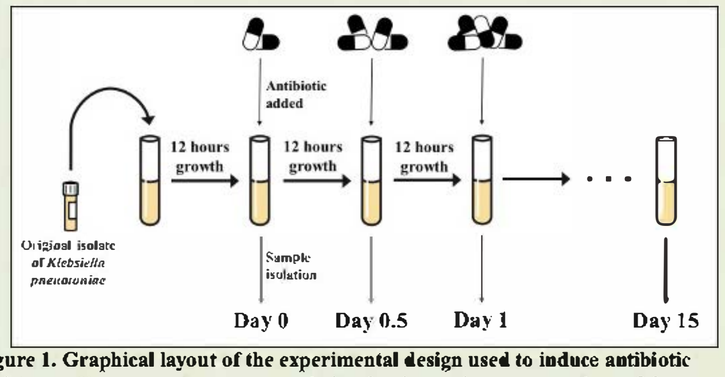

Figure 1. Graphical layout of the experimental design used to induce antibiotic resistance. *K. pneumoniae* was grown in low but increasing concentrations of the antibiotic cephalothin over a period of 15 days.

To assess how the flocculent phenotype affects resistance to antibiotics, *K. pneumoniae* flocs were first isolated from planktonic cells. Individual flocs were then exposed overnight to a range of antibiotic concentrations. To determine whether living cells were present within flocs after antibiotic treatment, the metabolic dye resazurin was added to treated flocs. This dye is irreversibly reduced and changes color from blue to pink in the presence of metabolically active cells. This color change can then be measured using a spectrophotometric method to determine if living bacterial cells are present within flocs after being treated with antibiotic. This also allows for the minimum inhibitory concentration (MIC) of a certain antibiotic to be measured.

Results

Acquisition of Antibiotic Resistance Leads to the Development of a Flocculent Phenotype

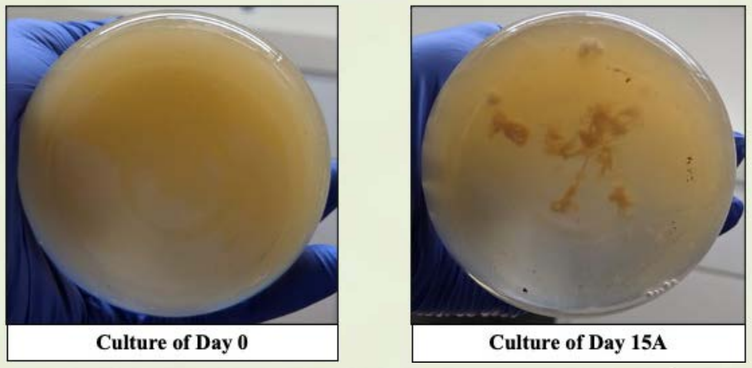

Figure 2. Adoption of a flocculent phenotype by a resistant strain. Representative pictures of cultures of susceptible and resistant strains, with Day 15A displaying large, flocculent aggregates after 15 days of antibiotic treatment.

Flocculent Structures Confer Additional Resistance to Antibiotics

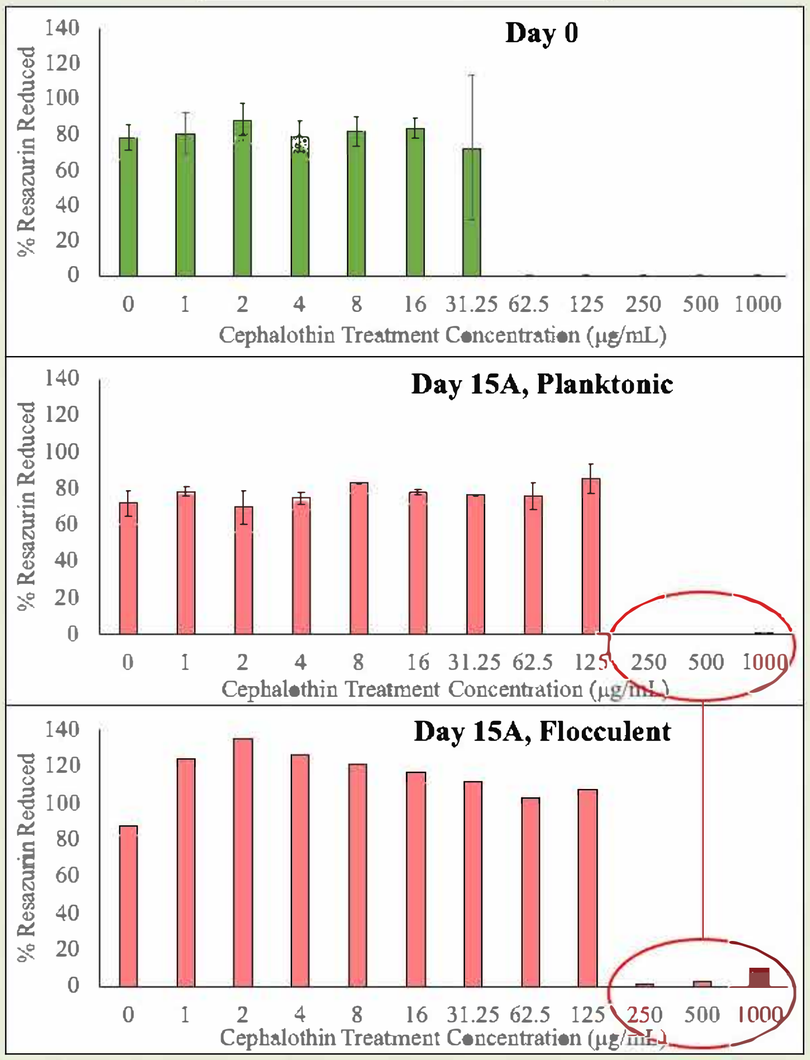

Figure 3. Preliminary data indicates flocculent structures allow bacteria to survive in higher-than-normal concentrations of antibiotic. A novel resazurin assay was conducted to quantify the amount of viable bacteria present in a range of antibiotic concentrations, where an increased amount of living cells leads to a higher percentage of reduced resazurin. Highlighted in red are results indicating that living cells are present when contained in a floc.

Results (cont.)

Development of a Novel Resazurin Assay Allows for Accurate MIC Measurements

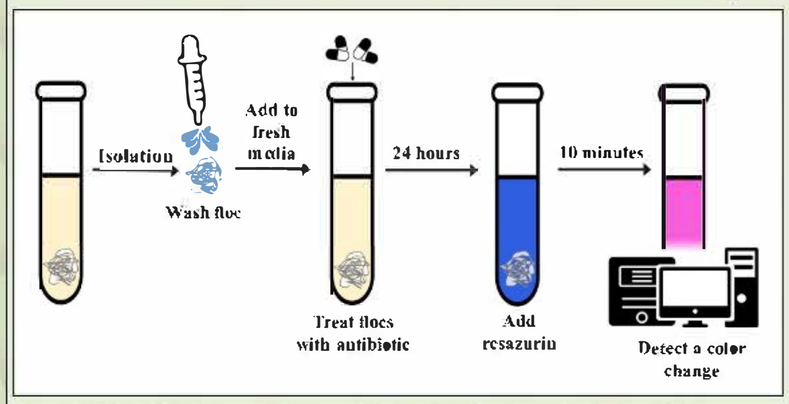

Figure 4. Graphical layout of a novel resazurin assay to measure bacterial growth. Resazurin is a metabolic dye that irreversibly changes color from blue to pink in the presence of living bacteria. This color change can be detected and quantified using a spectrophotometric method to determine the amount of living bacteria present.

Conclusions

- Induced cephalothin-resistant *K. pneumoniae* strains demonstrated an at least four-fold increase in the amount of cephalothin needed to inhibit bacterial growth.
- Antibiotic resistance development in five *K. pneumoniae* strains was also tied to the acquisition of a flocculent phenotype, which is a rarely-seen and understudied type of biofilm.
- Preliminary data suggests that flocculent structures are able to confer additional resistance to cephalothin.
- Prescence within a flocculent aggregate resulted in a substantially high minimum bactericidal concentration (MBC) of cephalothin, which is expected to be much higher than that of cephalothin for planktonic bacteria.
- A novel assay testing for additional antibiotic resistance conferred by flocculent structures has been outlined in the current study. Further optimization and trials is a key area of future development.

Future Directions

- Characterization of flocculent aggregates by microscopy, culturing, and plating techniques.
- Development of a physical or chemical treatment that can be utilized to destroy flocculent structures and potentially restore antibiotic susceptibility of bacteria trapped inside of flocs.
- Further optimization of a resazurin-based assay for testing the antibiotic resistance of flocculent strains of bacteria.
- Whole-genome sequencing will be performed to identify the genetic mutations responsible for the appearance of a flocculent phenotype.

Acknowledgements

Funding from the UNF Transformational Learning Opportunity Grant was used to support the present research. I would also like to acknowledge Montserrat Roberts, Fjona Dulli, Olivia Wright, Jasmine Anderson, and Michael Durnin for their work and dedication to our 15-day experiment.