Elimination of Antibiotic Resistant *Klebsiella pneumoniae* Using CRISPR-Cas9 System

Erin M. May, MLT (ASCP), Noah C. Fowler, MLT (ASCP)
Mentor: Matthew J. Nicholaou, DrPH, MT (ASCP)

ABSTRACT

Antimicrobial resistance has become a growing threat worldwide. Using CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats) gene editing technology, it may be possible to eliminate these resistant microorganisms with extreme precision. The goal of this study was to kill an ESBL (extended-spectrum beta-lactamase) ATCC 700603 quality control strain of *Klebsiella pneumoniae* using CRISPR-Cas9 technology. Continuing their promising research done with a CRISPR-Cas9 system in methicillin resistant Staphylococcus aureus, another MLS department research group designed plasmids for a new bacterial model, ESBL *K. pneumoniae,* which can cause the lung infection pneumonia. These plasmids targeted a housekeeping gene, DeaD, needed for survival and the bla(SHV-1) gene coding for beta-lactame resistance. *Klebsiella pneumoniae* was transfected with these CRISPR-Cas9 plasmids through electroporation. The CRISPR-Cas9 plasmids, effective in targeting these genes, caused double-stranded DNA breakage resulting in cell death (as demonstrated in other model systems). A non-specific GFP plasmid was transfected into *K. pneumoniae* to act as a positive control. Two plasmids targeting DeaD and bla(SHV-1) respectively, were transfected into *K. pneumoniae* to observe the targeted genes effect on colony count. Cultures were grown on Trimethoprim antibiotic selective media. Successful transfection of the CRISPR-Cas9 plasmids led to decreased colony count of *K. pneumoniae* when plated on selective media. Ultimately, these CRISPR-Cas9 plasmids can be fused with bacteriophage DNA to form a phagemid, which can then be tested as a novel therapeutic for treating antimicrobial resistant organisms.

INTRODUCTION

As of 2017 it is estimated that a minimum of 2 million illnesses and 23,000 deaths occur each year as a direct result of antibiotic resistance in the United States (CDC, 2017). Antibiotic resistance develops as misuse of antibiotics and continued evolution occurs in a bacterial genome. An outbreak of *K. pneumoniae*, in China has been identified as hypervirulent, highly drug-resistant, and highly transmissible even to healthy individuals (Gu *et al*., Lancet, 2017).

CRISPR is the adaptive immune system response of bacteria to help detect and destroy DNA from invaders by specifically cleaving or degrading the target DNA. The use of modern CRISPR technology has been suggested as a solution to the threat of antibiotic resistance due to the extreme precision of targeting specific sequences in the bacterial genome. The accidental or intentional altering of a bacterial genome utilizing CRISPR leads to cell death (Gomaa *et al*., American Society for Microbiology, 2014).

RESULTS

Table 1. Transformation efficiencies in gray and yellow *Klebsiella pneumoniae*

	Gray		Yellow	
	Run 1	Run 2	Run 1	Run 2
GFP 170 ng/mL	1.07×10^5	2.79×10^4	2.50×10^4	1.06×10^4

Two separate runs were performed for both colony types with the 1µL of blank (sterile water), GFP, DeaD, or bla(SHV-1) plasmid electroporated with 50µL of colony specific *K.pneumoniae* after which 10µL, 30µL, and 50µL amounts were plated. Interpretation of colony counts for plates occurred at 24 hrs. The concentration below GFP indicates concentration CRISPR-Cas9 DNA in that plasmid. This shows how successful transformation was after electroporation.

Figure 1: Gray *K. pneumoniae* culture plates
CFU (colony forming units) on Trimethoprim (30µg/mL) agar from 50 µL of gray *K. pneumoniae* and 1µL of sterile water, DeaD, and GFP electroporation suspensions plated to show growth differences.

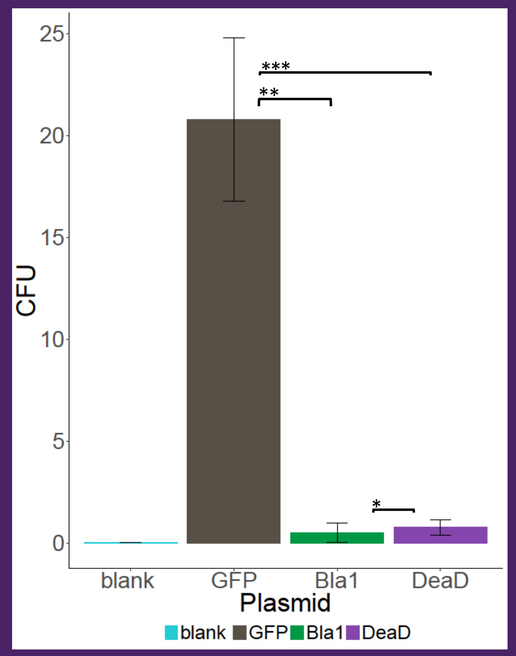

Figure 2: Mean CFU (colony forming units) per µl *K. pneumoniae*
Each target is shown. The negative blank control (blue). The positive GFP control (gray). The Bla1 (green) and the Dead (purple).
* p-value of 0.496
** p-value of 0.000963
*** p- value of 0.00100
Error bars indicate standard deviation from the mean.

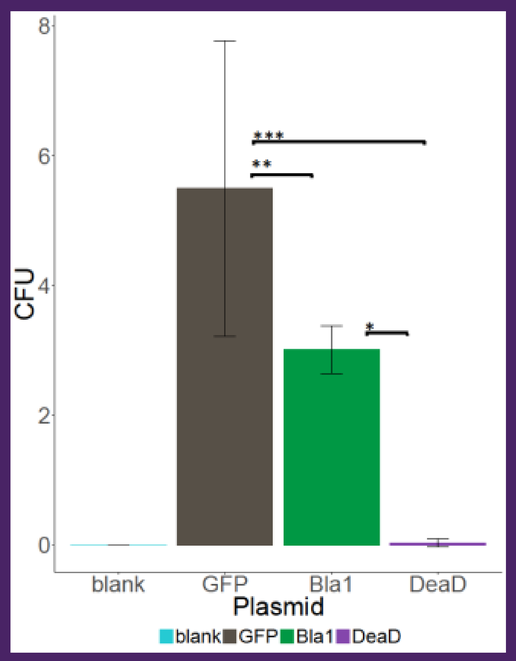

Figure 3: Mean CFU per µL in yellow *K. pneumoniae*
Each target is shown. The negative blank control (blue). The positive GFP control (gray). The Bla1 (green) and the Dead (purple).
* p-value of 0.000162
** p value of 0.135
*** p-value of 0.0142
Error bars indicate standard deviation from the mean.

All p values were calculated using a two-tailed Two Sample t-test assuming normal distribution, independence of variables, and equal variances. The ANOVA test done for each group had a p-value less than the alpha level 0.05, thus we concluded that the target plasmids were not the same.

Targeting and removal of the DeaD housekeeping gene successfully reduced growth in both colonies showing that is crucial for the survival of both organisms. The yellow colony up-took the bla(SHV-1) plasmid but did not show significantly reduced growth compared to the the positive control GFP, which means the bla(SHV-1) is not necessary for the survival of the yellow organism.

METHODS

CRISPR plasmids specific to *K. pneumoniae* (ATCC 700603) were created by the University of Utah CORE lab and were designed to target specific sequences in the housekeeping DeaD gene, beta-lactam resistance bla(SHV-1) gene, and a non-specific positive control GFP. Each plasmid also contained a Trimethoprim resistance gene which helped to select for positive transformants when grown on 30 µg/mL Trimethoprim agar. For the "blank", sterile water was added instead of plasmid which served as a negative control when plated on the Trimethoprim media.

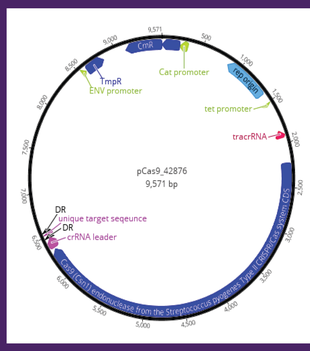

Figure 4: pCas9 Plasmid Design
Sequence specific to the targeting done in this research.

Two separate distinct colonies grew from the ATCC 700603 *K. pneumoniae* strain. One colony was named "gray" and the other "yellow". A MIC (minimal inhibitory concentration) panel was done, confirming that the colonies were *K. pneumoniae*. The gray colony was found to be resistant to Trimethoprim and the yellow one susceptible.

Electrocompetent gray and yellow *K. pneumoniae* each had a blank, GFP, DeaD, and bla(SHV-1) cuvette electroporated; each cuvette was plated on 30 µg/mL Trimethoprim media plates (warmed for 1 hour in air incubator at 47ºC) in 10µL, 30µL, and 50µL amounts.

Klebsiella pneumoniae was made electrocompetent by taking a colony less than 48 hours old and growing it up in 15 mL of SOB (super optimal broth) broth for at least 18 hours on a rotating air incubator at 37ºC with speed set to 250 rpm. With an optical density of 0.50 – 0.70, cells were spun down at room temperature on a Thermo Scientific ST16 Benchtop Centrifuge at 3,800 rpm for 10 minutes. After decanting, cells were re-suspended in 8 mL ice cold 10% glycerol, 2 mL were then placed into four 2 mL aliquots. Aliquots were spun on a Sorvall Legend Micro 21R microcentrifuge (4700 rpm, 10 min., 4ºC). Cells were decanted and re-suspended in 1 mL ice cold 10% glycerol and aliquots were combined in pairs. Aliquots were spun down at the same speed, time, and cold conditions; after each centrifugation step, aliquots were combined in pairs after decanting and re-suspending in 1 mL ice cold 10% glycerol. On the final resuspension of the last aliquot, 100 µL of bacterial suspension was pipetted into 10 separate fresh aliquots that were stored at -80ºC until used.

For transformation using electroporation, 50 µL of competent cells and 1µL of the specific CRISPR-Cas9 plasmid were combined, placed in a 0.1 cm Gene Pulser/MicroPulser cuvette, and electrically shocked (1,700 V, 25 µF, 200 Ω, time interval 4.0-4.4) using a Bio-Rad MicroPulser electroporation machine. The suspension was added to 500 µL of SOC (super optimal broth with catabolite) recovery broth and placed in an air incubator at 47ºC for 1 hour before being plated. Plates were placed in an air incubator at 47ºC to grow for 24 hours at which time the plates were read.

DISCUSSION

The yellow strain may have not had the bla(SHV-1) incorporated into it's genome as a crucial gene for survival like the gray strain did. This could be due the yellow strain harboring this gene on a pathogenicity island. The yellow strain could have also reverted back to a wild type strain rendering the ATCC 700603 type specific CRISPR-Cas9 system ineffective.

Further testing can be run to determine if resistance to beta-lactam drugs have been reduced. For future studies a bacteriophage could be fused with these CRISPR-Cas9 plasmids that target these crucial genomic sequences to form a phagemid which can then be tested as a novel therapeutic for treating antimicrobial resistant organisms.

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WEBER STATE UNIVERSITY
Dumke College of Health Professions
DEPARTMENT OF MEDICAL LABORATORY SCIENCES