2015

Klebsiella Pneumoniae Assimilates to Increasing Concentrations of Cephalothin by Differential Outer Membrane Porin Expression and Increased Production of Capsular Polysaccharides

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KLEBSIELLA PNEUMONIAE ASSIMILATES TO INCREASING CONCENTRATIONS OF

CEPHALOTHIN BY DIFFERENTIAL OUTER MEMBRANE PORIN EXPRESSION AND

INCREASED PRODUCTION OF CAPSULAR POLYSACCHARIDES

by

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A thesis submitted to the Honors Program

in partial fulfillment of the requirements for

Honors in the Major – Biology

UNIVERSITY OF NORTH FLORIDA

HONORS PROGRAM

December, 2015

Certificate of Approval

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Dedication

Mom, Dad, Co 5, Bao, and Nikla – thank you for always supporting and encouraging me to pursue my greatest potentials.

Acknowledgements

I would like to thank Dr. Terri Ellis for her patience, inspiration, and guidance throughout my time at the University of North Florida. She has been my professor and mentor through some of the most memorable experiences in my academic career. Because of your teachings, I feel I am ready to tackle the next big step in my career. I have developed valuable research and presentation skills through your encouragement and being exposed to international professional scientific communities. Thank you for being such a unique mentor who inspires critical thinking, creativity, and confidence.

Additionally, I would also like to extend my gratitude to Dr. Charles Goulet and Dr. Yusuke Kamiyoshihara, whom I worked with closely in the Klee Laboratory at the University of Florida. Thank you for being patient with me as you taught me how to pipette for the first time and teaching me what PCR stood for. Your inspiration has instilled in me the intellectual curiosity I value so much in my career. To Dr. Abba Zubair, thank you for allowing me to study with you at the Mayo Clinic and exposing me to the world of stem cell therapy. This has shaped the course I aim to take with my career. I am so grateful to have met you.

I deeply appreciate the enthusiasm and energy that LouAnne Hawkins has put into undergraduate research. Thank you for always inspiring your students to rise above the norm and reach their maximum potential. Your confidence in all of us has allowed me to truly realize what I am capable of. I am especially appreciative of the guidance you have given during the writing of my Honors Thesis.

The extensive work of this research project would not have been possible without the financial support from the UNF Transformational Learning Opportunity grant. It is also because of this grant that I am able to attend and present at the American Society for Microbiology General Meeting.

Finally, I would like to extend my endless appreciation to my family and friends. Your unconditional love and encouragement has allowed me to continue pursuing my dream of a career in medicine.

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Abstract

Klebsiella pneumoniae is an opportunistic Gram-negative nonmotile bacteria that causes nosocomial infections. In these bacteria, nutrients as well as antibiotics are able to diffuse through the outer membrane via outer membrane porins, transmembrane protein channels. Loss of outer membrane porins, increased capsule production, and a highly mucoid phenotype are commonly observed among antibiotic resistant isolates of this pathogen. The goal of this study was to investigate changes in outer membrane porin expression and capsular polysaccharide production by *K. pneumoniae* as it transitions to a β-lactam antibiotic resistant phenotype. *Klebsiella pneumoniae* strain 43816, which is susceptible to β-lactam antibiotics such as cepthalothin, was exposed to increasing concentrations of cephalothin over a 14-day period. During this period, daily samples of the bacteria were assessed for capsule synthesis, gene expression analysis, and changes in the physical appearance of the culture. At the end of the 14-day exposure, cells had assimilated to survive in 7.5 μg/mL of cephalothin, and had taken on a highly mucoid phenotype. High levels of capsular polysaccharides synthesis were confirmed by the uronic acid assay. Minimum inhibitory concentration assay showed treated cultures had become highly resistant to cephalothin. Two-step RT-qPCR demonstrated that this assimilated bacteria exhibited upregulation of *magA*, which has been linked to invasive, mucoid forms of *Klebsiella* infection. Additionally, cultures at the end of the treatment consistently showed downregulation of *ompK35* and *ompK36* and upregulation of *ompK26*. These data demonstrate that low-level exposure to cephalothin can induce significant changes in cellular phenotype that may impact both the resistance and virulence profile.

Introduction

   Klebsiella pneumoniae is a Gram-negative nonmotile opportunistic bacterium frequently responsible for nosocomial infections. This bacteria is abundant in the normal flora of humans, particularly in mucosal surfaces such as the nasopharynx and intestinal tract. Development of infection by opportunistic pathogens only begins once the immune system has been compromised. In the clinical realm, it is well known for being the culprit of community-acquired bacterial pneumonia. *K. pneumoniae* is also responsible for other infections, including urinary tract infections, soft tissue infections, and septicemia (Podschun 1998). It has been reported that *K. pneumoniae* is responsible for an estimated 20% of bacterial sepsis cases. Due to young children's immature immune system, patients in pediatric wards where systemic infections can easily set in are especially susceptible to *K. pneumoniae* (Neuhauser 2003).

   Recent data published by the Center for Disease Control has shown that in the past decade, infections by *K. pneumoniae* has significantly increased and that up to 50% of the strains in these cases have developed resistance to modern antibiotics (CDC 2013, Landman 2007). These clinical isolates are resistant to a wide spectrum of antibiotics, even antibiotics reserved as a last resort due its harsh effects on the liver. There has been an abundance of research on *K. pneunomiae* in its resistant state, but no study has focused on its transition from a susceptible to resistant state. Studying the pathogenesis associated with *K. pneumoniae* will provide a fundamental understanding of its mechanism and allow for alternative treatments of infection to be developed.

   Antibiotic resistance mechanisms are related to alterations within the bacterial membrane. The structure of Gram-negative bacteria involves two membranes, the outer membrane and the inner membrane. The outer membrane serves as a barrier and protects internal components of

bacteria from harmful molecules. On the outer membrane, there are proteins called outer membrane porins (OMP). These β-barrel proteins are the gateway for which antibiotics can transit through the outer membrane and go on to disrupt biochemical pathways, such as cell wall synthesis, essential to the survival of bacteria. Outer membrane porins are also essential for bacteria as it allows for diffusion of nutrients across the outer membrane. Loss of OMPs has been associated with cephalosporin antibiotic resistance acquisition in various bacterial strains (Hernandez-Alles 2000). This family of antibiotics contain a β-lactam ring that inhibits proper assembly of the bacterial cell wall once diffused through the porins. When exposed to cephalosporins, *K. pneumoniae* are able to downregulate the expression of these porins. The downregulation of OMPs can enhance the survival of bacteria and allow for activation of defense mechanisms that breaks down the structure of antibiotics (Martinez-Martinez 1999).

Klebsiella pneumoniae utilizes a variety of defense mechanisms to combat the deleterious effects of antibiotics diffusing into the periplasmic space. Outer membrane porin loss allows for the removal of a gate through which antibiotics can pass. Upregulation of efflux pumps will result in rapid removal of antibiotics into the extracellular space (Bialek-Davenet 2015, Padilla 2010). Extended spectrum β-lactamases, which are highly characteristic in resistant clinical isolates of *K. pneumoniae*, work to cleave and inactivate β-lactam antibiotics such as cephalothin. Finally, the increased production of capsular polysaccharides coats the bacterial cell with a barrier that is much less permeable to antibiotics. The combination of these membrane alterations have been known for the development of antibiotic resistance within *K. pneumoniae*.

Specific loss of OMPs has been shown to be a key feature within antibiotic resistant strains of *K. pneumoniae*, among other numerous species (Domenech-Sanchez 2003, Wang 2009). Of particular interest in the current study are the following outer membrane porins: OmpK26,

OmpK35, and OmpK36. OmpK35 and OmpK36 have been shown to be absent in *K. pneumoniae* strains resistant to antibiotics, particularly antibiotics within the family of cephalosporins (Domenech-Sanchez 2003, Kaczmarek 2006, Tsai 2011). OmpK35 and OmpK36 are major nonspecific outer membrane porins that allow for diffusion of numerous nutrients as well as deleterious antibiotics across the cell membrane and into the periplasmic space. In contrast, OmpK26 has been shown to be produced in abundance once OmpK35 and OmpK36 are lost. OmpK26 is a compensatory porin that sustains the fitness of *K. pneumoniae* and allows it to possess antibiotic resistance (Garcia-Sureda 2011). The alterations to these three outer membrane porins will be investigated as *K. pneumoniae* transitions to antibiotic resistant state.

Capsular polysaccharides aid in resistance largely due to decreasing the permeability of antibiotics through the outer membrane and into the periplasmic space. Clinically resistant strains of *K. pneumoniae* exhibit a hypermucoid phenotype that aid in conferring resistance to a wide spectrum of antibiotics as well as being responsible for causing liver abscesses. A gene cluster with 18 genetic components has been proposed to control production of capsular polysaccharides (Yeh 2006). *MagA* is a virulence gene most commonly linked to invasive mucoid forms of *K. pneumoniae* infection (Fang 2004). Other genes of interest in this present study include *rmpA* and *wza*. *RmpA* has been identified as being important in regulating capsular polysaccharide biosynthesis and *wza* is vital in the transport of capsular polysaccharides (Cheng 2010, Everard 2010). Transcriptional investigation of these three genes will aid in understanding the shift of capsular polysaccharide biosynthesis as *K. pneumoniae* transitions to an antibiotic resistant state.

The antibiotic that will be used in this study is cephalothin from the cephalosporin family of antibiotics. Cephalothin is an antibiotic commonly used in clinical settings to treat bacterial

infections and resistance acquisition occurs frequently. The purpose of this study is to investigate changes outer membrane porin expression and capsule polysaccharide production by *K. pneumoniae* as it transitions to a β-lactam antibiotic resistant state. As *K. pneumoniae* is exposed to increasing concentrations of cephalothin, it was hypothesized that gene expression levels of all three OMPs will fluctuate and allow for cephalothin assimilation. Furthermore, capsular polysaccharide production was expected to increase over time as an antibiotic defense mechanism. It was found that the cephalothin assimilation *K. pneumoniae* cultures exhibited downregulation of OmpK35 and OmpK36 while upregulating OmpK26. Furthermore, assimilated cultures obtained a mucoid phenotype with highly elevated levels of capsular polysaccharide production. Investigating these cellular and physiological changes allows for the understanding of the precise time point during which antibiotic resistance is developed.

Materials and Methods

Bacterial Culture and Induction of Resistant Strain. *Klebsiella pneumoniae* lab strain 43816 (ATCC) was grown overnight in 5mL LB media and incubated in a Thermo Scientific rotational shaker at 37ºC (Figure 1). From the overnight culture, 50 µL was removed and added to four separate culture tubes. Two culture tubes contained LB media with cephalothin at a concentration of 1.0 µg/mL. The remaining two tubes only contained LB media and served as the control. The culture was allowed to grow until early logarithmic phase ($OD_{600} = 1.0$) and total RNA was purified using Qiagen RNEasy Kit and Qiagen RNAprotect Bacteria Reagent. After 12 hours of the initial inoculation, 50 µL of bacterial was removed and added to four new test tubes containing fresh 5 mL LB media. The conditions remained the same. Bacterial cultures were once again allowed to grow to an early logarithmic phase and total RNA was extracted. At 24 hours after the initial inoculation, 50 µL of each sample was added to a new set of test tubes containing fresh 5 mL of LB media. The concentration of cephalothin was then increased by 0.5 µg/mL. This 24 hour procedural cycle was repeated daily, totaling to 14 days of cephalothin treatment.
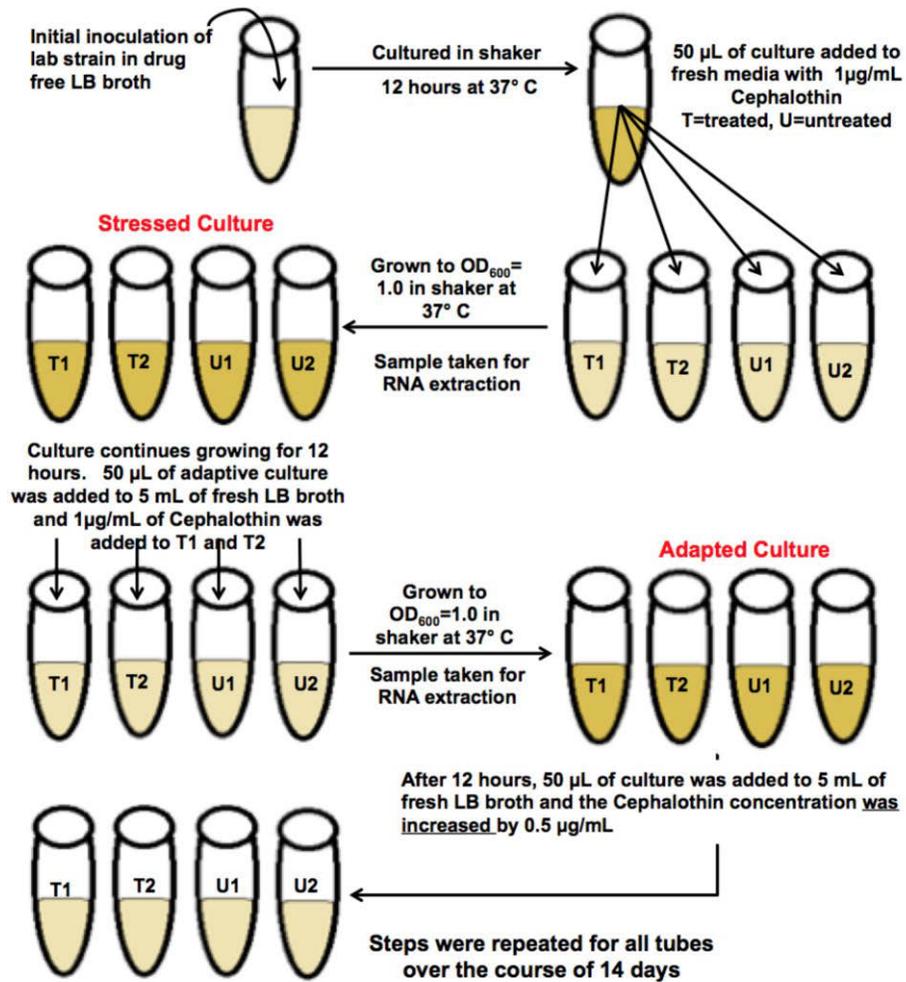
Figure 1 Induction of *Klebsiella pneumoniae* assimilation to increasing concentrations of cephalothin.

Gene Expression Quantification With Two-Step RT-qPCR. Total RNA from each sample was used to synthesize cDNA with NEB ProtoScript II Reverse Transcriptase and random hexamers primer. Gene of interest primers (Table 1) and synthesis cDNA was used to perform RT-PCR in combination with Sigma-Aldrich LuminoCt SYBR Green qPCR ReadyMix. Utilizing *GapA* for normalization, fold changes in gene expression were calculated through the $2\Delta\Delta C_T$ method (2001).

Table 1 Specific primers used in two-step RT-qPCR amplification of target interest genes in *Klebsiella pneumoniae* 43816.

Target Gene	Primer Sequence
magA	
Forward	5'—TGGCCAGTCCGAAAGTGAACGAAT—3'
Reverse	5'—TGCCAACAATTCCCGTTTCTGCTG—3'
ompK26	
Forward	5'—GAACAACGCCGGCAAAGATGATGA—3'
Reverse	5'—AGCTGCGGGCATAGACATAGTTCA—3'
ompK35	
Forward	5'—TTCGACAACGGTATCGCACTGTCT—3'
Reverse	5'—AGTACATGACGGCCGCATAGATGT—3'
ompK36	
Forward	5'—TCAGCGTGGTTTCGCATACTCTA—3'
Reverse	5'—TATTTCAGACCGCCGGTGTAGGTT—3'
rmpA	
Forward	5'—ACTGGACTACCTCTGTTTCATATTAC—3'
Reverse	5'—ATCCTGCAGTCAACCAATACTC—3'
wza	
Forward	5'—GGATCACCCGGAATTGACTAC—3'
Reverse	5'—TTCCAGCTACTTGGCACCTTAC—3'

Minimum Inhibitory Concentration Assay to Confirm Assimilation to Cephalothin.

Cephalothin treated, untreated, and *Klebsiella pneumoniae* lab strain 43816 cultures were

utilized in this MIC assay. In a 96-well polystyrene plate, cephalothin concentration range of 500

µg/mL to 0.49 µg/mL were used, where each step was a serial dilution. Bacterial samples were

diluted in 300 µL of LB media in each well and incubated for 10 hours at 37 ℃ without shaking.

The growth of *K. pneumoniae* at varying cephalothin concentrations was assessed using a

spectrophotometric plate reader.

Capsular Polysaccharide Extraction. Samples were grown overnight from glycerol stock with

cephalothin and 500 µL treated with 100 µL of 1% Zwittergent 3-14 detergent in 100 mM citric

acid (pH 2.0) for 30 minutes at 50 ℃, with vortexing. The bacteria were then pelleted by

centrifugation (14,000 RPM, 5 minutes) and 300 µL of supernatant was then transferred to a new

tube. Room temperature absolute ethanol was added to a final concentration of 80%. The CPS

was allowed to precipitate at 4 ℃ for 30 minutes and then centrifuged (14,000 RPM, 5 minutes).

The supernatants were decanted thoroughly and precipitates were allowed to dry on layer of

laboratory absorbents. Samples were allowed to resuspend overnight in 250 µL of 100 mM HCl.

This protocol was an adaptation from Domenico et al. (1989).

Quantification of Uronic Acid. Xanthan gum was used as a positive control in place of bacterial cultures (Mojica 2007) and a standard curve was generated. As adapted from Blumenkrantz et al. (1973), 0.2 mL of overnight samples were added to 1.2 mL of H_2SO_4/Tetraborate solution. All new tubes used in this assay were kept refrigerated beforehand. The samples were mixed well and heated in a water bath at 100 ℃ for 5 minutes. The tubes were then removed and cooled in an ice-water bath with 20 µL of a meta-hydroxydiphenyl in NaOH solution added. All samples were incubated for 10 minutes at room temperature and the samples' absorbances were measured at 520 nm using a UNICO Spectrophotometer.

Results

  As antibiotic concentrations increased, we observed changes to the culture morphology. This observation started at Day 2 (corresponding to a cephalothin concentration of 1.5 µg/mL) with increasing intensity at each subsequent treatment day and higher doses of cephalothin. Cephalothin treated bacterial cultures started producing small precipitates at early logarithmic growth phase, and finally exhibiting a distinct mucoid phenotype at the end of the 12-hour period (Figure 2). A distinct mucoid phenotype was apparent on the sides of the test tube and possessed a viscous characteristic.

Figure 2 Cephalothin treated bacterial cultures exhibited a mucoid phenotype (right), compared to the untreated cultures (left). Viscous residues adhering to the sides of the growth test tube indicated the mucoid phenotype.

Klebsiella pneumoniae lab strain 43816 was used to start the treated and untreated cultures. From the overnight culture of *K. pneumoniae* 43816, samples were treated with a non-inhibitory concentration of cephalothin and allowed to grow to early logarithmic growth phase. Twelve hours after the initial inoculation, cultures were transferred to fresh LB media containing the same concentration of cephalothin. Another 12 hours later, cultures were transferred to new LB media and the concentration of cephalothin was increased. This cycle of culture transfers and antibiotic treatment was repeated for a total of 14 days (Figure 1).

Bacterial cultures treated with cephalothin exhibited a greatly reduced growth rate, indicative of a high stress level (Figures 3a and 3b). As early as the second day of treatment, treated cultures required as long as 400 minutes to reach the early logarithmic growth phase ($OD_{600} = 1.0$) while untreated cultures only required approximately 120-160 minutes. For the entire duration of the 14-day treatment, treated cultures required more time to reach the required optical density. Initially, treated cultures required upwards of an extra 227 minutes, on average, to reach early logarithmic growth phase. This gap was eventually reduced to a difference as little as 25 minutes as the treated started to assimilate. However, at the higher cephalothin concentration (6.5 µg/mL), the difference in growth time between treated and untreated cultures was as much as 240 minutes.

a
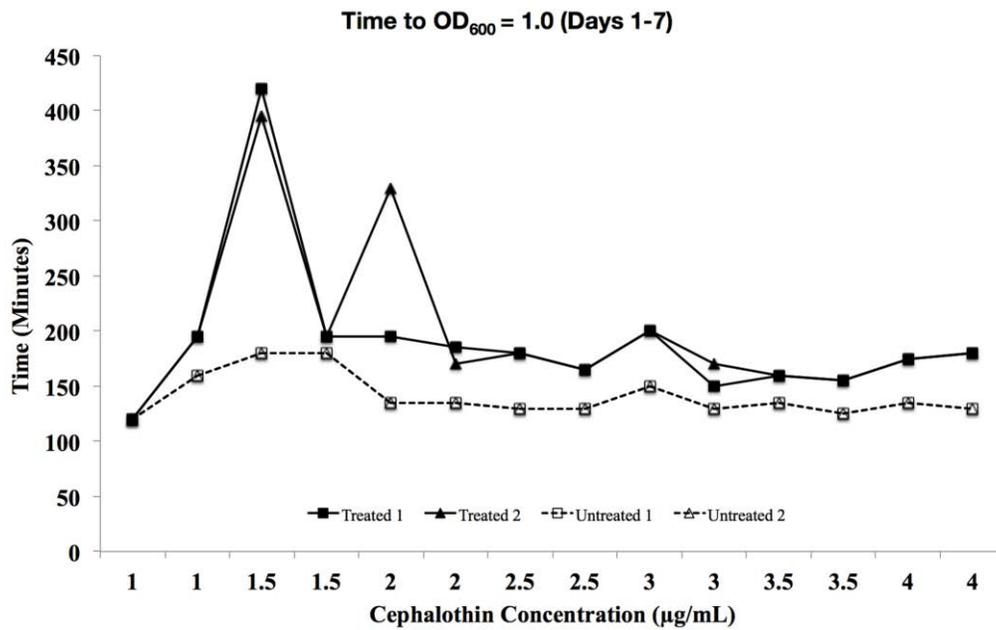
b

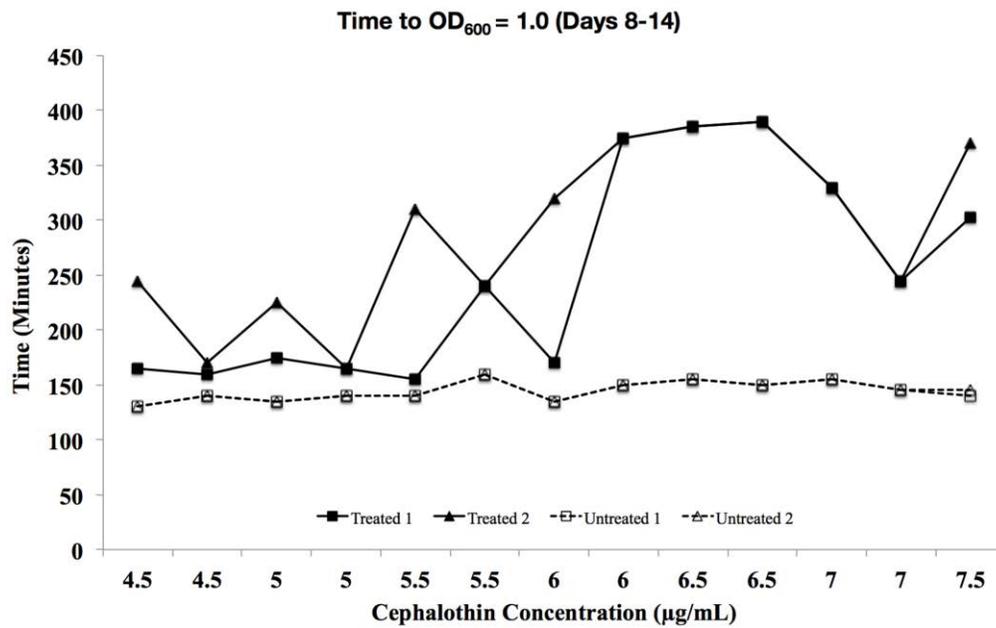Figure 3 Amount of time (minutes) bacterial culture required to reach early logarithmic growth phase ($OD_{600} = 1.0$) was lengthened in treated samples. Data shown represents Days 1-7 (a) and Days 8-14 (b). Each point on the x-axis represents a 12-hour interval, where cultures were transferred to fresh LB media and cephalothin was renewed. The concentration of cephalothin was increased every 24 hours.

A minimum inhibitory concentration assay was performed using both Day 14 assimilated cultures and Day 14 untreated cultures. Both Day 14 assimilated cultures exhibited efficient growth in a cephalothin dosage they were last exposed to (Figure 4). At half of that concentration, both Day 14 assimilated cultures grew almost as well as the *K. pneumoniae* 43816 control. These results suggest that the antibiotic treated *K. pneumoniae* cultures had assimilated to growing in a cephalothin rich environment.
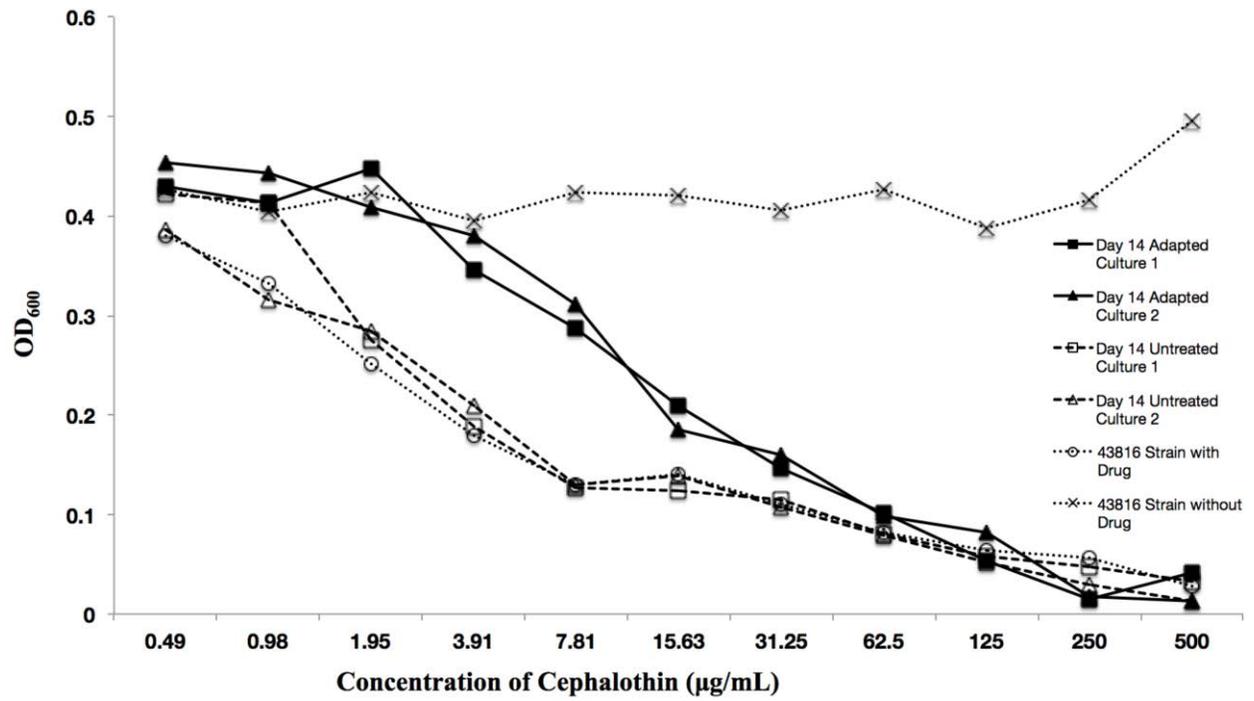
Figure 4 Minimum Inhibitory Concentration assay confirmed treated cultures have assimilated to a cephalothin rich environment. Absorbance values were measured 10 hours after inoculation of cultures.

In order to investigate transcriptional activity of antibiotic resistance associated genes, two-step RT-qPCR was performed. The two major nonspecific outer membrane porins, OmpK35 and OmpK36, prevents the entry of cephalothin when downregulated while upregulation of the specific porin OmpK26 allows for diffusion of only nutrients. Initially, all three outer membrane porin genes were highly upregulated in the treated samples (Figure 5). As the cephalothin drug concentration increased throughout the 14-day treatment, the gene expression levels fluctuated and mostly showed trend of downregulation. At the end of the 14-day antibiotic treatment, OmpK35 and OmpK36 were both downregulated. Contrarily, OmpK26 was highly upregulated. This is consistent with what is known about the major outer membrane porins. OmpK35 and OmpK36 is typically downregulated in antibiotic resistant clinical strains of *K. pneumoniae* while OmpK26 is upregulated to help maintain fitness. These results suggest the cephalothin treated *K. pneumoniae* cultures was assimilating to the antibiotic rich environment through transcriptional regulation of essential outer membrane porins.
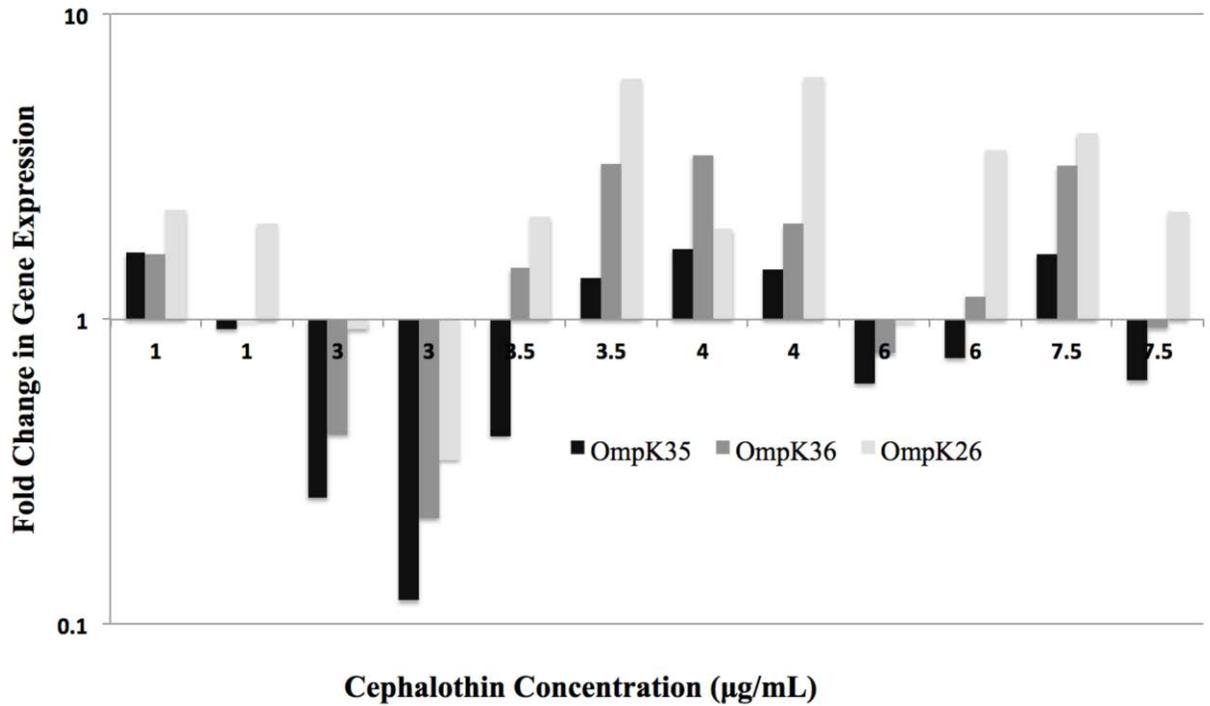
Figure 5 Fold change in gene expression of major outer membrane porin genes. Data shown indicates the average fold change in gene expression of treated cultures, compared to untreated cultures. Each data point on the x-axis represents an antibiotic treatment interval.

As the concentration of cephalothin increased over the course of the 14-day treatment, the mucoid phenotype became increasingly more apparent. The initial stress caused by exposure to a low dose of cephalothin induced the upregulation of *rmpA* and *wza* transcripts, but no upregulation of *magA* was observed (Figure 6). Towards the middle of the treatment at a cephalothin concentration of 3.5 µg/mL, *magA* had a fold change in gene expression of 25.57 while *rmpA* and *wza* had a fold change of 2.93 and 2.21, respectively. All three genes associated with the biosynthesis of capsular polysaccharides still remained upregulated by the end of the 14-day treatment.

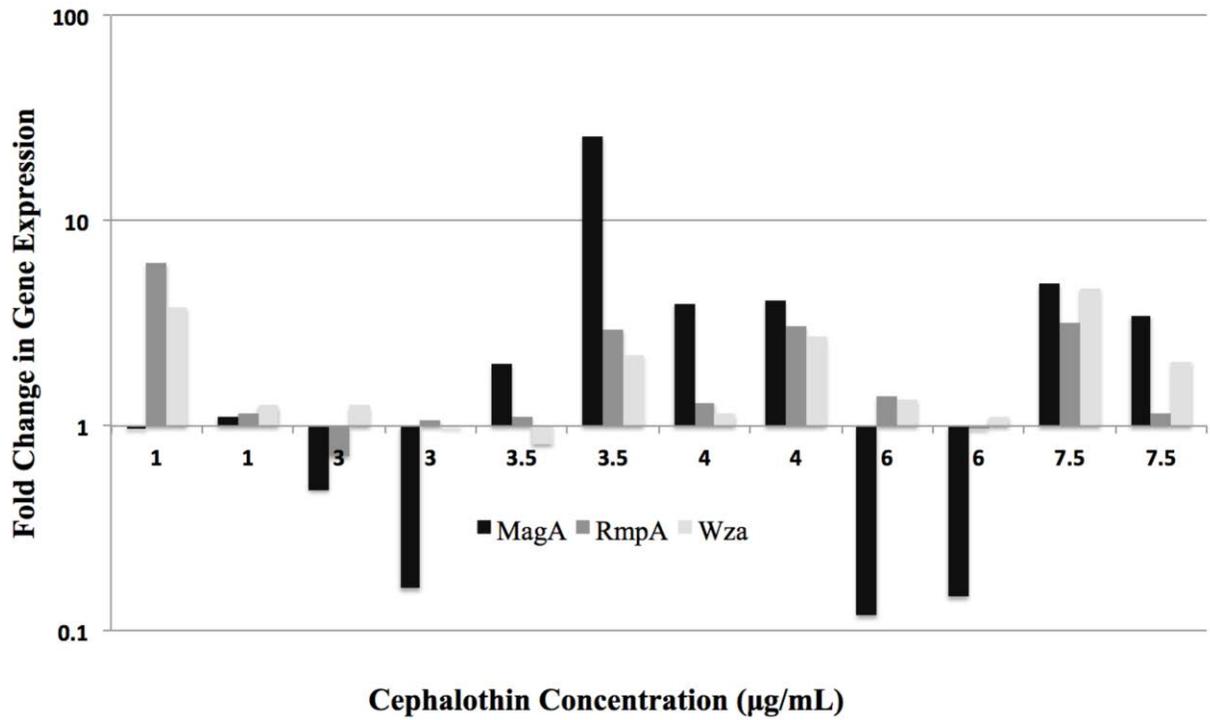Figure 6 Fold change in gene expression of genes associated with capsular polysaccharide biosynthesis. Data shown indicates the average fold change in gene expression of treated cultures, compared to untreated cultures. Each point on the x-axis represents an antibiotic interval.

Clinical strains of *Klebsiella pneumoniae* that are resistant to a spectrum of antibiotics typically exhibit a highly mucoid phenotype. Elevated levels of capsular polysaccharides provide an impermeable barrier inhibiting antibiotic passage through the outer membrane. Capsular polysaccharides were extracted from *K. pneumoniae* cultures in the 14-day treatment in order to assess biosynthesis levels. Uronic acids are present in tandem repeats within capsular polysaccharides. Spectrophotometric absorbance quantitation of uronic acid using a wavelength of 520nm allowed for the indirect measurement of capsular polysaccharide production by each bacterial culture (Figure 7). Initially, uronic acid levels were low in both treated and untreated samples. Day 11 and Day 14 (corresponding to cephalothin concentrations of 6 and 7.5 µg/mL, respectively) exhibited greatly elevated levels of uronic acid. Treated cultures that showed upregulation of capsular polysaccharide gene transcripts also had increased uronic acid levels. These data suggest that in response to higher concentrations of cephalothin, *K. pneumoniae* produced higher amounts of capsular polysaccharide, as indicated by elevated levels of uronic acid.
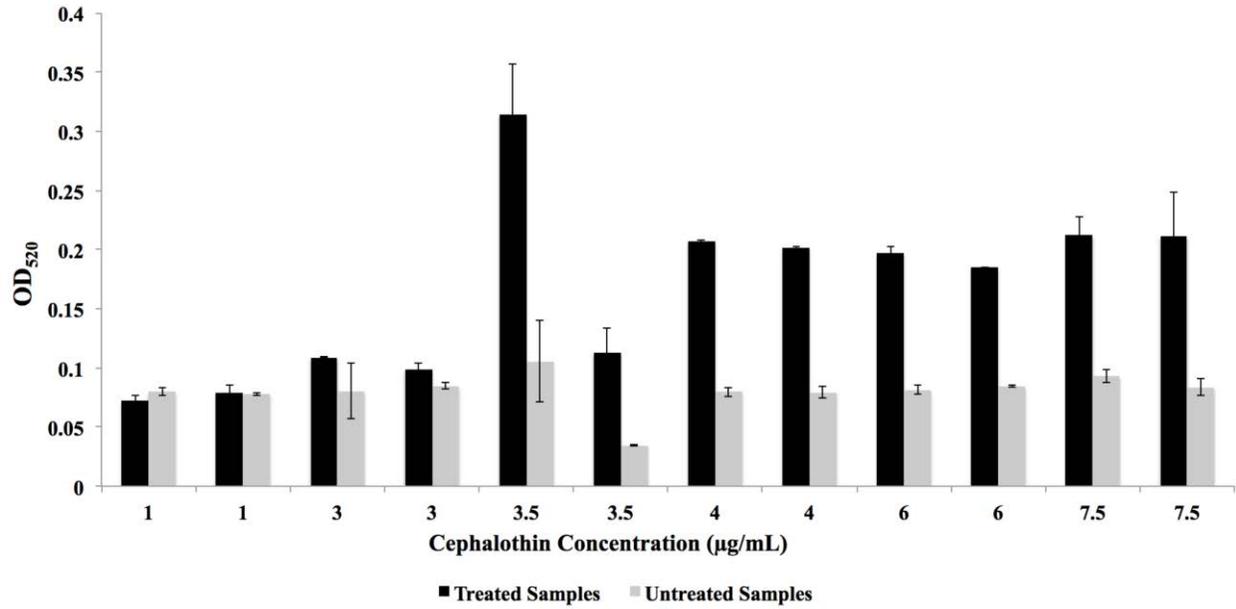
Figure 7 Cephalothin treated samples contained a higher concentration of uronic acid, indicative of elevated capsular polysaccharide biosynthesis. Data shown represents the average of treated and untreated samples. Each data point on the x-axis represents an antibiotic treatment interval.

Discussion

Nosocomial infections by *Klebsiella pneumoniae* are widely studied in the clinical realm. These pathogens typically thrive in the clinical setting due to increased presence of patients that are immunocompromised. Even with an aggressive antibiotic regimen prescribed, the immune system is unable to completely rid of the opportunistic strains, leading to antibiotic resistance development and proliferation of resistant *K. pneumoniae*. Assimilations such as increased β-lactamase activity, differential regulation of outer membrane porins, upregulation of efflux pumps, and increased production of capsular polysaccharides all influence antibiotic resistance in *K. pneumoniae.* Similarly, other bacterial species, namely *Escherichia coli*, utilize assimilations such as acquiring plasmids coding for β-lactamases to evolve antibiotic resistance (Adler 2013). Here, we have shown that laboratory induced cephalothin resistant *Klebsiella pneumoniae* gradually altered its outer membrane porin expression and capsular polysaccharide biosynthesis in order to increase survivability. The results of this study have also hinted at a critical point during which cephalothin resistance developed.

In order to induce antibiotic resistance, *Klebsiella pneumoniae* 43816 was initially exposed to a non-inhibitory concentration of cephalothin and then the concentration was gradually increased. The treatment of *Klebsiella pneumoniae* with increasing concentrations of cephalothin greatly reduced its growth rate. Throughout the entire duration of the 14-day antibiotic treatment, both treated cultures required a longer amount of time to reach an OD_{600} of 1.0. This can suggest that the bacteria were possibly stressed and that surviving cells were forced to utilize assimilative mechanisms until it was able to proliferate and enter a logarithmic growth phase. As early as Day 2, treated cultures started showing formation of faint precipitates and a slight mucoid phenotype. The mucoid phenotype was much more pronounced and distinct towards the middle of the 14-

day treatment, where thick residues formed and stuck to the sides of the growth test tubes. Elevated capsular polysaccharide production as confirmed by uronic acid quantitation was consistent with this observation. By the end of the 14-day antibiotic treatment, cultures treated with cephalothin exhibited a very thick and slimy coating on the sides of the growth test tube above the LB media level. It has been previously shown that antibiotic presence at MIC levels is sufficient to induce hyper production of capsular polysaccharides (Held 1995). Other bacterial species such as *Acinetobacter baumannii* have also been shown to respond to presence of antibiotics by significantly increasing production of capsular polysaccharide as one defense mechanism (Geisinger 2015). *K. pneumoniae* strains with this 'hypermucoviscous' phenotype have been classified to be associated with numerous invasive diseases, or community-acquired *K. pneumonia* bacteraemia (Lee 2006).

In the clinical realm, antibiotic resistant strains of *Klebsiella pneumoniae* commonly cause abscesses, particularly liver abscess. Capsular serotype K1 of *Klebsiella pneumoniae* containing the loci with *magA* and *wza* has been identified as one of the leading serotypes associated with primary pyrogenic liver abscess (Chuang 2006, Fang 2010). The gene *wza* has been identified as having an important role in the transport of capsular polysaccharides (Evrard 2010). *Wza* codes for an integral outer membrane protein facilitating the transport of capsular polymers across the bacterial membrane to its extracellular space (Dong 2006). It has also been suggested that *rmpA* is an important regulator and aids in initiating capsular polysaccharide biosynthesis (Cheng 2010). The synergistic effect and prevalence of both *magA* and *rmpA* induce the 'hypermucoviscous' phenotype and is responsible for numerous cases of *K. pneumoniae* bacteraemia (Chang 2013, Yu 2006). Their prevalence can be largely due to capsular polysaccharides significantly inhibiting the actions of antimicrobial peptides and the innate

immune system (Campos 2004).

In this study, initial exposure to cephalothin stressed *K. pneumoniae* and at the transcriptional level, many of the genes of interest were upregulated. As concentrations of cephalothin increased, gene expression levels fluctuated until Day 6 (corresponding to a cephalothin concentration of 6 µg/mL), where there is consistent differential expression of outer membrane porin genes linked to antibiotic resistance. At the end of the 14-day treatment, cephalothin treated *K. pneumoniae* cultures consistently upregulated *ompK26* while downregulating *ompK35* and *ompK36.* Assimilation of cephalothin was confirmed by an MIC assay and qPCR data suggested that *K. pneumoniae* likely became resistant to cephalothin around Day 6. Downregulation of *ompK35*/*ompK36* greatly reduced the ability of cephalothin to diffuse into the periplasmic space and inhibit growth while the upregulation of *ompK26* helped to maintain fitness.

A distinct mucoid phenotype was also observed starting on Day 6. Genes associated with biosynthesis of capsular polysaccharides, *magA, rmpA,* and *wza*, were highly upregulated at this time point and persisted until the end of the 14-day treatment. Uronic acid quantitation also showed increased presence of capsular polysaccharides in cephalothin treated cultures starting at Day 6. These factors help to explain that *K. pneumoniae* had started to become assimilated to a cephalothin rich environment as a denser network of capsular polysaccharides can reduce the diffusion of cephalothin across the bacterial outer membrane. After all, resistance is futile.

The findings of this study indicated that lower doses of cephalothin could initiate dramatic cellular and physiological changes within *Klebsiella pneumoniae* that leads to increased survival and antibiotic resistance. A typical antibiotic regimen prescribed by physicians includes 14-day doses of respective antibiotics. However, many patients often do not conclude the treatment

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regimen and can fall ill to another bacterial infection. Subsequent treatments of more antibiotics fail to work due to the acquisition of antibiotic resistant strains. Furthermore, these patients pose a significant risk to other immunocompromised patients, as they are likely to spread these antibiotic resistant strains.

References

Adler M, Anjum M, Andersson DI, Sandegren L. 2013. Influence of acquired β-lactamases on the evolution of spontaneous carbapenem resistance in *Escherichia coli*. *J Antimicrob Chemother*. 68: 51-59.

Bialek-Davenet S, Lavigne J-P, Guyot K, Mayer N, Tournebize R, Brisse S, Leflon-Guibot V, Nicolas-Chanoine M-H. 2015. Differential contribution of AcrAB and OqxAB efflux pumps to multidrug resistance and virulence in *Klebsiella pnuemoniae*. *J Antimicrob Chemother*. 70: 81-88.

Blumenkrantz N, Asboe-Hansen G. 1973. New method for quantitative determination of uronic acids. *Analytical Biochemistry*. 54: 484-489.

Campos MA, Vargas MA, Regueiro V, Llompart CM, Alberti S, Bengoechea JA. 2004. Capsule polysaccharide mediates bacterial resistance to antimicrobial peptides. *Infection and Immunity*. 72(12): 7107-7114.

Control, C. f. D. 2013. Vital Signs: carbapenem-resistant *Enterobacteriaceaea*. MMWR Morbidity and Mortality Weekly Report: 62: 165-170.

Chang L, Bastian I, Warner M. 2013. Survey of *Klebsiella pneumoniae* bacteraemia in two South Australian hospitals and detection of hypermucoviscous phenotype and *magA/rmpA* genotypes in *K. pneumoniae* isolates. *Infection*. 41: 559-563.

Cheng HY, Chen YS, Wu CY, Chang HW, Lai YC, Peng HL. 2010. RmpA regulation of capsular polysaccharide biosynthesis in *Klebsiella pneumoniae* CG43. *J. Bacteriol*. 192(12): 3144-3258.

Chuang Y-P, Fang C-T, Lai S-Y, Chang S-C, Wang J-T. 2006. Genetic determinants of capsular serotype K1 of *Klebsiella pneumoniae* causing primary pyogenic liver abscess. *J Infect Dis*. 193: 645-654.

Domenico P, Diedrich DL, Cunha BA. 1989. Quantitative extraction and purification of exopolysaccharides from *Klebsiella pneumoniae*. *Journal of Microbiological Methods*. 9: 211-219.

Domenico P, Schwartz S, Cunha BA. 1989. Reduction of capsular polysaccharide production in *Klebsiella pneumoniae* by sodium salicylate. *Infect. Immun.* 57(12): 3778-3782.

Domenech-Sanchez A, Martinez-Martinez L, Hernandez-Alles S, Conejo MdC, Pascual A, Tomas JM, Alberti S, and Benedi VJ. 2003. Role of *Klebsiella pneumoniae* OmpK35 porin in antimicrobial resistance. *Antimicrobial Agens and Chemotherapy*. 47(10): 3332-3335.

Dong C, Beis K, Nesper J, Brunkan AL, Clarke BR, Whitfield C, Naismith JH. 2006. The structure of Wza, the translocon for group 1 capsular polysaccharides in *Escherichia coli*, identifies a new class of outer membrane protein. *Nature*. 444(7116): 226-229.

Evrard B, Balestrino D, Dosgilbert A, Bouya-Gachancard J-LJ, Charbonnel N, Forestier C, Tridon A. 2010. Roles of capsule and lipopolysaccharide O antigen in interactions of human monocyte-derived dendritic cells and *Klebsiella pneumoniae*. *Infection and Immunity*. 78(1): 210-219.

Fang C-T, Chuang Y-P, Shun C-T, Chang S-C, Wang J-T. 2004. A novel virulence gene in *Klebsiella pneumoniae* strains causing primary liver abscess and septic metastatic complications. *J Exp Med*. 199(5): 697-705.

Fang C-T, Lai S-Y, Yi W-C, Hsueh P-R, Liu K-L. 2010. The function of *wzy*_K1 (*magA*), the serotype K1 polymerase gene in *Klebsiella pneumoniae cps* gene cluster. *The Journal of Infectious Diseases*. 201: 1268-1269.

Garcia-Sureda L, Domenech-Sanchez A, Barbier M, Juan C, Gasco J, and Alberti S. 2011. OmpK26, a novel porin associated with carbapenem resistance in *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy*. 55(10): 4742-474.

Geisinger E, Isberg R. 2015. Antibiotic modulation of capsular exopolysaccharide and virulence in *Acinetobacter baumannii*. *PLoS Pathog*. 11(2): e1004691.

Held TK, Adamczik C, Trautmann M, Cross AS. 1995. Effects of MICs and sub-MICs of antibiotics on production of capsular polysaccharide of *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother*. 39(5): 1093-1096.

Hernandez-Alles S, Conejo M, Pascual A, Tomas JM, Benedi VJ, and Martinez-Martinez L. 2000. Relationship between outer membrane alterations and susceptibility to antimicrobial agents in isogenic strains of *Klebsiella pneumoniae*. *The Journal of Antimicrobial Chemotherapy*. 46: 273-277.

Kaczmarek FM, Dib-Hajj F, Shang W, and Gootz TD. 2006. High-level carbapenem resistance in a *Klebsiella pneumoniae* clinical isolate is due to the combination of bla-ACT-1 beta-lactamase production, porin OmpK35/36 insertional inactivation, and down-regulation of the phosphate transport porin PhoE. *Antimicrobial Agents and Chemotherapy*. 50(10): 3396-3406.

Landman D, Bratu S, Kochar S, Panwar M, Trehan M, Doymaz M, and Quale J. 2007. Evolution of antimicrobial resistance among *Pseudomonas aeruginosa, Acinetobacter baumannii* and *Klebsiella pneumoniae* in Brooklyn, NY. *The Journal of Antimicrobial Chemotherapy*. 60: 78-82.

Lee H-C, Chuang Y-C, Yu W-L, Lee N-Y, Chang C-M, Ko N-Y, Wang L-R, Ko W-C. 2006. Clinical implications of hypermucoviscosity phenotype in *Klebsiella pneumoniae* isolates: association with invasive syndrome in patients with community-acquired bacteraemia. *Journal of Internal Medicine*. 259: 606-614.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods*. 25: 402-408.

Mojica K. Elsey D. Cooney MJ. 2007. Quantitative analysis of biofilm EPS uronic acid content. *Journal of Microbiological Methods*. 71: 61-65.

Martinez-Martinez L, Pascual A, Hernandez-Alles S, Alvarez-Diaz D, Suarez AI, Tran J, Benedi VJ, and Jacoby GA. 1999. Roles of beta-lactamases and porins in activities of carbapenems and cephalosporins against *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy*. 43(7): 1669-1673.

Nelson K, Whittam TS, Selander RK. 1991. Nucleotide polymorphism and evolution in the glyceraldehyde-3-phosphate dehydrogenase gene (*gapA*) in natural populations of *Salmonella* and *Escherichia* coli. *Proceedings of the National Academy of Sciences of the USA*. 88: 6667-6671.

Neuhauser MM, Weinstein WR, Rydman R, Danziger LH, Karam G, and Quinn JP. 2003. Antibiotic resistance among gram-negative bacilli in the US intensive care units: implications for fluoroquinolone use. *The Journal of the American Medical Association*. 289: 885-888.

Padilla E, Llobet E, Domenech-Sanchez A, Martinez-Martinez L, Bengoechea JA, Alberti S. 2010. *Klebsiella pneumoniae* AcrAB efflux pump contributes to antimicrobial resistance and virulence. *Antimicrob Agents Chemother*. 54(1): 177-183.

Podschun R, and Ullmann U. 1998. *Klebsiella spp.* as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clinical Microbiology Reviews*. 11: 589-603.

Tsai YK, Fun CP, Lin JC, Chen JH, Chang FY, Chen TL, and Siu LK. 2011. *Klebsiella pneumoniae* outer membrane porins OmpK35 and OmpK36 play roles in both antimicrobial resistance and virulence. *Antimicrob Agents Chemother*. 55(4): 1485-1493.

Wang XD, Cai JC, Zhou HW, Zhang ZR, and Chen GX. 2009. Reduced susceptibility to carbapenems in *Klebsiella pneumoniae* clinical isolates associated with plasmid-mediated beta-lactamase production and OmpK36 porin deficiency. *Journal of Medical Microbiology*. 58: 1196-1202.

Yeh K-M, Lin J-C, Yin F-Y, Fung C-P, Hung H-C, Siu L-K, Chang F-Y. 2010. Revisiting the importance of virulence determinant *magA* and its surrounding genes in *Klebsiella pneumoniae* causing pyogenic liver abscesses: exact role in serotype K1 capsule formation. *Journal of Infectious Diseases*. 201: 1259-1267.

Yeh K-M, Chang F-Y, Fun C-P, Lin J-C, Siu LK. 2006. *MagA* is not a specific virulence gene for *Klebsiella pneumoniae* strains causing liver abscess but is part of the capsular polysaccharide gene cluster of *K. pneumoniae* serotype K1. *Journal of Medical Microbiology*. 55: 803-804.

Yu W-L, Ko W-C, Cheng K-C, Lee H-C, Ke D-S, Lee C-C, Fung C-P, Chuang Y-C. 2006. Association between *rmpA* and *magA* genes and clinical syndromes caused by *Klebsiella pneumoniae* in Taiwan. *Clinical Infectious Diseases*. 42: 1351-1358.

Vita

Nghi Lam was born in Ho Chi Minh City, Vietnam and lived there for 5 years before immigrating to Florida. He was raised in Coral Springs, FL by his parents, Tuyen and Van Lam, and his aunt, Phuong Lam, alongside his brother, Bao Lam. Before enrolling at the University of North Florida, Nghi received his first Bachelor's Degree from the University of Florida where he majored in History. At UF, he was a competitor on the university's cycling team. He also spent majority of his free time volunteering at the UF College of Medicine Equal Access Clinic, Habitat for Humanity, and on international medical relief missions in The Gambia and Vietnam.

Nghi graduated from the University of North Florida in Jacksonville, FL with Honors in the Major with a Bachelor of Science in Biology. He concentrated in the Biomedical Sciences while minoring in Public Health as well as Clinical and Applied Movement Sciences. He has been an active volunteer at Volunteers in Medicine and the National MS Society while also coaching at risk adolescents regarding fitness and maintaining a healthy lifestyle. In the summer of 2015, Nghi biked across the United States on a trek that spanned nearly 4,000 miles from Yorktown, VA to San Francisco, CA. It was a life-changing experience that aimed to fulfill the mission of raising research funding and awareness for multiple sclerosis.

Nghi has accepted a Post-Baccalaureate Cancer Research Training Award from the National Institutes of Health. He will be a research fellow within the National Cancer Institute investigating genetic risk variants contributing to melanomagenesis. After concluding his training at the NIH, Nghi aspires to attend medical school to fulfill his dream of a career in academic medicine.