Title

Investigating Antibiotic Resistant Uropathogens in Merced: Sources and Resources

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Investigating Antibiotic Resistant Uropathogens in Merced: Sources and Resources

A dissertation in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Quantitative and Systems Biology

by

Candace Cole

Committee in charge:

Professor Marcos E. García-Ojeda, Chair
Professor Miriam Barlow, Advisor
Professor Mark Sistrom
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2022

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2022

I dedicate this to my parents, Leticia and Richard Cole.
I am forever grateful for the sacrifices they made to get me here.

I dedicate this to my grandma, Rafaela Guzman, who believed in me every step of the way.
I only wish she were here to see this.

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List of Abbreviations

CDC, Center for Disease Control
E. coli, *Escherichia coli*
UPEC, Uropathogenic *E. coli*
DHMMC, Dignity Health Merced Medical Center
PCR, Polymerase Chain Reaction
ESBL, Extended Spectrum β-lactamase
NCBI, National Center for Biotechnology Information
bla, β-lactamase gene
K. pneumoniae, *Klebsiella pneumoniae*
U.S., The United States of America
p-value, measure of statistical significance
FDR, false discovery rate
NIH, National Institute of Health
bp, base pair
DNA, Deoxyribonucleic Acid
NEB, New England Biolabs
BLAST, Basic Local Alignment Search Tool
ANOVA, Analysis of Variance
Z-test, statistical test of population means
PC, Phi coefficient
UTI, Urinary Tract Infection
TS33, Type III secretion system
PCA, principal components analysis
Non-UPEC, samples not considered UPEC
DH5α, electrocompetent *E. coli* strain for molecular cloning
COST, vector and vector construct containing different combinations of blaCTX-M-15, blaOXA-1, blaSHV-2, and blaTEM-10
pBR322, cloning plasmid vector containing ampicillin and tetracycline selectable markers
n-BLAST, nucleotide BLAST
SOC, Super Optimal Broth with catabolite expression
LB, Lysogeny Broth
LB-tet, Lysogeny Broth with 1:200 tetracycline
MH, Mueller-Hinton agar or broth
CRO, Ceftriaxone
IPM, Imipenem
FOX, Cefoxitin
AMC, Amoxicillin and Clavulanic Acid
CTT, Cefotetan
CAZ, Ceftazidime
AM, Ampicillin
MIC, Minimum Inhibitory Concentration
MHII, Cation adjusted Mueller-Hinton Broth
EUCAST, European Committee on Antimicrobial Susceptibility Testing
CLSI, Clinical & Laboratory Standards Institute
mm, millimeters
E240K, Glutamic acid substitution for lysine at position 240

List of Symbols

ß, beta
α, alpha
%, percent of one hundred
°, denotes degrees
µ, Micro
*, denotes statistical significance

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Abstract

Antibiotic resistant infections are a public health threat. According to the CDC, the incidence of antibiotic resistant infections has increased 50% in the last five years[1]. Urinary tract infections (UTIs) are the most commonly occurring bacterial infection and resistance among them is highly prevalent. Lack of mandatory reporting of these infections, and a vast array of treatment options, place UTIs at the frontiers of newly evolved resistance. To investigate this important human health threat, I performed three studies. First, I performed a surveillance study of antibiotic resistance trends in uropathogenic *E. coli* (UPEC) at Dignity Health Mercy Medical Center (DHMMC) in Merced, and compared them to resistance trends found nationwide. I found higher incidence of resistance at DHMMC than nationwide. Second, to identify causes for this elevated resistance, I investigated potential environmental sources of antibiotic resistant infections at DHMMC. In doing so, I developed a rapid PCR screen for identifying UPEC using a suite of six genetic markers significantly associated with UPEC. Using this screen, I found evidence of UPEC in all environmental sites surveyed, most associated with livestock for retail meat. Third, to identify the fitness effects of antibiotic resistance genes upon bacteria being treated with antibiotics, and to further infer the effects of antibiotic consumption on the selection of the antibiotic resistance genes, I created a customizable plasmid vector construct library. This library contains all possible combinations of the four most identified resistance genes at DHMMC, and it can be used to evaluate their fitness, relative to one another, in the presence and absence of different antibiotics. In summary, I characterized the antibiotic resistance trends at DHMMC, identified potential sources of the resistance, and developed an experimental tool to evaluate the ongoing selection from antibiotic consumption resulting in the resistance trends at DHMMC.

Chapter 1: Introduction

Urinary tract infections (UTIs) are the most common type of bacterial infection in the developed world[2]. UTI from catheterization is the most common nosocomial infection[3]. A UTI is characterized as the presence of more than 10^5 bacteria in the urine. These bacteria can come from an external source or the native gut flora. Uropathogenic *E. coli* (UPEC) are the most common causative agent of UTIs. Women are more prone to UTIs due to a short urethra and proximity to fecal matter[4]. The severity of UTIs range from asymptomatic to complicated, which can result in kidney infection. Typical treatment for UTI is 3-7 day treatment with β-lactam antibiotics[4]. β-lactam antibiotics have been in use since the 1940s, and they continue to be the most widely used antibiotics due to their high effectiveness, ease of delivery, and low toxicity[5,6]. The longstanding use of β-lactam antibiotics has led to the emergence of resistant strains in clinical care settings[7]. UTIs are becoming increasingly hard to treat due to resistance to multiple types of antibiotics, including β-lactams[8].

The current empirical treatment for UTIs is up to the prescribing physician and is based on loose guidelines of broad spectrum antibiotics including fluroquinolones, cephalosporins, and aminoglycosides, often without antibiotic susceptibility testing[9–11]. The treatment of UTIs should take into consideration the current antimicrobial susceptibility patterns, and predictability, of the causative agent to avoid ineffective treatment[11].

The continuous use of β-lactam antibiotics has led to the selection and evolution of successful β-lactamase genes present in UPEC [12]. B-lactamase genes produce extended spectrum β-lactamase (ESBL) enzymes that work by hydrolyzing β-lactam antibiotics, rendering them ineffective. The most common β-lactamase genes bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, and bla_{OXA} have been previously identified in UPEC, and result in resistance to β-lactam antibiotics[13,14].The increasing antibiotic resistance in UTIs is of great concern to public health. The emergence of the ESBL $bla_{CTX\text{-}M\text{-}15}$ in community acquired UTIs has resulted in ineffective treatment with the later generation β-lactam antibiotics, substantially limiting the available antibiotic treatment options[12,15].

There are many roadblocks to solving the problem of resistance in UPEC that result from holes in the technical and intellectual status quo of the antibiotic research community.

1. We do not understand the relative contributions of local vs. world-wide antibiotic consumption on the occurrence of antibiotic resistance in defined locations, such as hospitals and communities.

2. We do not understand the role of environmental reservoirs of resistant UPEC in human infections. It is important to understand the source of UPEC causing these infections as a promising route to limit the spread of UPEC within a community.

3. We do not understand the relative contributions of the myriad of available resistance genes to resistance in UPEC. It is important to understand the agents of selection that drive the dissemination of antibiotic resistance among UPEC.

My dissertation studies have centered on overcoming these technical and intellectual hurdles. Using innovative approaches, I have initiated research in previously unaddressed, and highly important areas of the antibiotic resistance problem as follows:

1. Currently, there is no comparative data on antibiotic resistance in community acquired UTIs[16]. Previous guideline for the treatment of UTIs are compromised by changing patterns of antibiotics resistance in UPEC[16]. It is critical to know the current local antibiotic resistance patterns in UPEC to determine the appropriate antibiotic treatment. I created an innovative bioinformatics approach that serves as a model for how this problem should be addressed. I created a database of clinical isolate genomes from the United States to identify resistance trends nationwide and compare to local trends. This database allowed us to identify unique regional trends that indicate higher local resistance in Merced, CA. These findings suggest strong local selection in Merced, and prompted us to investigate potential sources of selection.

2. Additionally, there has been no formal investigation into the relatedness of environmental and hospital UPEC isolates, and no good approach for performing this study has been previously described. To determine potential sources of UPEC in surrounding environment, I developed a rapid PCR approach to identify UPEC. Using six UPEC associated genes, I was able to identify UPEC in local environmental samples associated with retail meat production using this PCR approach.

3. There are no set of laboratory strains or plasmid constructs available for controlled studies of the contributions and interactions of individual resistance genes, or how these interact in combination with each other. I designed a plasmid vector expressing four clinically relevant antibiotic resistance genes I identified at the DHMMC. Using this plasmid vector, I created a construct library with different combinations of genes observed at the local hospital as a tool to measure their relative fitness contributions.

To address the shortcomings contributing to the rise of antibiotic resistant UPEC, we partnered with a local hospital to identify the abundance of resistance genotypes compared to nationwide genotypes. We have a collection of UPEC isolates collected between 2013-2019 from Dignity Health Mercy Medical Center (DHMMC) in Merced, California. We have identified the frequency of these common β-lactamase genes within this collection and found indicators of high resistance within these UPEC[17]. These indicators include high frequency of genes, which confer elevated resistance to later generation β-lactam antibiotics and detected more frequent genetic associations between genes that confer resistance to inhibitor combination therapies. We need to identify the local source of the antibiotic resistant UPEC. With that information we can develop a way to determine how the resistance trends at DHMMC have evolved and been selected. This in turn may enable us to employ effective antimicrobial stewardship.

We sought to identify the source of the antibiotics resistant UPEC at DHMMC by examining local environmental reservoirs. UTIs have historically been thought to be caused by bacteria entering the urethra from an external source or infections caused by patient's native gut flora[2,4]. UPEC identified in retail meat and on animal farms are similar to human UTI isolates with regard to virulence genes, antibiotic resistance genes, and clonal relatedness[18,19]. This is likely due to the heavy use of antibiotics, including medically important antibiotics, in agriculture for the prevention of disease and growth promotion in food animals[20]. Recent studies have linked animal food consumption with UTI occurrence. Due to the increasing number of UTI outbreaks, UTIs are now hypothesized to be primarily a foodborne infection. DHMMC serves Merced, California, which is one of the top six producing agriculture counties in California. Merced's primary products are dairy, almonds, chicken, and cattle[21]. The scale of agricultural production in Merced and abundance of antibiotic resistant UTIs at DHMMC, supports the inquiry of UPECs in agriculture. We identified a suite of genes significantly associated with UPEC (*chuA, fyuA, papA, traT, yrbH, yqeK*) that enable us to rapidly identify local sources of UPEC. We investigated the surrounding agricultural environments associated with animal food production to determine if they are a reservoir for hospital UPEC at DHMMC. We identified the same UPEC associated genes and common β-lactamase genes in the environmental samples as identified in hospital UPEC. This suggests a link between agricultural animal food production and UPEC in the local hospital, perhaps a shared reservoir.

We determined the frequency of these common β-lactamase genes within UPEC at DHMMC and identified a potential environmental reservoir for these UPEC. It is also important to understand the impact of selection by antibiotic consumption on the antibiotic resistance trends we identified. Antibiotic consumption is the use of antibiotics for numerous applications, including clinical and industrial. The link between antibiotic consumption and antibiotic resistance is well documented and is the primary driver of antibiotic resistance[22]. The use of broad-spectrum antibiotic treatment not based on culture identification and resistance phenotype can result in ineffective clearance of the infection-causing bacteria[23]. The use of antibiotics creates evolutionary selective pressure on bacteria to develop and spread antibiotic resistance. In the presence of antibiotics, selected bacteria can resist antibiotic treatment, replicate, and become a resistant population[23]. Previous experimental work on the antibiotic selection on resistance genotypes proposed to leverage this selective pressure, in the form of sequential administration of different antibiotics to select for any particular resistance genotype of the sixteen TEM alleles tested[24]. However, this work was limited in its focus on the TEM gene when in actuality this is only one of several genes resulting in β-lactam resistance.

Previous work has also shown that different resistance genes are genetically linked and can spread together at different frequencies[17]. We identified the most common β-lactamases at DHMMC and developed a similar system for studying the fitness effects of those genes alone and in all possible combinations. We designed a plasmid vector that expresses four common ESBL genes CTX-M-15, OXA-1, SHV-2, and TEM-10 (COST) identified in UPEC at DHMMC. Using this vector, we constructed a library that contains sixteen potential genotype combinations of these common ESBL genes with differential antibiotic resistance phenotypes. This vector and subsequent vector construct library are a novel tool that can be used to determine the effect of antibiotic consumption on the ongoing local selection of antibiotic resistance genes in UPEC. In turn, that information may yield more effective approaches for prescribing antibiotics than currently exist.

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Chapter 2: Distribution of β-Lactamase Genes in Clinical Isolates from California Central Valley Hospital Deviates from the United States Nationwide Trends

Distribution of β-Lactamase Genes in Clinical Isolates from California Central Valley Hospital Deviates from the United States Nationwide Trends

Candace Guzman-Cole[1], Fabian Santiago[2], Sona Garsevanyan[1], Suzanne Sindi[2] and Miriam Barlow[1,*]

Abstract: The evolution and dissemination of antibiotic resistance genes throughout the world are clearly affected by the selection and migration of resistant bacteria. However, the relative contributions of selection and migration at a local scale have not been fully explored. We sought to identify which of these factors has the strongest effect through comparisons of antibiotic resistance gene abundance between a distinct location and its surroundings over an extended period of six years. In this work, we used two repositories of extended spectrum β-lactamase (ESBL)-producing isolates collected since 2013 from patients at Dignity Health Mercy Medical Center (DHMMC) in Merced, California, USA, and a nationwide database compiled from clinical isolate genomes reported by the National Center for Biotechnology Information (NCBI) since 2013. We analyzed the stability of average resistance gene frequencies over the years since collection of these clinical isolates began for each repository. We then compared the frequencies of resistance genes in the DHMMC collection with the averages of the nationwide frequencies. We found DHMMC gene frequencies are stable over time and differ significantly from nationwide frequencies throughout the period of time we examined. Our results suggest that local selective pressures are a more important influence on the population structure of resistance genes in bacterial populations than migration. This, in turn, indicates the potential for antibiotic resistance to be controlled at a regional level, making it easier to limit the spread through local stewardship.

Keywords: ESBL; selection; Enterobacteriaceae; antibiotic resistance; stewardship

Introduction

β-lactam antibiotics have been in use since the discovery of penicillin in the 1940s, and they continue to be the most widely used antibiotics due to their high effectiveness, ease of delivery, and low toxicity[1,2]. The longstanding use of β-lactam antibiotics has led to the emergence of resistant strains in clinical care settings[3]. The continuous selection and evolution of β-lactamase genes by β-lactam antibiotic use has led to the diversification of successful β-lactamase genes: *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, and *bla*$_{OXA}$[4]. β-lactamase genes produce extended spectrum β-lactamase (ESBL) enzymes that work by hydrolyzing β-lactam antibiotics, rendering them ineffective. *bla*$_{TEM}$ and *bla*$_{SHV}$ were the first β-lactamase enzymes identified in 1963 and 1972, respectively, and were implicit in outbreaks in the 1990s[5–7]. Today, *bla*$_{SHV}$ composes 10% of ESBLs, and *bla*$_{TEM}$ has become somewhat less common in the U.S.[8]. *bla*$_{CTX-M}$ was first identified in 1989, and was identified with increasing frequency throughout the 1990s[9]. By the 2000s, the frequency of *bla*$_{CTX-M}$ enzymes surpassed those of *bla*$_{TEM}$ and *bla*$_{SHV}$. Although first discovered in 1976, *bla*$_{OXA}$ enzymes have been increasing in prevalence due to the frequent association of *bla*$_{OXA-1}$ with *bla*$_{CTX-M-15}$[10,11]. Today, *bla*$_{CTX-M}$ enzymes are the most identified ESBLs, and have displaced *bla*$_{TEM}$ and *bla*$_{SHV}$ in many individual hospitals[6,9,12–14]. However, this trend is not uniform across publications originating from different surveillance locations[11,15,16]. Bajpai et al. (2017) found *bla*$_{TEM}$ to be the most abundant ESBL enzyme in a single hospital,

although other reports detail different ESBL gene frequencies[17]. In the United States, few recent nationwide surveillance studies have specifically examined the frequencies of specific ESBL genes. One recent survey of 26 hospitals identified *bla*$_{TEM}$ as the most abundant ESBL enzyme in clinical isolates (47%), followed by *bla*$_{CTX-M}$ (36%), *bla*$_{SHV}$ (35%), and *bla*$_{OXA}$ (20%)[18].

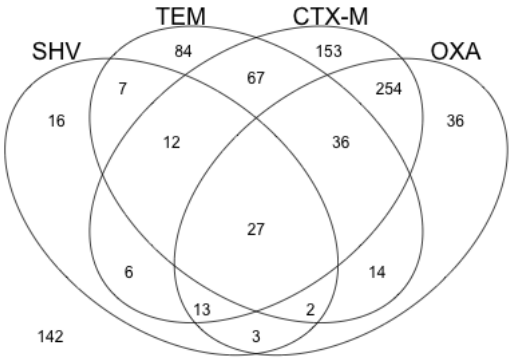
Regional variance in the frequencies of ESBLs enables the assessment of which factors are contributing the most to ESBL frequencies. Due to the strong selection that bacteria experience from antibiotics and the rapid migration of bacteria that occurs in human populations, selection and migration were the two factors we chose to investigate. To understand the relative contributions of selection and migration, it was important to obtain and compare updated ESBL gene frequencies. We chose to compare the frequencies of ESBLs in a local repository of ESBL positive isolates collected from a single hospital, with average frequencies nationwide across the U.S. obtained from ESBL-positive clinical isolates whose genomic sequences have been deposited in the NCBI Isolates Browser Database.

When comparing genetic variations over two populations, there are four possible outcomes depending on the stability of gene frequencies within a site and comparisons of those frequencies between sites. First, gene frequencies that are stable over time within a population and non-uniform across populations indicate low migration between bacterial populations and that selection for resistance within a given population is strong and constant. However, if the gene frequencies are unstable over time within a population and non-uniform across populations, this would indicate alternating local selective pressures and rapid migration as "immigrants would increase the mutation supply rate[19]" and would compete with "better-adapted residents maintaining the population away from the local fitness optimum[20]". Stability over time and uniformity between populations suggest rapid continuous migration between populations and strong consistent selection resulting in a highly resistant and optimized strain[20]. Finally, unstable (alternating) frequencies over time and uniformity between populations indicate strong alternating selective pressures in large areas (or populations). Moreover, this signal also indicates rapid migration because variation between populations averages out as immigration leads to a decrease in genetic differentiation between populations[21]. We compared ESBL gene frequencies from Dignity Health Mercy Medical Center (DHMMC) and the rest of the U.S. over a period of six years as follows.

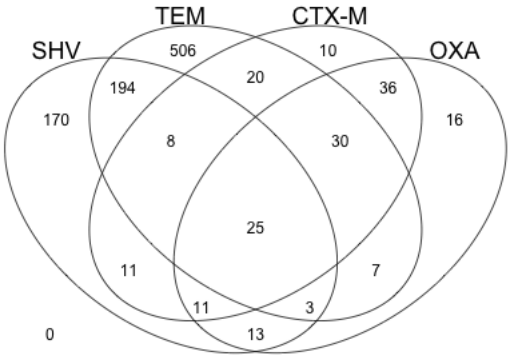
Results

Regional Gene Frequencies

We performed a molecular surveillance study of common the β-lactamases *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$, and *bla*$_{OXA}$ among isolates. At DHMMC, the most common ESBL gene we identified was *bla*$_{CTX-M}$, followed by *bla*$_{OXA}$, *bla*$_{TEM}$, and *bla*$_{SHV}$ (Figure 1-1a). Their yearly frequencies are provided in Table 1. Mathematical analysis of those frequencies over time revealed no significant differences over months or agricultural seasons. However, there were some significant differences (p-value < 0.05) in yearly frequencies (Table 1-1, SI Tables 1-4). *bla*$_{SHV}$ and *bla*$_{TEM}$ frequencies were stable over time. *bla*$_{CTX-M}$ frequencies increased after the first year in 2014 and remained stable over time (SI Table 3). *bla*$_{OXA}$ frequencies significantly decreased in 2016 from previous years but returned to stable in 2017 (SI Table 4).



(**a**)



(**b**)

Figure 1-1. Venn diagrams of *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$, and *bla*$_{OXA}$ combinations from both repositories. (**a**) Venn diagram of the resistance genes found in the clinical isolates from DHMMC. There were 142 isolates without any of these resistance genes (inconclusive data). (**b**) Venn diagram of the resistance genes found in the nationwide database of ESBL clinical isolates.

DHMMC	***bla*$_{SHV}$**	***bla*$_{TEM}$**	***bla*$_{CTX-M}$**	***bla*$_{OXA}$**
2013 (*n*=106)	9.4 (4.6, 16.7)	37.7 (28.5, 47.7)	52.8 (42.9, 62.6)	52.8 (42.9, 62.6)
2014 (*n*=88)	8.0 (3.3, 15.7)	29.5 (20.3, 40.2)	75.0 (64.6, 83.6)	54.5 (43.6, 65.2)
2015 (*n*=255)	7.5 (4.5, 11.4)	29.8 (24.3, 35.8)	71.4 (65.4, 76.8)	49.8 (43.5, 56.1)
2016 (*n*=207)	12.6 (8.4, 17.9)	24.6 (18.9, 31.1)	62.8 (55.8, 69.4)	35.7 (29.2, 42.7)
2017 (*n*=126)	9.5 (5.0, 16.0)	23.8 (16.7, 32.2)	61.1 (52.0, 69.7)	36.5 (28.1, 45.6)
2018 (*n*=90)	13.3 (7.1, 22.1)	28.9 (19.8, 39.4)	63.3 (52.5, 73.2)	37.8 (27.8, 48.6)

Table 1-1. The yearly frequency of *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$, and *bla*$_{OXA}$ from DHMMC. Each frequency is presented with a 95% confidence interval. The number of isolates is given in the first column in parenthesis. The confidence intervals are given in parenthesis in all the other columns.

We then measured the frequencies at which these genes co-occurred in each isolate population. Statistical analysis suggests a genetic linkage (or correlation) between resistance genes in isolates from DHMMC (Table 1-2). Two statistical methods (Pearson's chi-square test and the phi coefficient) revealed a significant positive correlation between *bla*$_{TEM}$ and *bla*$_{SHV}$ (p-value < 0.05) and between *bla*$_{CTX-M}$ and *bla*$_{OXA}$ (p-value < 0.05). There are significant negative correlations between *bla*$_{TEM}$ and *bla*$_{CTX-M}$ (p-value < 0.05) and *bla*$_{TEM}$ and *bla*$_{OXA}$ (p-value < 0.05).

When stratified by species, the resistance genes in *E. coli* and *K. pneumoniae* isolates from the DHMMC clinical isolates show unique correlations (Table 2). In *E. coli* isolates, *bla*$_{CTX-M}$ and *bla*$_{OXA}$ are positively correlated with one another (p-value < 0.05) and negatively correlated with *bla*$_{TEM}$ (p-value < 0.05). However, in *K. pneumoniae*, all the

resistance genes are positively correlated with each other (p-value < 0.05) but significance is lost for bla_{TEM} and bla_{OXA} after the false discovery rate (FDR)-controlling procedure.

Species	Markers	Chi-Square *p*-Value	PC	PC *p*-Value
All (*n*=872)	bla_{SHV}:bla_{TEM}	3.75×10^{-9} *	0.20	2.74×10^{-9} *
	bla_{SHV}:bla_{CTX-M}	6.37×10^{-1}	0.02	6.37×10^{-1}
	bla_{SHV}:bla_{OXA}	1.08×10^{-1}	0.05	1.08×10^{-1}
	bla_{TEM}:bla_{CTX-M}	1.49×10^{-3} *	−0.11	1.46×10^{-3} *
	bla_{TEM}:bla_{OXA}	3.00×10^{-6} *	−0.16	2.68×10^{-6} *
	bla_{CTX-M}:bla_{OXA}	8.59×10^{-30} *	0.38	5.24×10^{-32} *
E. coli (*n*=787)	bla_{SHV}:bla_{TEM}	5.22×10^{-1}	0.02	5.23×10^{-1}
	bla_{SHV}:bla_{CTX-M}	2.58×10^{-1}	−0.04	2.59×10^{-1}
	bla_{SHV}:bla_{OXA}	4.89×10^{-1}	−0.02	4.89×10^{-1}
	bla_{TEM}:bla_{CTX-M}	1.24×10^{-6} *	−0.17	1.07×10^{-6} *
	bla_{TEM}:bla_{OXA}	1.40×10^{-9} *	−0.22	9.50×10^{-10} *
	bla_{CTX-M}:bla_{OXA}	9.22×10^{-26} *	0.37	1.53×10^{-27} *
K. pneumoniae (*n*=85)	bla_{SHV}:bla_{TEM}	3.36×10^{-4} *	0.39	2.34×10^{-4} *
	bla_{SHV}:bla_{CTX-M}	1.97×10^{-5} *	0.46	8.15×10^{-6} *
	bla_{SHV}:bla_{OXA}	6.84×10^{-3} *	0.29	6.44×10^{-3} *
	bla_{TEM}:bla_{CTX-M}	2.58×10^{-5} *	0.46	1.13×10^{-5} *
	bla_{TEM}:bla_{OXA}	3.84×10^{-2}	0.22	3.88×10^{-2}
	bla_{CTX-M}:bla_{OXA}	5.69×10^{-6} *	0.49	1.72×10^{-6} *

Table 1-2. Linkage analysis summary for DHMMC isolates. The p-value for a chi-square test for linkage, the phi coefficient, and the associated p-value are presented for each resistance marker pair comparison. The number of isolates for each species is given in parenthesis. An asterisk (*) indicates a statistically significant comparison after the FDR-controlling procedure (q* = 0.025) for both the chi-square test and the phi coefficient.

U.S. Database Gene Frequencies

We conducted an analogous surveillance study using a nationwide database of ESBL clinical isolates from the NIH Pathogen Detection Isolates Browser (Figure 1-1b). Nationwide, the resistance gene frequencies were different from the DHMMC repository. The most common ESBL gene was bla_{TEM}, followed by bla_{SHV}, bla_{CTX-M}, and bla_{OXA}. Those frequencies are provided in Table 1-3. Analysis of these gene frequencies over time also showed no significant differences in the frequencies of common ESBL genes in the U.S despite annual changes in the sources of isolates (SI Table 9). over a period of months. However, there were significant differences (*p*-value < 0.05) in the yearly frequencies for bla_{SHV}, bla_{TEM}, and bla_{CTX-M} (Table 1-3, SI Tables 5-8). bla_{SHV} significantly increased in 2015, followed by a significant decrease in 2016, but returned to stable in 2017 (SI Table 5). bla_{TEM} frequencies significantly decreased in 2017 and 2018 from previous years (SI Table 6). There was a non-significant decrease in bla_{CTX-M} frequency in 2015 from previous years. The 2015 bla_{CTX-M} frequency was significantly different than that of 2018 due to an increase that year (SI Table 7).

Nationwide U.S.	*bla*$_{SHV}$	*bla*$_{TEM}$	*bla*$_{CTX-M}$	*bla*$_{OXA}$
2013 (*n*=6)	16.7 (0.4, 64.1)	83.3 (35.9, 99.6)	0.0 (0.0, 45.9)	0.0 (0.0, 45.9)
2014 (*n*=179)	4.5 (1.9, 8.6)	82.7 (76.3, 87.9)	14.0 (9.2, 19.9)	16.8 (11.6, 23.1)
2015 (*n*=268)	66.8 (60.8, 72.4)	74.6 (69.0, 79.7)	8.2 (5.2, 12.2)	9.7 (6.4, 13.9)
2016 (*n*=190)	19.5 (14.1, 25.8)	84.7 (78.8, 89.5)	16.8 (11.8, 22.9)	13.2 (8.7, 18.8)
2017 (*n*=251)	50.6 (44.2, 56.9)	66.9 (60.7, 72.7)	13.9 (9.9, 18.9)	11.6 (7.9, 16.2)
2018 (*n*=166)	50.0 (42.2, 57.8)	66.9 (59.2, 74.0)	22.3 (16.2, 29.4)	18.7 (13.1, 25.4)

Table 1-3. The yearly frequency of *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$, and *bla*$_{OXA}$ from the U.S. Nationwide Database. Each frequency is presented with a 95% confidence interval. The number of isolates is listed in the first column in parenthesis.

In the nationwide database, the co-occurrence of resistance genes differs from that of our local samples from DHMMC (Table 1-4). We observed a negative association between *bla*$_{TEM}$ and the other resistance markers (p-value < 0.05), and a positive association between *bla*$_{CTX-M}$ and *bla*$_{OXA}$ (p-value < 0.05). The negative association of *bla*$_{TEM}$ with both *bla*$_{CTX-M}$ and *bla*$_{OXA}$ was almost double that found at DHMMC for all species (Tables 1-2 and 1-4). When stratified by species, *E. coli* upholds these trends, but in *K. pneumoniae*, *bla*$_{SHV}$ is negatively correlated with the other resistance genes (p-value < 0.05) and *bla*$_{CTX-M}$ is positively correlated *bla*$_{OXA}$ (p-value < 0.05).

Species	Markers	Chi-Square *p*-Value	PC	PC *p*-Value
All (*n*=1060)	bla_{SHV}:bla_{TEM}	7.03×10^{-43} *	−0.42	6.29×10^{-47} *
	bla_{SHV}:bla_{CTX-M}	2.13×10^{-1}	−0.04	2.14×10^{-1}
	bla_{SHV}:bla_{OXA}	2.81×10^{-1}	−0.03	2.81×10^{-1}
	bla_{TEM}:bla_{CTX-M}	1.31×10^{-9} *	−0.19	9.80×10^{-10} *
	bla_{TEM}:bla_{OXA}	3.31×10^{-17} *	−0.26	1.02×10^{-17} *
	bla_{CTX-M}:bla_{OXA}	1.01×10^{-99} *	0.65	7.63×10^{-129} *
E. coli (*n*=559)	bla_{SHV}:bla_{TEM}	2.03×10^{-6} *	−0.20	1.68×10^{-6} *
	bla_{SHV}:bla_{CTX-M}	1.48×10^{-1}	−0.06	1.49×10^{-1}
	bla_{SHV}:bla_{OXA}	1.52×10^{-1}	−0.06	1.52×10^{-1}
	bla_{TEM}:bla_{CTX-M}	6.72×10^{-35} *	−0.52	2.90×10^{-40} *
	bla_{TEM}:bla_{OXA}	1.85×10^{-64} *	−0.72	2.38×10^{-89} *
	bla_{CTX-M}:bla_{OXA}	1.83×10^{-53} *	0.65	1.05×10^{-68} *
K. pneumoniae (*n*=501)	bla_{SHV}:bla_{TEM}	6.81×10^{-10} *	−0.28	3.45×10^{-10} *
	bla_{SHV}:bla_{CTX-M}	4.69×10^{-5} *	−0.18	4.23×10^{-5} *
	bla_{SHV}:bla_{OXA}	7.37×10^{-3} *	−0.12	7.30×10^{-3} *
	bla_{TEM}:bla_{CTX-M}	4.73×10^{-1}	0.03	4.74×10^{-1}
	bla_{TEM}:bla_{OXA}	5.68×10^{-1}	0.03	5.69×10^{-1}
	bla_{CTX-M}:bla_{OXA}	4.37×10^{-48} *	0.65	1.04×10^{-61} *

Table 1-4. Linkage analysis summary for the Nationwide Database Isolates. The *p*-value for a chi-square test for linkage, the phi coefficient, and the associated *p*-value are presented for each resistance marker pair comparison. The number of isolates for each species is given in parenthesis. An asterisk (*) indicates a statistically significant comparison after the FDR-controlling procedure ($q^* = 0.025$) for both the chi-square test and the phi coefficient.

Comparison of DHMMC and U.S. Populations

We then performed a formal statistical comparison between the frequencies of resistance genes in the DHMMC repository and the U.S. database to determine significant differences (Table 1-5). In the nationwide database, bla_{SHV} and bla_{TEM} occur more frequently (all *p*-values < 0.05) than at DHMMC. At DHMMC, bla_{CTX-M} and bla_{OXA} occur at higher frequencies than they do nationwide (all *p*-values < 0.05). A further breakdown of

the gene frequencies by species and FDR controlling revealed no significant frequency difference in bla_{SHV} within species between datasets. bla_{TEM} occurs in 57–68% more *E. coli* isolates in the U.S. database than *E. coli* isolates at DHMMC (p-values < 0.05). There is no significant difference in bla_{TEM} frequency in *K. pneumoniae* isolates. $bla_{CTX\text{-}M}$ occurs much more frequently in *E. coli* and *K. pneumoniae* isolates from DHMMC than in the U.S. database (all p-values < 0.05). $bla_{CTX\text{-}M}$ occurs in 47–58% more *E. coli* isolates and 33–52% more *K. pneumoniae* isolates at DHMMC than isolates from the nationwide U.S. database. This trend is similar but less drastic for bla_{OXA}, which occurs in 26–36% more *E. coli* isolates and 26–44% more *K. pneumoniae* isolates than isolates nationwide.

Species	Marker	F_M	F_N	F_M—F_N	p-Value	95% CI
All	bla_{SHV}	9.9	41.0	−31.2	2.79×10^{-53} *	(−35.2,−27.2)
	bla_{TEM}	28.6	74.8	−46.3	1.34×10^{-91} *	(−50.7,−41.8)
	$bla_{CTX\text{-}M}$	65.1	14.2	50.9	2.40×10^{-117} *	(46.6,55.2)
	bla_{OXA}	44.2	13.3	30.8	6.58×10^{-52} *	(26.9,34.8)
E. coli	bla_{SHV}	2.8	2.5	0.3	7.44×10^{-1}	(−1.5,2.0)
	bla_{TEM}	25.9	89.1	−63.2	1.14×10^{-115}	(−68.6,−57.8)
	$bla_{CTX\text{-}M}$	65.8	12.7	53.1	1.75×10^{-83}	(47.7,58.5)
	bla_{OXA}	43.6	12.5	31.1	4.16×10^{-34}	(26.1,36.1)
K. pneumoniae	bla_{SHV}	75.3	84.0	−8.7	4.86×10^{-2}	(−17.4,−0.1)
	bla_{TEM}	52.9	58.9	−5.9	3.05×10^{-1}	(−17.3,5.4)
	$bla_{CTX\text{-}M}$	58.8	16.0	42.9	1.46×10^{-18}	(33.3,52.4)
	bla_{OXA}	49.4	14.2	35.2	2.65×10^{-14}	(26.2,44.3)

Table 1-5. Percent frequency differences between resistance markers at DHMMC and the Nationwide Database. The frequency of a resistance marker from DHMMC is denoted FM, and the frequency of a resistance marker from the National Database is denoted by FN. An asterisk (*) indicates a statistically significant comparison after the FDR-controlling procedure (q* =0.025). The last column provides the 95% confidence interval for the percent difference for a particular resistance marker between the two datasets.

Discussion

Our results indicate the relative importance of selection and migration in small and large regions. The stability of resistance genes over time in a distinct community that differ from the nationwide frequencies strongly suggests that local selective pressures have a larger impact on frequencies than migration. DHMMC is unique with regards to the presence of $bla_{CTX\text{-}M}$ and bla_{TEM} genes. At DHMMC, $bla_{CTX\text{-}M}$ occurs more frequently than in the nationwide database, while bla_{TEM} occurs less frequently at DHMMC. The negative correlation at DHMMC between bla_{TEM} and $bla_{CTX\text{-}M}$ and between bla_{TEM} and bla_{OXA} implies incompatibilities between bla_{TEM} and at least one of the other genes. Since $bla_{CTX\text{-}M}$ and bla_{OXA} are commonly linked with each other, it is not surprising that bla_{TEM} is negatively associated with both of them, and genetic incompatibilities may exist for only one of those pairings. As those genetic compatibilities were not observed throughout the U.S., it is likely that they are the product of local selective pressures. This negative relationship results mainly from *E. coli* isolates; this relationship is not observed in *K. pneumoniae* isolates. This result indicates that either the genetic background of *K. pneumoniae* eliminates the incompatibilities of these genes, or that the antibiotic exposures of these pathogens is

different from *E. coli.* Additionally, at DHMMC, there appears to be strong selection for $bla_{CTX\text{-}M}$, which may be displacing bla_{TEM} likely due to antagonism between these genes[22].

In terms of antimicrobial stewardship, our results suggest resistance may be modulated at a regional level, facilitating the implementation of effective strategies to limit and control selection of the antibiotic resistance genes. However, we also found evidence that resistance must be modulated differently for separate species, which may be difficult to conduct because of the environmental presence of antibiotics and the use of empiric therapies without species identification.

High bla_{SHV} frequencies are unique to our nationwide dataset. However, when we considered *E. coli* and *K. pneumoniae* isolates separately, DHMMC and nationwide frequencies of bla_{SHV} are similar. The greater number of *K. pneumoniae* isolates in the nationwide database likely accounts for the overall higher nationwide frequencies of bla_{SHV} (Simpson's paradox[23]). A high bla_{TEM} frequency is also unique to our nationwide dataset. There are 57–68% more *E. coli* isolates with bla_{TEM} nationwide than isolates from DHMMC. *E. coli* isolates nationwide have a negative correlation between bla_{TEM} and the other resistance genes, meaning these isolates are likely to have bla_{TEM} and no other resistance genes. In *K. pneumoniae* isolates nationwide, there is a negative correlation between bla_{SHV} and the other resistance genes, meaning these isolates are likely to have bla_{SHV} and no other resistance genes, which is the opposite of the *K. pneumoniae* isolates from DHMMC. ESBLs were initially derived from bla_{SHV} and bla_{TEM}, explaining the relatively high bla_{TEM} frequencies nationwide, as bla_{TEM} has proceeded to fixation[24]. bla_{SHV} and bla_{TEM} have been responsible for most ESBL infections since at least the 1980s, so it is reasonable for them to be widely distributed in a large-scale dataset[25].

We observed stable gene frequencies over time within the DHMMC population which differ from nationwide frequencies. This implies there is low migration or low survival of immigrants between populations, and that the selective forces within the DHMMC population are strong and constant. Overall, there is strong evidence that local selective pressures have a much stronger effect on the frequencies of ESBLs in local populations. This suggests that communities and specific regions have the potential to effectively manage ESBL frequencies through intentional antibiotic stewardship practices.

Materials and Methods

Hospital Isolates

Clinical patient isolates (n = 872) were collected from patients at DHMMC in Merced, CA, USA, from 2013 to 2018. These isolates were flagged as ESBL using Vitek 2 (bioMérieux, Inc. Hazelwood, MO, USA). These patient samples were collected from urine, blood, sputum, and wounds.

Molecular Methods

Genomic DNA was isolated using the boil preparation method by adding a single colony to 100 µL sterile water and boiling at 100 °C for 15 min. From this 100 µL solution, 1 µL was used in the PCR reaction for the respective genes with the primers listed in Table 1-6. Multiplex PCR was used to determine the presence of $bla_{CTX\text{-}M}$, bla_{TEM}, and bla_{OXA}. Detection of bla_{SHV} was run in a separate reaction. Each PCR reaction consisted of 1 µL of template DNA, 10 µM of each primer, and Taq 2X master mix (NEB) at a final concentration of 1X, and the reactions were run under the following conditions: initial denaturation at 94 °C for 10 min, 30 cycles of 94 °C for 40 s, 60 °C for 40 s, 72 °C for 1 min, and a final elongation at 72 °C for 7 min [26]. PCR amplicons were run out on 2% agarose gel at 100 V for 30 min and visualized using a ChemiDoc™ Touch Imaging System. (Figures S1 and S2).

Gene	Primer Sequence (5′ to 3′)	Product Size (bp)
bla_{SHV}	Forward GCCTGTGTATTATCTCCCTGTTAG Reverse TCCCGGCGATTTGCTGATTCC	813
bla_{TEM}	Forward TGACGCCGGGCAAGAGCA Reverse AAGGGCCGAGCGCAGAAGTG	424
bla_{OXA}	Forward AGCGCCAGTGCATCAACAG Reverse GCAAAACCCAAACAACAGAAA	300
bla_{CTX-M}	Forward CGGCCGCGGTGCTGAAGAA Reverse GCTGCCGGTTTTATCCCCCACAA	482

Table 1-6. List of primers pairs used to identify bla_{SHV}, bla_{TEM}, bla_{OXA}, and bla_{CTX-M} and their expected product size.

U.S. Database

We obtained clinical isolate genomes from the NCBI RefSeq database[27], using the NIH Isolate Browser[28] to identify clinical isolates of *E. coli* and *K. pneumoniae* from the United States from 2013 to 2018. Using the Comprehensive Antibiotic Resistance Database (CARD)[29], we identified isolate genomes containing ESBL genes to compile an ESBL clinical database (n = 1060) using the BLAST+ program. In combination with a 98% identity cut-off to positively identify the frequency of bla_{TEM}, bla_{OXA}, bla_{CTX-M}, and bla_{SHV}, we applied an additional base pair match cutoff for each gene to limit partial gene matches. For bla_{TEM}, we required a base pair (bp) match at or above 753 bp; for bla_{OXA}, we required 831 bp; for $bla_{CTX-M,}$ we required 876 bp; and for bla_{SHV}, we required 861 bp. The metadata for nationwide clinical isolate genomes were downloaded from the NIH Isolate Browser and included date, species, and location. The list of genome assemblies used to perform this analysis can be found in the Supplementary Materials.

Statistical Analysis

We used one-way analysis of variance (ANOVA) to compare the means of the resistance gene frequencies to identify significant differences between frequencies across months. We compared the same months across years, different months within the same year, different months across all years, and bins of 2, 3, 4, and 6 months. We tested for significant differences between the means of the resistance gene frequencies across years at DHMMC and the nationwide database using a Z-test [30]. We tested for significant differences in the proportions of a resistance marker between isolates from DHMMC and the nationwide database using a Z-test. Pairwise linkage among the resistance alleles in each of the two clinical isolate populations, DHMMC and the nationwide database, was assessed using a chi-square test[31]. The phi coefficient (PC) was used as a chi-square measure of directional deviation from the null relationship of independent assortment[32]. The PC has the desired property of accounting for sample size (often >500 in this work) and because it has a known sampling distribution, it allows us to compute significance and to form confidence intervals. Controlling for multiple statistical tests was conducted via the FDR-controlling procedure[33], a Bonferroni-type multiple testing procedure, with a false discovery control level of q^* = 0.025. Only those results that remained significant after

using FDR are reported as significant. All analyses were performed using the Statistics and Machine Learning Toolbox of MATLAB R2020a[34].

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antibiotics10050498/s1. Table S1: Pairwise yearly frequency comparison for *bla*$_{SHV}$ in the DHMMC database, Table S2: Pairwise yearly frequency comparison for *bla*$_{TEM}$ in the DHMMC database, Table S3: Pairwise yearly frequency comparison for *bla*$_{CTX-M}$ in the DHMMC database, Table S4: Pairwise yearly frequency comparison for *bla*$_{OXA}$ in the DHMMC database, Table S5: Pairwise yearly frequency comparison for *bla*$_{SHV}$ in the Nationwide database, Table S6: Pairwise yearly frequency comparison for *bla*$_{TEM}$ in the Nationwide database, Table S7: Pairwise yearly frequency comparison for *bla*$_{CTX-M}$ in the Nationwide database, Table S8: Pairwise yearly frequency comparison for *bla*$_{OXA}$ in the Nationwide database, Figure S1: 2% agarose gel of *bla*$_{CTX-M}$, *bla*$_{TEM}$, and *bla*$_{OXA}$ PCR products, Figure S2: 2% agarose gel of *bla*$_{SHV}$ PCR products, Table S9: Number of clinical isolates annually by state.

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Chapter 3: Identifying candidate UPEC genes for rapid identification of UPEC in the environment and the clinic

Abstract

Urinary tract infections (UTIs) caused by uropathogenic *E. coli* (UPECs) are the most common bacterial infection. Recently, UPECs have been hypothesized to be transmitted in food, originating from manure-based fertilizer[1]. We are investigating UPECs in a single hospital, Dignity Health Mercy Medical Center (DHMMC) and in surrounding agricultural environments to determine if they are a reservoir for hospital UPEC isolates in Merced, California. We identified several UPEC associated genes (*chuA, fyuA, papA, traT, yrbH*)[2,3] from those environmental samples that are also present in the hospital isolates. We also identified the same resistance genes as observed in the hospital (bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, bla_{OXA}) in environmental samples from surrounding cattle feed lots and dairy farms. These results suggest that there may be overlap between hospital and environmental populations of *E. coli.* Taken together, these results implicate agricultural sources as likely local reservoirs of antibiotic resistant UTIs and indicate that antibiotics may exacerbate their transmission.

Introduction

Urinary Tract Infections (UTI) are the most common type of bacterial infection in the world with an estimated 130-175 million cases annually[4,5]. In the United States, there are 6-8 million UTI cases every year[4]. UTIs have become hard to treat due to increasing resistance to multiple antibiotics[2]. UTIs are primarily caused by uropathogenic *E. coli* (UPEC) that are genetically and phenotypically distinct from commensal *E. coli* and diarrheagenic *E. coli* (ExPEC)[4]. UPEC are characterized by their virulence factors that allow them to colonize the bladder which include pathogenicity (*yrbH*), adhesion (*papA*), iron uptake (*chuA, fyuA*) and protectin/serum resistance (*traT*)[2]. UTIs were originally thought be caused by bacteria entering the urethra (ascending infection) from an external source or infections caused by patient's native gut flora[4,6]. UPEC is the main cause of community-acquired UTIs, causing 80-90% of cases[7]. UPEC have recently been identified in retail meat and on animal farms that are similar to human UTI isolates with regard to virulence genes, antibiotic resistance genes, and clonal relatedness[8,9]. Recent studies have linked animal food consumption with UTI and due to the increasing number of UTI outbreaks, UTIs are now hypothesized to be foodborne.

The increasing antibiotic resistance in UTIs is of great concern to public health. Understanding the source of UPEC causing these infections is a promising route to limit the spread of UPEC within a community as well as the dissemination of antibiotic resistance among UPEC. We have a collection of UPEC isolates collected between 2013-2019 from Dignity Health Mercy Medical Center (DHMMC) in Merced, California. Merced is one of the top six producing agricultural counties in California whose main exports are dairy, almonds, chicken, and cattle[10]. The magnitude of agricultural influence in Merced and abundance of antibiotic resistant UTIs, warrants our investigation of UPECs in agriculture. Using a suite of UPEC associated genes and antibiotic resistance genes identified at DHMMC, we are investigating surrounding agricultural environments associated with animal food production to test the hypothesis that they are a reservoir for hospital UPEC at DHMMC.

Function	Gene	Function	Gene
Fimbriae	fimH[2,12,13,16]	Type III Secretion System (T3SS) Not present in UPEC strains	eivI[25]
Adhesion/Pilus	papA[2]		eivJ2[25]
Iron Acquisition	feoB[2]		epaR[25]
	iutA[2,12,13,26]		epaS1[25]
	fyuA[2,12]		yqeF[25]
Hemoglobin Receptor	chuA[2,12]		yqeG[25]
Toxin Genes	traT[2,12]		yqeH[25]
	CNF-1[14,16]		yqeI[25]
Other	yrbH (D-arabinose 5- phosphate isomerase)[3]		yqeJ[25]
	C3509 (ATP-binding protein of an ABC transport system)[3]		
	aer (aerobactin siderophores)[12,16]		

Table 2-1. Candidate genes for identifying UPEC isolates.

Methods

E. coli genomic database

I compiled two databases to identify potential UPEC associated genes. We obtained *E. coli* genomes from the NCBI RefSeq database (as of October 2020)[11] that fit into the following *E. coli categories*: ten commensal strains, ten ExPEC strains, and twenty-one UPEC strains. The list of genome assemblies used to perform this analysis can be found in the Supplementary Materials.

We identified a list of 22 genes associated with UPEC based on their abundance in numerous studies which can be found in Table 2-1[2,3,12–16]. We also included genes which are part of a type III secretion system previously identified to not be part of the UPEC genome to act as negative controls[17]. We compiled a n-BLAST “subject” dataset of UPEC genes of interest to identify their frequency within our *E. coli* database. Our

BLAST “subject” dataset was made of representative PCR amplicons of the genes in Table 2-1 from primers previously used in the literature.

The genes were investigated for their correlation to UPEC belong to the following categories that are characteristic to UPEC virulence: Fimbrial adhesion for attachment to the bladder, iron acquisition for surviving in the iron-limited urinary tract, serum resistance to avoid immune response, LPS biosynthesis and bacterial membrane transport.

Clinical UPEC database

We created a second database of sequenced genomes from our collection of UPEC-enriched clinical isolates. These isolates were collected over a six-year period from the local hospital and sequenced using Illumina Hi-seq paired-end sequencing. This database is composed of 116 genomes with 74 UPEC isolates and 42 non-UPEC isolates. UPEC for this database was considered *E. coli* species identified in urine sample. The sample types for these isolates include blood, sputum, urine, wound, and N/A. Isolates were compiled into a database and a BLAST search was performed with our UPEC “subject” dataset to identify the frequency of our genes of interest.

Correlation Analysis and PCA of UPEC genes

We performed Pearson’s correlation analysis and principal component analysis (PCA) on the genes of interest to identify significant positive and negative UPEC correlations. A representative UPEC point was plotted on the PCA to illustrate where a UPEC isolate would lie with respect to the principal components. We used the results from PCA of each of our datasets to identify genes that were correlated with UPEC. Genes were then selected based on the magnitude of correlation, either positive or negative, and the significance of that correlation ($p<0.05$). The genes we selected clustered near the representative UPEC point on the PCA plots and had significant positive correlations with UPEC. For a negative control, we chose a gene that did not cluster with UPEC on the PCA plots and was significantly negatively associated with UPEC.

Function	Gene	Isolates Positive for Gene	Isolates Negative for Gene	UPEC Correlation	*p*-value
Aerobactin siderophore	aer	14	26	0.314	0.048*
ATP-binding protein of an ABC transport system	C3509	30	10	0.231	0.152
Hemoglobin receptor	chuA	23	17	0.354	0.025*
Toxin Gene	CNF-1	7	33	0.461	0.003*
	traT	13	27	0.374	0.018*
Iron acquisition	feoB	40	0	---	---
	fyuA	21	19	0.651	0
	iutA	14	26	0.314	0.048*
Fimbrial adhesion	fimH	40	1	0.224	0.165
Part of Type III secretion system not found in UPEC	eivI	7	33	-0.197	0.222
	eivJ2	14	26	-0.21	0.194
	epaR	15	25	-0.258	0.108
	epaS1	15	25	-0.258	0.108
	kduI	40	0	---	---
	yqeF	40	0	---	---
	yqeG	40	0	---	---
	yqeH	24	16	-0.51	0.001*
	yqeI	22	18	-0.503	0.001*
	yqeJ	22	18	-0.503	0.001*
	yqeK	22	18	-0.503	0.001*
Pilus gene	papA	5	35	0.378	0.016*
D-arabinose 5-phosphate isomerase	yrbH	19	21	0.551	0.000*

Table 2-2. Correlation Analysis of candidate UPEC genes from *E. coli* database. An asterisk (*) indicates a statistically significant *p*-value (< 0.05).

Function	Gene	Isolates Positive for Gene	Isolates Negative for Gene	UPEC Correlation	*p*-value
Aerobactin siderophore	aer	85	31	0.275	0.003*
ATP-binding protein of an ABC transport system	C3509	99	17	0.195	0.036*
Hemoglobin receptor	chuA	94	22	0.139	0.137
Toxin Gene	CNF-1	26	90	0.276	0.003*
	traT	91	25	0.259	0.005*
Iron acquisition	feoB	116	0	---	---
	fyuA	90	26	0.197	0.034*
	iutA	92	24	0.279	0.002*
Fimbrial adhesion	fimH	100	16	0.115	0.22
Part of Type III secretion system not found in UPEC	eivI	32	84	0.104	0.268
	eivJ2	34	82	0.052	0.582
	epaR	45	71	0.121	0.195
	epaS1	45	71	0.121	0.195
	kduI	105	11	0.185	0.047*
	yqeF	104	12	0.156	0.094
	yqeG	105	11	0.185	0.047*
	yqeH	46	70	0.134	0.151
	yqeI	45	71	0.121	0.195
	yqeJ	46	70	0.097	0.298
	yqeK	45	71	0.121	0.195
Pilus gene	papA	3	113	-0.103	0.27
D-arabinose 5- phosphate isomerase	yrbH	88	28	0.204	0.028*

Table 2-3. Correlation Analysis of candidate UPEC genes from DHMMC database. An asterisk (*) indicates a statistically significant *p*-value (< 0.05).

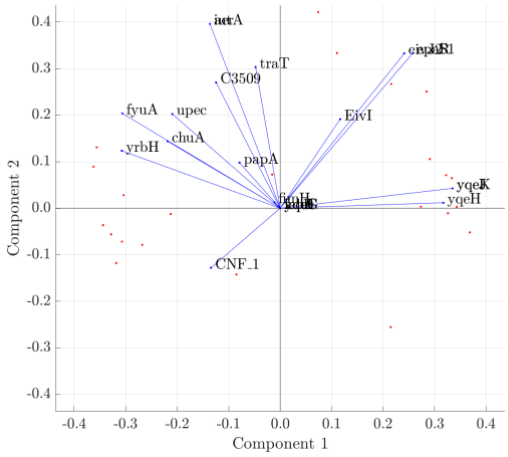

Figure 2-1. Principal components analysis of UPEC genes in *E. coli* database: principal components 1 and 2. UPEC have a negative association with component 1 and a positive association with component 2.

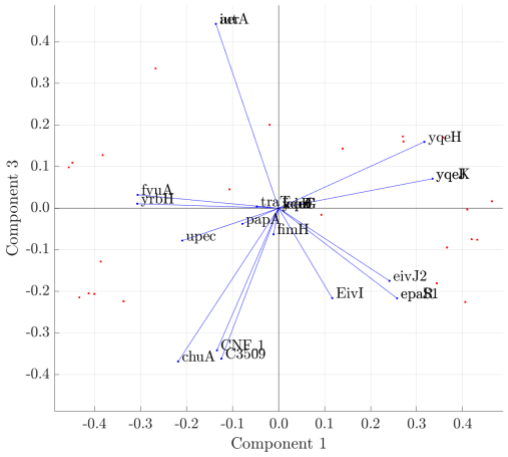

Figure 2-2. Principal components analysis of UPEC genes in *E. coli* database: principal components 1 and 3. UPEC have a negative association with components 1 and 3.

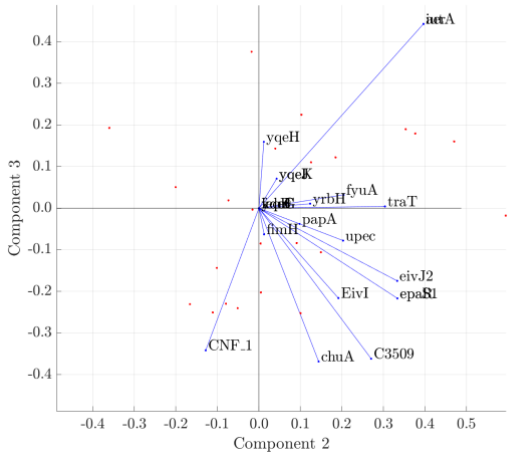

Figure 2-3. Principal components analysis of UPEC genes in *E. coli* database: principal components 2 and 3. UPEC have a positive association with component 2 and a negative association with component 3.

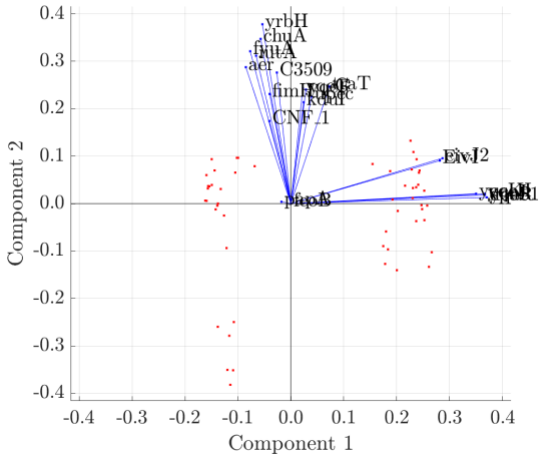

Figure 2-4. Principal components analysis of UPEC genes in DHMMC database: principal components 1 and 2. UPEC have a positive association with components 1 and 2.

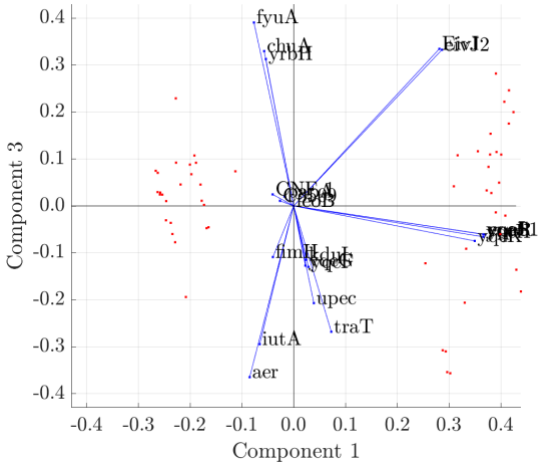

Figure 2-5. Principal components analysis of UPEC genes in DHMMC database: principal components 1 and 3. UPEC have a positive association with component 1 and a negative association with component 3.

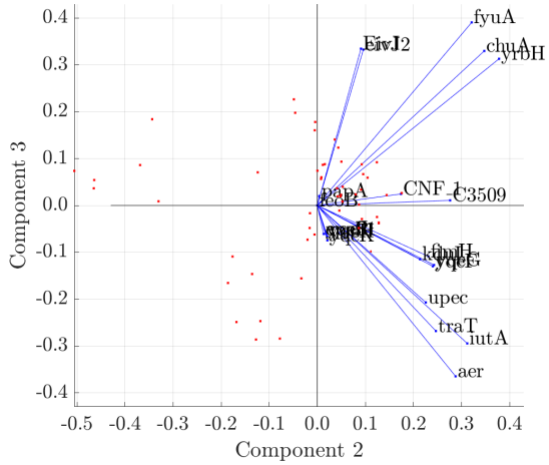

Figure 2-6. Principal components analysis of UPEC genes in DHMMC database: principal components. UPEC have a positive association with component 2 and a negative association with component 3.

We designed primers based on six genes positively correlated (Figures 2-1 through Figure 2-6) with UPEC and one gene negatively associated with UPEC, to act as a negative control.

Gene	Forward 5'→3'	Reverse 5'→3'	Product (bp)
chuA[3]	GCTACCGCGATAACTGTCAT	TGGAGAACCGTTCCACTCTA	221
fyuA[2]	TGATTGACCCCGCGACGGGAA	CGCAGTAGGCACGATGTTGTA	785
papA[2]	ATGGCAGTGGTGTTTTGGTG	CGTCCCACCATACGTGCTCTTC	739
traT[2]	GGTGTGGTGCGATGAGCACAG	CACGGTTCAGCCATCCCTGAG	288
yrbH[3]	TTGCACCAACAACGTCTACC	TCTGCGTCTTCTACCATCAC	259
yqeK[17]	TGCCCATGATGTTGTTTGCG	TGGACATTGAGTTTTCGCAGAT	140

Table 2-4. List of PCR primer pairs of UPEC-associated genes and their expected product size

Results

UPEC gene frequencies in genomic databases

We performed a n-BLAST search of the UPEC genes in our *E. coli* genomic database compiled from commensal, ExPEC, and UPEC genomes from the NCBI RefSeq database. A gene was considered present if the length match spanned the full gene length and had a percent identity of at least 98%. In our *E. coli* genomic database, we identified chuA in 15 UPEC isolates (71%), 8 Non-UPEC isolates (40%), fyuA in 17 UPEC (81%), 4 Non-UPEC (20%), papA in 5 UPEC (24%), 0 Non-UPEC (0%), traT in 10 UPEC (48%), 3 non-UPEC (15%), yrbH in 15 UPEC (71%), 4 non-UPEC (20%), yqeK in 6 UPEC (29%), 16 non-UPEC (80%) (Table 2-2).

We performed a BLAST search of the UPEC genes in our clinical UPEC database compiled of sequenced genomes from our collection of UPEC-enriched clinical isolates from DHMMC. A gene was considered present if the length match spanned the full gene length and had a percent identity of at least 98%. In our clinical UPEC database, we identified chuA in 63 UPEC isolates (85%), 31 non-UPEC (74%), fyuA in 62 UPEC (84%), 28 Non-UPEC (67%), papA in 1 UPEC (1%), 2 Non-UPEC (5%), traT in 64 UPEC (87%), 27 non-UPEC (64%), yrbH in 61 UPEC (82%), 27 non-UPEC (64%), yqeK in 32 UPEC (43%), 13 non-UPEC (31%) (Table 2-3).

UPEC and ESBL resistance gene frequencies in environmental samples

We performed PCR of our UPEC genes on six samples from each of our three environmental sites. Presence of any genes in any of the replicates were considered present at the site sampled. We identified chuA, yrbH, and traT in all environmental sites. We did not identify fyuA, papA, and yqeK in any environmental sites.

We wanted to compare the antibiotic resistance genotypes in environmental samples to the genotypes in UPEC-enriched clinical isolate collection[18] which may also support animal food source reservoirs for UPEC. We performed PCR to identify the following genes bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{OXA}. We identified bla_{SHV} in all environmental sites, bla_{TEM}, bla_{CTX-M}, and bla_{OXA} were identified in 2 out of 3 environmental sites. The two sites with bla_{TEM}, bla_{CTX-M}, and bla_{OXA} were dairy farms while the site without these genes was a cattle feed lot.

Discussion

The suite of genes chosen to identify UPEC in our environmental isolates are responsible for characteristic pathogenicity of UPEC and colonization of the urinary tract. chuA and fyuA are involved in the uptake of iron from host cells and successful biofilm formation in the iron-deplete urinary tract[19]. chuA is a heme transporter protein UPEC use to acquire iron from heme[20]. fyuA in responsible for iron uptake and essential for biofilm formation. papA is part of a pathogenicity island that is responsible for pilus formation during initial attachment of UPEC to the urinary tract[7]. traT protects UPEC from the host immune response by providing serum resistance contributing to the overall pathogenicity of UPEC[2]. yrbH encodes an isomerase required for the biosynthesis of the lipopolysaccharide (LPS) layer present on all Gram-negative bacteria[21]. yqeK is a gene within a second type III secretion system operon, ETT2. ETT2 has differential expression across *E. coli* types[17]. The downregulation of ETT2 is associated with the decrease in flagellar proteins and an increase in fimbriae[22]. Each of these genes contributes to the numerous characteristics that make UPEC unique. Identification of any combination of these genes in an environmental sample lends support to the presence of UPEC.

There were differences in the abundance of UPEC-associated and antibiotic resistance genes in the environmental and clinical isolates. In the environmental samples, fyuA and papA were not identified despite their frequency in the clinical databases and significant correlation to UPEC. We did identify chuA, yrbH, and traT in all environmental sites which suggests there are UPEC in environmental associated with animal food production. We wanted to compare the antibiotic resistance genotypes in environmental samples to the genotypes in UPEC-enriched clinical isolate collection[18] which may also support animal food source reservoirs for UPEC. Two of the sites were positive for bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{OXA} and one site was positive for bla_{SHV} only. The sites positive for bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{OXA} were dairy farms while the site with bla_{SHV} only was a cattle feed lot. On average, dairy cattle receive larger amounts of more classes of antibiotics more frequently throughout their lives[23,24]. Our antibiotics resistance gene findings support the higher prevalence of antibiotics used in cattle for dairy or at dairy farms as opposed to use in beef cattle. Taken together, the results show that UPEC identified at animal food production sites are not moving directly into hospitals. Future research will be to enrich for *E. coli* in the environmental samples then sequence individual environmental isolates. Genomic sequences will be used determine environmental *E. coli* isolates relatedness to clinical isolates to identify their relatedness to one another. Other future directions will be to identify variation among the ESBL and UPEC genes on interest.

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Chapter 4: Designing a plasmid vector to test the selection of common ESBL genes

Abstract

The fitness effects of antibiotic resistance genes under selection and dissemination of resistant bacteria throughout infectious and environmental bacterial populations. The interplay between these genes and their fitness effects in combination with each other remain largely unknown and undescribed. The ability to develop further insights into the fitness effects that result from the expression of multiple resistance genes and the effects of antibiotic consumption on multiple drug resistance has been severely hindered by a lack of comprehensive expression libraries of resistance genes. We catalyze the advancement of our knowledge about the cause/effect relationships of antibiotic consumption and resistance, we have created a plasmid vector-based construct library expressed in DH5α *E. coli* with natural gene expression and differential antibiotic resistant phenotypes as a tool to test antibiotic consumption on selection of ESBL resistance genes. Based on the frequency and genetic linkage trends of the ESBL resistance genes identified at DHMMC, we designed the construct library to contain the sixteen potential gene combinations of $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, $bla_{\text{SHV-2}}$, and $bla_{\text{TEM-10}}$ (COST) identified at DHMMC. The COST library has been validated to express the appropriate differential phenotypes.

Introduction

Antibiotic consumption mainly involves the indiscriminate use of antibiotics in a myriad of applications. The link between antibiotic consumption and antibiotic resistance is well documented and is the primary driver of antibiotic resistance[1]. Between 2000 and 2015, global antibiotic consumption increased 65% with documented parallel increases in antibiotic resistance[1]. The United States is the top consumer of antibiotics among high income countries[1]. Consumption of cephalosporins (specifically, third generation cephalosporins) has decreased in high income countries while it has increased in low middle income countries. Consumption of third generation cephalosporins is associated with the emergence of ESBL-producing bacteria[1]. There have been marked increase in the use of broad-spectrum antibiotics has increased globally, irrespective of a country's income. The use of broad-spectrum treatment as a result of empirical therapies over theoretical therapies based on culture identification and resistance phenotype can result in ineffective clearance of the infection-causing bacteria[2]. This inappropriate use of antibiotics creates evolutionary selective pressure on bacteria to develop and spread antibiotic resistance chromosomally or on plasmids. In the presence of antibiotics, select bacteria can resist, reproduce, and generate resistant strains of themselves[2]. Treatment-induced resistance results from selection of existing resistant strains rather than the evolution of resistance in the predominant infection-causing strain within a patient[3]. Non-human use of antibiotics include their use for the prevention and treatment of animal disease and the promotion of growth or productivity in food producing animals[2]. Antibiotics are also used extensively on crops to improve productivity by preventing or curing disease[4].

Current research on antibiotic resistance relies on surveillance studies to identify antibiotic resistant genotypes and phenotypes in the clinic. While this is critical for identifying resistance and susceptibility patterns of clinical isolates, there are likely unidentified genes and or accessory elements contributing to these patterns not taken into consideration. The COST vector is a defined system with four clinically relevant genes and their natural regulatory components. Conclusions drawn from experiments with the COST vector can pinpoint the specific fitness contributions and resistance phenotypes by each COST gene or combination of COST genes. Fitness experiments

with the COST vector will explain why some certain resistance gene combinations are identified in the clinic and why others are not. Identifying the fitness of the COST constructs in clinically relevant antibiotics will elucidate the trends identified in surveillance studies of clinical isolates.

It is critical to understand the evolutionary selection antibiotic consumption has on antibiotic resistance trends. Previous experimental work on the antibiotic selection on resistance genotypes was able to leverage selective pressure, in the form of sequential administration of different antibiotics to select for any particular resistance genotype of the sixteen TEM alleles tested[5]. Previous work has also shown that different resistance genes are genetically linked and can spread together at different frequencies[6]. We designed a plasmid vector that expresses four common ESBL genes CTX-M-15, bla_{OXA-1}, bla_{SHV-2}, and bla_{TEM-10} (COST), a tetracycline selectable marker, and a restriction site for an additional gene. $bla_{CTX-M-15}$ is one of the most widely disseminated ESBL resistance gene worldwide[7]. $bla_{CTX-M-15}$ confers resistance to the third generation cephalosporins with enhanced hydrolytic activity against ceftazidime[8]. bla_{OXA-1} confers resistance to aminopenillins, ureidopenicillins, and narrow spectrum penicillins. bla_{OXA-1} frequently occurs in combination with $bla_{CTX-M-15}$ resulting in resistance to combination β-lactam-β-lactamase inhibitor therapy[9]. bla_{SHV-2} is a successful SHV variant that confers resistance to 3rd generation cephalosporins with limited hydrolytic ability of ceftazidime[10]. bla_{TEM-10} is one of the over one hundred variants of TEM variant that confers ceftazidime resistance. Using this vector, we were able to create a construct library that contains sixteen potential genotype combinations of these common ESBL genes observed in the clinic expressing differential antibiotic resistance phenotypes.

Methods

Cost plasmid vector sequence design

The pBR322 plasmid vector containing four ESBL genes, $bla_{CTX-M-15}$, bla_{OXA-1}, bla_{SHV-2}, and bla_{TEM-10} was designed using the Benchling platform[11]. Using NCBI BLAST, I found numerous representative genomes for each gene. For $bla_{CTX-M-15}$, I ran n-BLAST on the gene and identified and downloaded eleven *E. coli* and *K. pneumoniae* genomes which contained $bla_{CTX-M-15}$. Using Benchling, I made an alignment of the upstream and downstream regions flanking $bla_{CTX-M-15}$ gene between neighboring genes. This was done to ensure my alignment had all the regulatory regions for $bla_{CTX-M-15}$and to identify essential conserved regions of the gene in a plasmid or genome. The conserved regions contained the -35 and -10 regions, identified using the annotations from NCBI as well as conserved regions across genomes flaking $bla_{CTX-M-15}$. The consensus sequence derived from this alignment was used as the $bla_{CTX-M-15}$gene insert for the plasmid vector. This was done for bla_{OXA-1} and bla_{SHV-2}. For bla_{OXA-1}, I used twelve representative genomes from *E. coli* and *K. pneumoniae*. For bla_{SHV-2} I used seven representative genomes from *E. coli* and Klebsiella species (*K. pneumonia* and *K. michiganensis*)

For TEM-10 I used eight representative genomes from *E. coli*, *Acinetobacter baumannii*, *K. pneumonia,* and the cloning pBR322 vector. We planned to use the Amp resistance marker included in pBR322, bla_{TEM-1}, with all its regulatory elements as our TEM-10 insert by changing the sequence at where bla_{TEM-1} and bla_{TEM-10} differ. After downloading the genomes containing bla_{TEM-1} and bla_{TEM-10} and aligning them, I was able to identify the conserved regions and places where bla_{TEM-1} and bla_{TEM-10} differ. The consensus sequence was derived to include all conserved regulatory regions across genomes with respect to pBR322 and bla_{TEM-10} mutations.

The *bla*$_{TEM-1}$ sequence in pBR322 was edited to code for *bla*$_{TEM-10}$ while *bla*$_{SHV-2}$, *bla*$_{OXA-1}$ and *bla*$_{CTX-M-15}$ genes were inserted downstream of *bla*$_{TEM-10}$. The orientations of each gene were maintained from the plasmids in which they were identified. TEM-10 and *bla*$_{SHV-2}$ are located on the reverse strand while *bla*$_{OXA-1}$ and *bla*$_{CTX-M-15}$are on the forward strand.

Restrictions enzyme sites were identified using Benchling. I performed a vector wide search to identify New England Biolabs (NEB) restriction sites not found in pBR322 containing all gene inserts. From this list, I excluded restriction sites with methylation issues and limited the list further to those restriction enzymes whose reactions occur in the same reaction buffer which consisted of nine potential restriction enzymes. I searched the vector for sequences similar to those restriction sites, limiting my search to one to three nucleotide changes. I was able to identify and incorporate eight restriction sites before and after each gene with limited nucleotide changes (MfeII, SpeI, AvrII, SacI, AflII, SbfI, PacI, and KpnI). I ran another search of restriction sites in the vector to determine if newly inserted restriction sites created any issues (new restriction sites) and verified these sites occurred only once in the vector sequence. Finally, using Benchling's virtual digestion feature, I ran digestions to verify my restriction sites resulted in the appropriate constructs.

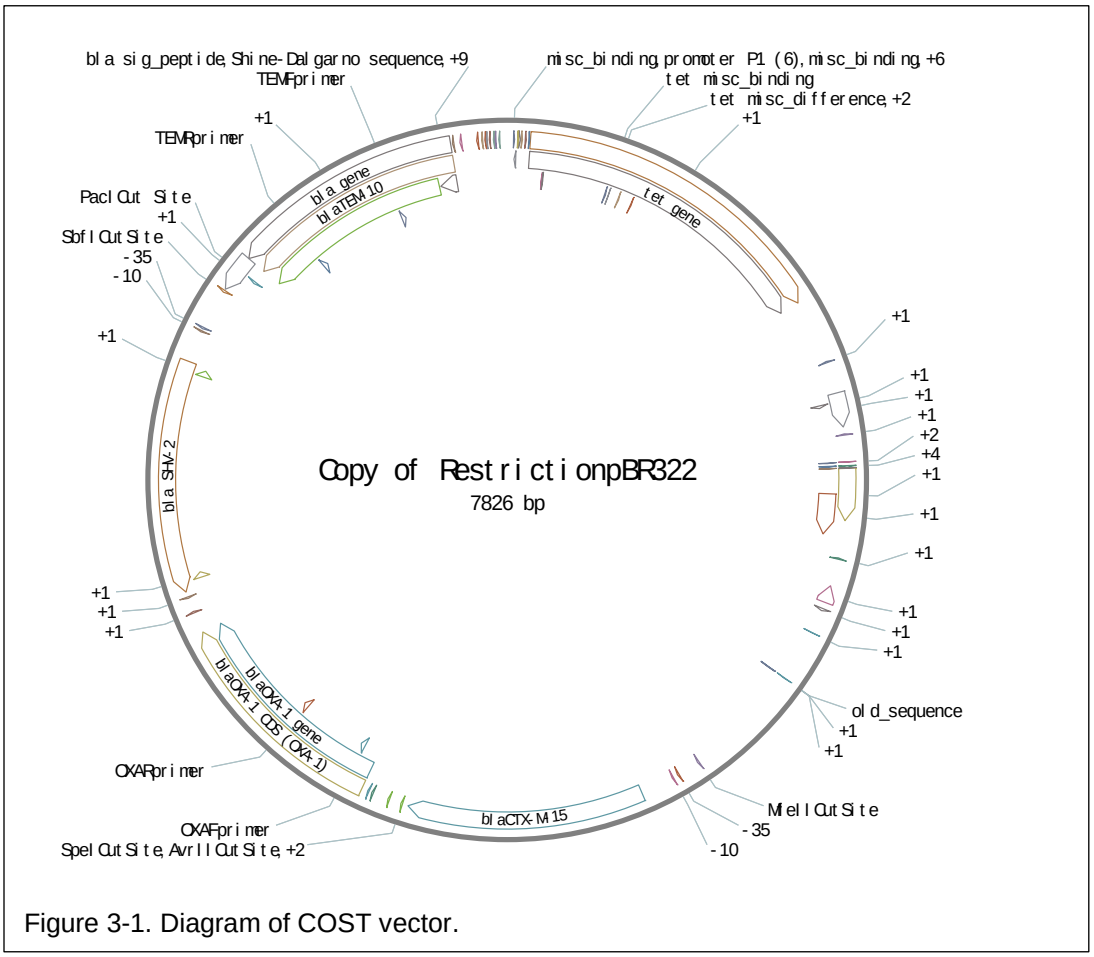

Figure 3-1. Diagram of COST vector.

An additional restriction site was added for a fifth gene or genetic marker to be tested in combination for future projects. I added the restriction site, BsaBI, near the tetracycline selectable marker, downstream of the gene that can be used as an

additional insert site once constructs have been created. Just after digesting the vector into the 16 constructs inserting the additional genetic marker or a barcode so the single construct libraries have unique identifiable regions for sequencing or qPCR.

COST plasmid vector library creation

I created a construct library to be able to test fitness and selection of the different ESBL genes and different combination of ESBL genes. I performed sequential restriction digestions of the COST vector, removing a single gene at a time resulting in 16 different combinations of ESBL genes expressed in *E. coli*: COST, COT, COS, CST, OST, CO, CS, CT, OS, OT, ST, C, O, S, T, and Empty.

Twenty mL of COST expressed in DH5 alpha *E. coli* in LB Miller broth was spun down into a pellet at 16,000 rpm in 30 second increments until all 20mL of culture was used to make the pellet. The pellet was then processed using the NEB Monarch Mini Plasmid Prep Kit to extract plasmid DNA. Gel electrophoresis was used to confirm the presence of plasmid DNA. 2µg of plasmid DNA was used with 2µL of 2 restriction enzymes were used in double digestion of plasmid DNA to remove a single ESBL gene. Restriction digestions where incubated at 37°C for 1 hours followed by spin column clean-up using the NEB Monarch PCR clean up kit to stop the digestion and remove gene cut from vector. Gel electrophoresis was performed to confirm appropriate digestion sizes based on construct digested and gene removed. Blunting was performed to clean up digested over-hanging ends of the vector to make ligation easier. The blunting reaction was incubated at room temperature for 30 minutes then 70°C for 10 minutes to inactivate the blunting enzymes. 17µL of blunted DNA was used to ligate vector ends together (2µL ligase buffer, 1µL ligase). The ligation reaction was incubated at room temperature for 2 hours, then 65°C for 10 minutes to inactivate ligase. Spin columns were used to remove salts and ethanol prior to transformation. 20µL of ligated vector DNA was added to spin columns, centrifuges at 800x*g* for 2 minutes. Between 5-15µL of vector DNA was added to 40µL of competent DH5α *E. coli* cells, electroporated, and incubated in 2mL SOC media at 37°C on a rotor for 1 hour. 100-200µL of transformed cells were plated on LB-tet plates overnight. Single colonies were picked for PCR to verify the correct genes were present and then streaked on LB tet plates to obtain single pure colonies. A single pure colony was used to inoculate LB tet broth and incubated at 37°C overnight. The broth culture was spun down at 3500x*g* for 10 minutes to pellet cells. 1mL of glycerol was used to resuspend pellet then stored at -80°C for long term storage. This was done for all sixteen constructs.

Construct	Genes	Size (bp)
COST	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{SHV-2}$, *bla*$_{TEM-10}$, *tetR*	7826
COT	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{TEM-10}$, *tetR*	6651
COS	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{SHV-2}$, *tetR*	6800
CST	*bla*$_{CTX-M-15}$, *bla*$_{SHV-2}$, *bla*$_{TEM-10}$, *tetR*	6805
OST	*bla*$_{OXA-1}$, *bla*$_{SHV-2}$, *bla*$_{TEM-10}$, *tetR*	6686
CO	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *tetR*	5625
CS	*bla*$_{CTX-M-15}$, *bla*$_{SHV-2}$, *tetR*	5779
CT	*bla*$_{CTX-M-15}$, *bla*$_{TEM-10}$, *tetR*	5630
OS	*bla*$_{OXA-1}$, *bla*$_{SHV-2}$, *tetR*	5660
OT	*bla*$_{OXA-1}$, *bla*$_{TEM-10}$, *tetR*	5515
ST	*bla*$_{SHV-2}$, *bla*$_{TEM-10}$, *tetR*	5666
C	*bla*$_{CTX-M-15}$, *tetR*	4411
O	*bla*$_{OXA-1}$, *tetR*	4493
S	*bla*$_{SHV-2}$, *tetR*	4640
T	*bla*$_{TEM-10}$, *tetR*	4491
Empty	*tetR*	3472

Table 3-1. List of COST construct library abbreviations, genes present, and vector size.

ESBL resistance phenotype assay of COST vector construct library: Disk diffusion

I performed a Disk Diffusion assay to confirm ESBL activity and differential expression of ESBL genes in the different plasmid constructs. I streaked the following constructs onto LB-tet plates: COST, COT, CST, COS, OST, CO, OS, OT, O, and the empty vector. Constructs were grown at 37ºC overnight then single colonies were selected to inoculate Mueller-Hinton (MH) Broth in a standing culture overnight at 37°C. 100µL of each MH broth culture was used to inoculate MH plates and spread evenly across the plates with sterile glass beads. Two MH plates of each culture were inoculated. Plates were left to dry for 10 minutes. The antibiotics and their concentrations in Table 3-2. were stamped on the inoculated MH plates. Plates were grown for 18 hours at 37°C and then zones of inhibition were measured.

Antibiotic Disk	Concentration (µg/µL)	Class and Generation
Ceftriaxone (CRO)	30	Cephalosporin, 3rd
Imipenem (IPM)	10	Carbapenem, 1st
Cefoxitin (FOX)	30	Cephamycin, 2nd
Cefepime (FEP)	30	Cephalosporin, 4th
Amoxicillin+ Clavulanic Acid (AMC)	30	Penicillin, 3rd + inhibitor
Cefotetan (CTT)	30	Cephamycin, 2nd
Ceftazidime (CAZ)	30	Cephalosporin, 3rd
Ampicillin (AM)	10	Penicillin, 3rd

Table 3-2. Antibiotic abbreviations, concentrations, class, and generation of antibiotics disks used for Mueller-Hinton Disk Diffusion assay.

Construct	CRO 30	IPM 10	FOX 30	FEP 30	AMC 30	CTT 30	CAZ 30	AM 10
Empty	31 mm	30 mm	30 mm	32 mm	21 mm	31 mm	30 mm	8 mm
COT	0	28	31	11	10	27	12	0
CO	0	26	29	12	9	29	14	0
OST	16	31	32	20	16	32	0	0
CST	6	30	31	15	16	31	10	7
COST	0	26	30	12	15	28	10	0
OS	16	30	32	21	15	30	20	0
COS	0	30	32	13	11	30	14	0
OT	33	29	32	33	21	33	13	0
O	35	27	30	36	19	30	29	8

Table 3-3. Mueller-Hinton Disk Diffusion susceptibility assay results. The antibiotic susceptibility results from each construct tested. The zone of inhibition is the diameter measured in millimeters (mm). A susceptible result is indicated by green, an intermediate result is indicated by yellow, and a resistant result is indicated by red according to CLSI and EUCAST standards.

ESBL resistance phenotype assay of COST vector construct library: Minimum inhibitory concentration

I performed a preliminary minimum inhibitory concentration (MIC) assay to further identify different resistance phenotypes among the COST construct library. MICs are a preliminary step for growth rate assays as they determine the minimum concentration of antibiotic required to inhibit growth. Growth rate assays use the concentration just below the MIC to determine the inhibitory but sublethal concentration. This concentration can capture selection and be used to determine bacteria fitness in the presence of specific antibiotics. The purpose of preforming the preliminary MICs is to determine suitable antibiotic concentrations for each antibiotic to be tested with all constructs.

I performed MICs on all sixteen constructs using the following antibiotics and inhibitor combinations: Meropenem, Meropenem + avibactam (4µg/µL), Aztreonam, and Ertapenem. Constructs were streaked for isolation onto LB + tet plates overnight at 37°C. Single colonies were selected and used to inoculate 10mL of LB Miller broth + tet. Standing cultures were incubated overnight at 37°C. Absorbance readings were taken for each construct broth culture to determine the amount of each culture to add to obtain a concentration of 2 x 10^5 cells/mL of Mueller-Hinton II broth. Antibiotic concentrations were serially diluted using MHII broth cultures. Meropenem concentrations without the inhibitor ranged from 4096 to 0.0625 µg/µL. 180µL of MHII broth cultures and 20µL of

10X Meropenem were used to create the initial 4096 µg/µL concentration then serially diluted to 0.0625 across seventeen wells. Aztreonam, Ertapenem, and Meropenem + avibactam (4µg/µL) concentrations ranged from 1024 to 0.0625µg/µL. For Aztreonam and Ertapenem 180µl of MHII broth cultures and 20µL of 10X antibiotic concentrations were used to create the initial 1024 µg/µL concentration then serially diluted to 0.0625 µg/µL across fifteen wells. For Meropenem + Avibactam, 185µL of MHII broth cultures and 5µL of 40X Meropenem were used to create the initial 1024µg/µL concentration then serially diluted to 0.0625µg/µL across fifteen wells. 10µL of 20X avibactam was added to each well after serial dilutions were done to create a 4µg/µL concentration in each well. Control wells for each construct were used for each antibiotic treatment to serve as a positive control for construct growth.

MIC results were read at 18 hrs. MICs were determined to be the well with the lowest antibiotic concentration with no growth.

	Meropenem	Meropenem +Avibactam (4µg/µL)	Aztreonam	Ertapenem
Empty	<0.0625	<0.0625	<0.0625	<0.0625
COT	<0.0625	<0.0625	256	256
CO	<0.0625	<0.0625	128	<0.0625
OST	<0.0625	<0.0625	32	<0.0625
CST	<0.0625	<0.0625	256	<0.0625
COST	<0.0625	<0.0625	128	<0.0625
OS	<0.0625	<0.0625	4	<0.0625
COS	<0.0625	<0.0625	256	<0.0625
OT	<0.0625	<0.0625	32	<0.0625
O	<0.0625	<0.0625	<0.0625	<0.0625
CS	<0.0625	<0.0625	64	<0.0625
CT	<0.0625	<0.0625	256	<0.0625
ST	<0.0625	<0.0625	64	<0.0625
C	<0.0625	<0.0625	256	<0.0625
S	<0.0625	<0.0625	4	<0.0625
T	<0.0625	<0.0625	16	<0.0625

Table 3-4. MIC results for all constructs in Meropenem, Meropenem + Avibactam, Aztreonam, and Ertapenem. The colors of the boxes indicate the level of susceptibly to the antibiotics based in EUCAST 2022 and CLSI 2021 standards. Green indicates susceptibility, white indicates intermediate susceptibility, and red indicates resistance.

Sequencing primer design to confirm sequence of constructs

The COST construct library was created by performing sequential restriction digestions of the COST vector into all possible gene combinations. To determine the correct vector sequence has been maintained throughout restriction digestions, it is important to sequence all of the constructs. I designed a series of sequencing primers to be used on all constructs to confirm the sequence of each COST construct. These primers are designed to capture areas of variation between constructs resulting in different length PCR products to be sent for sequencing. Each primer has been tested in silico on all constructs. A list of primers is provided in Table 3-5.

Primer region	Forward 5'→3'	Reverse 5'→3'
TEM_TET	GGCCTATCACGAGGCCCTTTCG	GTGAATCCGTTAGCGAGGTGCCG
TEM	GCAACTGTGACGCTCAGTGGAACG	CGAAAGGGCCTCGTGATACGCC
SHV	GCAAAGTCACAGACCGCGGGAT	CCACTGAGCGTCACAGTTCCTGC
OXA	AACCCTTCCATCGAGGGGGACG	GCGGTCTGTGACTTTGCCGTCT
CTXM	CTGCGCTCTGCTGAAGCCAGTT	CGTCCCCCTCGATGGAAGGGTT
MISC_CTXM	GTAAAAAGGCCGCGTTGCTGGC	GTAACTGGCTTCAGCAGAGCGC
TET_MISC	CAGCAACGCGGCCTTTTTACGG	CTCGACCTGAATGGAAGCCGGC

Table 3-5. List of PCR sequencing primer pairs and the regions in the construct vector they amplify. These primers result in amplicons which span the length of all vector constructs.

Results

Disk Diffusion

The distinction between susceptible, intermediate, and resistant are determined by the European Society of Clinical Microbiology and Infectious Diseases (EUCAST) and the Clinical and Laboratory Standard Institute (CLSI) standards for disk diffusion based on the diameter of the zone of inhibition around the antibiotic disks listed in Table 3-3[12,13]. All constructs were susceptible to imipenem, cefoxitin, and cefotetan. All constructs are resistant to ampicillin. All constructs with *bla*$_{\text{CTX-M-15}}$are resistant to the third generation cephalosporins (ceftriaxone and ceftazidime). These results are indicated by a disk diameter of less than 19mm for ceftriaxone and ceftazidime. All but one of the constructs with *bla*$_{\text{SHV-2}}$ are resistant to 3rd generation cephalosporins. The OS construct shows intermediate resistance to ceftazidime. This intermediate result is indicated by a disk diameter of 20mm. Constructs with *bla*$_{\text{TEM-10}}$ confer resistance to ceftazidime in constructs without *bla*$_{\text{CTX-M-15}}$ or *bla*$_{\text{SHV-2}}$ (OT). Constructs with only *bla*$_{\text{OXA-1}}$ and *bla*$_{\text{OXA-1}}$ and *bla*$_{\text{TEM-10}}$ were susceptible to the most antibiotics. *bla*$_{\text{OXA-1}}$ conferred resistance to ampicillin and in combination with *bla*$_{\text{TEM-10}}$ resistance to ceftazidime.

Minimum inhibitory concentration

The distinction between susceptible, intermediate, and resistant are determined by the European Society of Clinical Microbiology and Infectious Diseases (EUCAST) and the Clinical and Laboratory Standard Institute (CLSI) standards for MIC based on the growth in the concentrations of the antibiotics listed in Table 3-4.

All constructs were susceptible to meropenem and meropenem + avibactam as indicated by inhibition of growth in the presence at concentrations below 0.0625µg/µL. All but one of the constructs were susceptible to ertapenem based on an MIC of less than 0.0625 µg/µL. The COT construct containing *bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, and *bla*$_{TEM-10}$ showed resistance to ertapenem with an MIC of 265µg/µL. The construct with no resistance genes and the construct with only *bla*$_{OXA-1}$ were susceptible to aztreonam with MICs of less than 0.0625µg/µL. The *bla*$_{OXA-1}$ and *bla*$_{SHV-2}$ construct (OS) and the *bla*$_{SHV-2}$ construct (S) both showed intermediate resistance to aztreonam with MICs of 4µg/µL. The remaining constructs were resistant to aztreonam.

Discussion

Disk diffusion

The results of the MH disk diffusion assay show expected differential gene expression across constructs. *bla*$_{CTX-M-15}$ is responsible for conferring resistance to 3rd generation cephalosporins. The MH disk diffusion results reflect this activity. *bla*$_{TEM-10}$ confers ceftazidime resistance. This is illustrated by the *bla*$_{TEM-10}$ construct (T) due to its susceptibility to all antibiotics except ceftazidime in which it is resistant. *bla*$_{OXA-1}$ confers resistance to aminopenillins, ureidopenicillins, and narrow spectrum penicillins. The limited resistance conferred by the *bla*$_{OXA-1}$ construct (O) is due to its broad-spectrum ß-lactamase activity, as opposed to extended spectrum activity. *bla*$_{SHV-2}$ confers resistance to 3rd generation cephalosporins. The intermediate susceptibility to ceftazidime by *bla*$_{OXA-1}$ and *bla*$_{SHV-2}$ (OS) is due to the combination of *bla*$_{OXA-1}$ not contributing resistance to ceftazidime and *bla*$_{SHV-2}$ has limited hydrolytic ability of ceftazidime. This is due to a lack of the E240K mutation which allows for catalyzation of the bulky side group in ceftazidime[14].

Minimum inhibitory concentration

Meropenem and ertapenem are carbapenem antibiotics which are proven effective against ESBL-producing bacteria which are resistant to penicillins and cephalosporins[15]. Susceptibility to meropenem for all constructs is in line with expected results. The overall susceptibility to ertapenem by all but one construct (COT) is in line with expected results. The high MIC for ertapenem seen with the COT construct is unusual and likely due to error (pipetting) and not a consequence of high resistance conferred by COT. This should be double checked by performing additional replicates of this experiment.

Aztreonam is a monobactam antibiotic for use against Gram-negative bacteria but is generally ineffective against ESBLs and *E. coli*. The results in Table 3-4 follow that trend with resistance by all constructs with the ESBL genes *bla*$_{CTX-M-15}$ and *bla*$_{TEM-10}$. The constructs with ESBL genes that show susceptibility or intermediate susceptibility are *bla*$_{OXA-1}$ and *bla*$_{SHV-2}$ (OS), *bla*$_{SHV-2}$ (S), and *bla*$_{OXA-1}$ (O). Constructs with *bla*$_{SHV-2}$ have intermediate resistance/susceptibility to aztreonam likely due to their inability to process the bulky sidechain of aztreonam[14]. The susceptibility to aztreonam by *bla*$_{OXA-1}$ (O) can be attributed to the fact that it is not an extended spectrum β-lactamase, but rather a broad-spectrum β-lactamase. *bla*$_{OXA-1}$ is often found in isolates with other ESBLs and confers additional resistance.

Differential phenotypes of COST

The COST plasmid vector library is a critical tool for testing the effects of antibiotic consumptions on the selection of four common ESBL genes. The COST vector construct library contains all combinations the ESBL genes and expresses differential resistance phenotypes those combinations. Future work would include testing the fitness of the different COST vector constructs in clinically relevant antibiotics will shed light on selection of individual genes and different combinations of genes. This will be essential in the face of the antibiotic resistance crisis that plagues the US and globe. Understanding how selection works is key to mitigate unnecessary deaths and to maintain the efficacy of current antibiotics.

The COST vector provides an elegant tool for understanding the selection of antibiotic resistance genotypes using multiple clinically relevant resistance genes. The expected results of work performed with the COST vector will allow for controlled studies addressing the relationship between antibiotic consumption and the selection of resistance genes and combinations of genes. Future fitness experiments with the COST vector are likely to identify antibiotics or combinations of antibiotics that will prove disadvantageous to problematic combinations of these genes. The results can be used to update guidelines for UTI treatment with antibiotics and inform effective antibiotic cycling protocols.

Prospectus

The research presented throughout this dissertation addresses two facets of public health concerns in Merced, CA, and provides an important tool for investigating a third. In the second chapter, antibiotic resistance trends from the local hospital, DHMMC, revealed elevated resistance compared the nationwide US. The elevated resistance was evident on several fronts. At DHMMC, there were higher frequencies of $bla_{CTX\text{-}M}$, which confers resistance to later generation antibiotics. There was also evidence of bla_{TEM} displacement by $bla_{CTX\text{-}M}$, which indicates selection for higher resistance genotypes. Nationwide, there were high frequencies of bla_{TEM} in combination with negative gene correlations between bla_{TEM} and bla_{SHV}, $bla_{CTX\text{-}M}$, and bla_{OXA}. These results suggest less multidrug resistance nationwide because most of the isolates have blaTEM and are less likely to have any other resistance genes. There were significant positive genetic correlations between bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, and bla_{OXA} in *K. pneumoniae* isolates which suggests multidrug-resistant strains at DHMMC. This is in opposition to trends nationwide where there were negative gene correlations between blaSHV and all other resistance genes. Overall, the antibiotic resistance trends in Merced deviate significantly from the nationwide U.S. In the second chapter, we investigated a potential source of these resistance trends at DHMMC.

Our clinical isolate collection from DHMMC is enriched with UTI samples caused by uropathogenic *E. coli*. Recent literature suggests UPEC are a foodborne illness caused by ingesting contaminated food products[16]. Since Merced is a top agriculture producer of dairy and cattle, we sought to investigate the presence of UPEC in local agriculture sites associated with animal food production. We identified a suite of genes (*chuA, fyuA, papA, traT, yrbH, yqeK*) significantly correlated with UPEC to rapidly identify UPEC. We collected environmental samples from two dairy farms and one cattle feed lot in Merced and found three out of the five genes positively correlated with UPEC at all sites. We also investigated the level of antibiotic resistance at these agriculture sites with respect to the trends we found at DHMMC. We identified bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, and bla_{OXA} at two of the three sites surveyed. The remaining site was positive for only bla_{SHV}. These results reveal the presence of UPEC and antibiotic resistance genes at agricultural sites surrounding Merced that are also found in UTI samples from the local hospital. The differences in abundance of the genes identified, suggest that there is not direct movement of UPEC from agricultural sites into DHMMC. It is worth noting, we only looked at one point along the food chain and can only make conclusions about the environment of dairy farms and cattle feed lots in Merced. Future research, would include collecting samples from food animals (cows and chickens) and environmental samples from a wide variety of agriculture sites, including chicken farms. Using the UPEC genes we identified, future research can quickly identify the presence of UPEC in a sample and proceed with genome sequencing for targeted bioinformatic and phylogenetic analysis.

Previous work done in the Barlow, lab was able to use antibiotics to drive selection of desired TEM genotypes[5]. Combining this previous work with the surveillance work done in chapter one, I designed a customizable plasmid vector of antibiotic resistance genes from the families of genes I identified at DHMMC. The plasmid vector has $bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}1}$, $bla_{SHV\text{-}2}$, and $bla_{TEM\text{-}10}$ (COST) under the natural expression of their own promoters flanked by unique restriction enzyme sites, a tetracycline selectable

marker, and a restriction site for an additional gene. I designed the COST plasmid vector as a tool for understanding the antibiotic resistance phenotypes of resistance genes in combination with each other and alone. I performed sequential restriction enzyme digestion of each gene to make constructs of the sixteen possible gene combinations expressed in *E. coli*. I tested the COST construct library using two antibiotic susceptibility assay, Mueller-Hinton disk diffusion and minimum inhibitory concentration, and confirmed the differential resistance phenotype in all constructs. The COST vector and subsequent vector construct library is an important tool to understand how resistance genes confer resistance in combination with one another and alone. Future research will make use of the COST vector and construct library to study the effects of antibiotic consumption on the selection of different resistance genotypes.

The research in this dissertation has highlighted the need for and created tools to address important public health concerns in Merced. This research highlights the importance of continued surveillance of antibiotic resistance genotypes, identified a potential reservoir for antibiotic resistant UPEC, and developed an important tool to test ongoing selection of observed antibiotic resistance genotypes.

While my work focused on serving the local community, it is important to the nation and world as the Merced area encompasses many aspects of the antibiotic resistance problem. This research has created approaches and tools for studying a local region that are important in that they can be translated nationwide and worldwide.

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Appendices

List of genomes for *E. coli* database with accession numbers

Commensal strain genomes:
GCF_000005845.2_ASM584v2_genomic.fna.gz
GCF_000333215.1_ASM33321v1_genomic.fna.gz
GCF_001566335.1_ASM156633v1_genomic.fna.gz
GCF_003546975.1_ASM354697v1_genomic.fna.gz
GCF_000010245.2_ASM1024v1_genomic.fna.gz
GCF_000714595.1_ASM71459v1_genomic.fna.gz
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GCF_000017765.1_ASM1776v1_genomic.fna.gz
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GCF_002843685.1_ASM284368v1_genomic.fna.gz

ExPEC strain genomes:
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GCF_000017745.1_ASM1774v1_genomic.fna.gz
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UPEC strain genomes:
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List of COST Vector Construct Library Sequences

>COST- pBR322 expressing CTX-M-15, OXA-1, SHV-2, TEM-10

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