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Genetic Determinants of Antibiotic Resistance and their Effect on Fitness as Determined by Growth Rate Assays

A Thesis submitted in fulfillment of
the Requirements for the degree

Of

Masters of Science in Quantitative and Systems Biology

At

The University of California Merced

By

Evin Doscher

2017

Signature page for Masters Thesis defense

I certify that I have read this Thesis and that, in my opinion, it is fully adequate in scope and quality as a Thesis for the degree of Masters of Science

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Approved for the University of California Merced Committee on Graduate Studies.

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Abstract

Antibiotic resistance is a major public health crisis in the United States and around the world, with at least 23,000 people dying each year in the U.S. from antibiotic resistant infections; it is a major cause of death. Technical advancements to facilitate studies and diagnosis of resistance are an important step for finding solutions to this problem. For this study, we partnered with a local community hospital Mercy Medical Center. Our study focuses on resistant isolates that have been identified as possessing the Extended Spectrum β-Lactamases (ESBLs). We have used a novel and sensitive method for measuring the fitness of these isolates. By using growth rates as a measurement for fitness we have discovered previously unknown relationships between co-expressed resistance genes. We identified that in the presence of ampicillin, isolates that have both CTX-M-15 and TEM-1 genes have a slightly higher mean growth rate than isolates that have the CTX-M-15 gene but not the TEM-1 gene leading us to believe that there may be a synergistic effect between CTX-M15 and TEM-1 for the resistance of ampicillin. Surprisingly we also found that isolates in the presence of cephalosporin showed a decrease in growth rates if they had the TEM-1 resistance gene. This suggests that the presence of TEM-1 has a slightly inhibitory effect on growth in the presence of cephalosporins, which is still not understood at a cellular level. We also found that in the presence of ampicillin-sulbactam (SAM) there was no difference in growth rates for isolates that were TEM-1 (+) with those that are TEM-1 (-). However there was a difference in mean growth rates when comparing CTX-M-15 (+) isolates and CTX-M-15 (-) isolates in the presence of SAM. Indicating that CTX-M-15 is interfering with resistance for SAM when TEM-1 is present. Growth rates are a reliable measure of fitness. They calculate the maximum growth by measuring the optical density of that culture over time. The sensitivity of these assays may lead to novel findings about the nature of antibiotic resistance evolution. Future studies of fitness outcomes using growth rate assays are recommended.

Introduction:

Historical Context

Modern Microbiology began in 1676 when Anton van Leeuwenhoek made observations of bacteria and microorganisms using a single lens microscope that he had built himself (Toledo-Pereyra 2009). In the 1800s Ferdinand Cohn founded the field of bacteriology with his classification of bacteria based on morphology with the four classifications, thread, spherical, short rod and spiral (Kirk and Gruber 2005). Louis Pasteur disproved the theory of spontaneous generation in 1859 and that launched the field of microbiology and validated it as a biological science.

Around the same period of time the German microbiologist Robert Koch devised four generalized principals known as Koch's Postulates. These postulates helped to create a causative relationship between a microbe and a disease(Gal 2008). Koch was also responsible for introducing the modern streak plate method. Streak plating allows the isolation of a pure strain from a single species of Microorganism(Gal 2008). When studying bacteria, a sample is obtained then cultured on a petri dish made of agar. Agar is a jelly like substance that provides both nutrients and structural support for bacterial growth. With proper streak plating technique it is possible to obtain individual colonies of identical bacterial cells for testing.

The history of antibiotic resistance begins with the discovery of the first antibiotic penicillin discovered in 1928 by Alexander Flemings; which he isolated from the

mold penicillium(Abraham and Chain 1988). Penicillin's clinical value comes from its capability of exploiting and destroying the unique features of the bacterial cell while leaving human eukaryotic cells unharmed (Alekshun and Levy 2007). One of the defining features of a bacterial cell is its cell wall, which acts as both a protective layer surrounding the cell and a rigid structure preventing the cell from bursting from osmotic pressure. The cell wall is composed of peptidoglycan, a polymer composed of sugars and amino acids. Two repeating residues β-(1,4) linked *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) make up the peptidoglycan layer. The cell wall is in a dynamic state of building and breaking down the peptidoglycan layer as the cell wall changes responding to cell growth and division.

One of the most common ways to classify pathogenic bacteria is through the use of a Gram stain. Danish bacteriologist Hans Christian Gram developed the gram stain technique in 1884(Spiegel, Amsel et al. 1983). It uses two dyes crystal violet dye and safranin to help visibly distinguish the physical and chemical properties of bacterial cell walls. This technique allowed bacteria to be grouped into two large categories, being gram positive and gram negative. Gram-positive bacteria absorb the crystal violet stain and appear purple under the microscope(Bartholomew and Mittwer 1952). This is due to a single thick peptidoglycan layer that can measure from 20-80nm in thickness. Gram-positive bacteria have an outer cell wall followed by an inner cell membrane. Common gram-positive bacteria include *Streptococcus*, *Staphylococcus* and *Listeria*. Gram-negative bacteria do not retain the

crystal violet stain after rinsing but do retain the counterstain safranin, which makes them appear pinkish red under a microscope(Bartholomew and Mittwer 1952). Gram-negative bacteria differ from gram-positive bacteria in that they have 3 membranes, an outer membrane then a cell wall and an inner membrane. The outer membrane contains Lipopolysaccharides (LPS), which help protect them from chemical attack, but also functions as an endotoxin that induce an immune response. The cell wall, which fits between the outer and the inner cell membranes, is much thinner than in gram-positive bacteria since it only measures 2-3nm in thickness. Common gram-negative bacteria are *Escherichia coli*, *Salmonella* and *Pseudomonas* (Bartholomew and Mittwer 1952).

One of the most well studied model organisms is *Escherichia coli* (*E. coli*), which is a gram-negative commensal bacterium that is found in the human gut. It is part of the normal flora of the gut and produces vitamin K2 and prevents the colonization of pathogenic bacteria in the gut. It's model organism status came from the ease at which it can be cultured and grown outside of the body. It is capable of metabolizing a wide variety of substrates and has a short replication period of around 20 minutes under optimal conditions. But *E.coli* can also lead to disease in humans causing urinary tract infections, gastroenteritis and Crohns disease(Lau, Kaufmann et al. 2008). Benign E. coli can only become pathogenic after gaining a virulence factor from another bacterium.

Penicillin is a β-lactam antibiotic that works by irreversibly binding to the enzyme DD-transpeptidase that is responsible for crosslinking the residues NAG and NAM(Demain and Sanchez 2009). The key feature of the penicillin molecule is its 4 membered beta-lactam ring that allows the molecule binding affinity. When this occurs the formation of peptidoglycan ceases and the cell wall ruptures leading to the death of the bacterial cell(Girardin and Philpott 2004).

In the 1940's, once Penicillin was in wide use, resistance strains of bacteria began to develop that could inactivate the antibiotic (Davies and Davies 2010). As a result of the widespread usage of penicillin, bacteria began to evolve resistance to penicillin. Bacteria evolved enzymes known, as β -lactamases; these enzymes were capable of inactivating penicillin by hydrolyzing the β lactam ring(Bush 1988). In the time following the discovery and commercialization of penicillin many more classes of antibiotic were introduced. The development of antibiotics ushered in a new era of health, where historically life-threatening infections became routinely curable. Even with the great clinical success of antibiotics, resistant infections still developed. What remained elusive was the mechanism of bacteria's rapid resistance(Bush 1988).

In 1945 the Italian pharmacologist Giuseppe Brotzu discovered a subgroup of β-lactamase called cephalosporins (Bo 2000). Cephalosporin antibiotics work like other β-lactam antibiotics in that they interfere with the synthesis of the

peptidoglycan later of bacteria cell walls. However it was found that they were less susceptible to the β-lactamase enzyme than other β-lactam antibiotics (Bo 2000).

In the mid 1950's Japanese researchers discovered transferable genetic elements capable of being transmitted through a population of bacteria by means of conjugation (2001). Conjugation is the transfer of genetic material between bacterial cells; this is facilitated by a pilus, which is a hair like appendage that extends from one cell to another. In this way resistance genes can be spread rapidly through bacterial communities. These genetic elements that are transmitted are called plasmids, which are small circular double stranded DNA molecules that are separate from the chromosome(Recchia and Hall 1995). Bacterial cells become capable of conjugation after receiving what is called an F plasmid from a donor bacterial cell. This F plasmid confers the recipient bacterial cell the ability to then form a pilus and transmit genetic elements via plasmid to other bacteria cells (Sorensen, Bailey et al. 2005). This form of genetic exchange is called horizontal gene transfer. These resistance mechanisms have persisted and ultimately have allowed seemingly benign infections such as urinary tract infections (UTIs) caused by *E.coli* to become a global public health threat(McDanel, Schweizer et al. 2017).

Among gram-negative bacteria, plasmid borne beta-lactamase genes have been the most common source of resistance. In particular the TEM β lactamase has been an especially common resistance gene. The TEM-1 resistance gene was first reported in 1965 from an *E. coli* isolate (Salverda, De Visser et al. 2010). TEM-1 showed genetic

similarities to SHV-1 β-lactamases (Salverda, De Visser et al. 2010). One of the defining features of TEM-1 was its ability to hydrolyze both penicillins and cephalosporins(Salverda, De Visser et al. 2010). Over 170 variants of the TEM allele have been isolated in hospitals around the world(Salverda, De Visser et al. 2010). As a result the pharmaceutical industry in the 1980's began introducing new β-lactam antibiotics(Salverda, De Visser et al. 2010).

CTX-M-1 was first discovered in 1989 in both Europe and South America (Davies and Davies 2010). The first publication of the new ESBL was from a patient with an ear infection and the isolate was found to be resistant to cefotaxime (Davies and Davies 2010). In the 1990's diversification of the CTXM-1 allele was recorded with new variants CTX-M-10 which was discovered in the Mediterranean and CTX-M-15 which was discovered in India (Davies and Davies 2010). CTX-M-15 was found later to have the best dissemination capacity of the CTX-M family, reaching Europe, Africa, Asia and North and South America(Davies and Davies 2010).

Chapter 2

Background

The curative power of antibiotics is well established, however resistance to these antibiotics undermines their effectiveness. Antibiotic resistance is a major public health crisis in the United States and around the world(Neu 1992). With at least 23,000 people dying each year in the U.S. from antibiotic resistant infections, it is a major cause of death(Harris, Pineles et al. 2017). Microbes, like all other living organisms, evolve over time. However short generation times and horizontal gene transfer allows beneficial resistance mechanisms to spread rapidly throughout bacterial populations(Harris, Pineles et al. 2017).

Approximately 150 million people around the world contract urinary tract infections(Schulz 2011) . The most common type of bacteria that causes urinary tact infections is uropathogenic *E. coli*(Schulz 2011). Uropathogenic *E. coli* have specific virulence factors that allow them to infect the urinary tract of humans(Schulz 2011). These bacteria have type one pili that allows them to attach to the urothelial surface allowing them to resist the force of urinary flow(Schulz 2011). Carbapenem antibiotics have historically been considered drugs of last resort(Evans, Hujer et al. 2017). But since 1992 Carbapenem resistant Enterobacteriaceae (CRE) has become common in the United States(Gupta, Limbago et al. 2011). The most common CRE is known as Klebsiella pneumoniae Carbapenemases, a Class A enzyme capable of hydrolyzing a large array of β-lactams(Gupta, Limbago et al. 2011). Antibiotics are usually an effective treatment against UTI's however with the rise of antibiotic

resistant infections their effectiveness has waned (Schulz 2011). Many strains of bacteria are resistant to multiple antibiotics, so even though treatment options exist, it can be challenging to identify them. The first example of CTX-M- type β lactamases was documented in Western Europe in 1992(Leflon-Guibout, Jurand et al. 2004). *E. coli* are responsible for the majority of bacteremia cases in England and Wales due to the overuse of antibiotics. Extended spectrum beta-lactamases containing CTX-M-enzymes cause difficult to treat UTI's in the UK.

Clinical testing is critical for the development of effective treatment options;
Disc diffusion, E-testing, and agar dilution are three common methods used to detect resistance among bacterial strains(Maalej, Meziou et al. 2011). Disc diffusion is a method that uses antibiotic infused disc wafers to test how effective or resistance an antibiotic is at killing a particular strain of bacteria(Maalej, Meziou et al. 2011). Discs are placed on an agar plate which has been coated with a bacterial lawn(Maalej, Meziou et al. 2011). Antibiotic effectiveness can be observed by how much clearing occurs around the disk. This clearing is known as the inhibition zone. Susceptible bacteria will show a large inhibition zone while ineffective antibiotics will have little to no inhibition zone(Maalej, Meziou et al. 2011).

E-testing is similar to disc diffusion in that it uses a predefined antibiotic infused strip but it differs in that the strip has a concentration gradient(Maalej, Meziou et al. 2011). The strip shows the lowest concentration at which the bacteria can no longer grow(Maalej, Meziou et al. 2011). The E-test helps determine the minimum

inhibitory concentration (MIC), which is the lowest concentration of antibiotic at which visible grow can be observed(Maalej, Meziou et al. 2011).

Another method to test for the MIC is broth dilution(Maalej, Meziou et al. 2011). This method involves making a 2 fold serial dilution in broth with different concentrations of antibiotic (Maalej, Meziou et al. 2011). These media are then inoculated with the bacteria of interest and a control plate is made without antibiotic(Maalej, Meziou et al. 2011) and incubated at 37 degrees C for up to 20 hours(Maalej, Meziou et al. 2011). The plate with the lowest concentration that showed no signs of growth is the MIC(Maalej, Meziou et al. 2011).

Determining MICs has immense clinical usefulness. It allows health care providers to assess the most useful antibiotics for treating a patient, avoid administering toxic levels of antibiotic while still providing an effective standard of care(Andrews 2001). In a clinical setting organisms are classified based on there resistance to antibiotics being “Resistant”, “Intermediate”, and “Susceptible” (RIS), these classifications are standardized by the Clinical Laboratory Sciences Institute (CLSI) of the United States which is in international agreement with International Organization for Standardization (ISO) (Rodloff, Bauer et al. 2008).

In this chapter we present an alternative method for assaying resistance. While MIC’s are clinically useful another method based upon a bacteria’s growth rate can be used as a way to measure fitness. The ability to estimate fitness makes it possible to utilize several mathematical models and evolutionary theory to understand more

deeply the mechanisms of resistance and the predicted outcomes of antibiotic treatment. Bacterial growth rates are a measure of the rate at which a bacterium goes through binary fission. Bacterial growth goes through 4 phases, lag phase, exponential phase, stationary phase, and death phase. Bacterial growth rates measure the exponential stage since this is the period in which the most growth occurs in the bacterial colony. Bacterial growth rates can provide evidence on how effective and antibiotic is at inhibiting growth of a particular antibiotic. A low (Canton, Gonzalez-Alba et al. 2012) bacterial growth rate in the presence of an antibiotic provides a measurement of low resistance to the antibiotic. Where as a high bacterial growth rate in the presence of an antibiotic provides a measurement of high resistance to an antibiotic. Growth rates are derived from optical density (OD) readings, which is a measure of how much light is scattered from a sample. A sample with a low OD reading has fewer cells than a sample with a high (OD) reading. Growth rates are a proxy for fitness and can be used to develop evolutionary models.

For this study, we partnered with a local community hospital. Mercy Medical Center is located on North G Street in Merced and opened in August of 2010. Our lab has partnered with mercy medical center and has been receiving patient isolates since 2013. These patient isolates have presented our lab with a deeper understanding of the evolutionary trends of antibiotic resistance, and provided us with novel insights into the mechanics of resistance genes that drive antibiotic resistant infections.

Our study focuses on resistant isolates that have been identified as possessing the Extended Spectrum β-Lactamases (ESBLs). These isolates are resistant to several, though not necessarily all β-lactams. The most simple way to classify β lactamases is through molecular structure. There are four main classes being A, B, C and D. Class A through C β lactamases are well understood chromosomally encoded and plasmid mediated enzymes. Only a cursory understanding of class D β lactamases exists, and they have only been identified in gram-negative bacteria as plasmid encoded beta lactamases. OXA β lactamases were some of the first β lactamases that were identified. These resistance genes eventually acquired the ability to confer resistance to cephalosporins and carbapenems. OXA β lactamases were named after their ability to hydrolyze oxacillin. TEM is the most common beta lactamase, which accounts for approximately 90 percent of ampicillin resistance in *E. coli (Canton, Gonzalez-Alba et al. 2012)*. TEM β lactamase's are found in *E. coli*, *K. pneumoniae*, and many other gram-negative bacteria (Canton, Gonzalez-Alba et al. 2012). Conformational changes in the active site of the enzyme, caused by amino acid substitutions results in the resistance phenotype observed in extended spectrum beta lactamase's (ESBL) (Canton, Gonzalez-Alba et al. 2012). There are over 140 TEM enzymes that have been documented(Canton, Gonzalez-Alba et al. 2012). Amino acid substitutions found at 104, 164, 238 and 240 produce the TEM phenotype(Canton, Gonzalez-Alba et al. 2012).

The developments of CTX-M's are seen as a new paradigm in the evolution of resistance genes (Canton, Gonzalez-Alba et al. 2012). While CTX-M's were first

discovered in 1989 they did not become a major contributor to resistance until the 2000s (Canton, Gonzalez-Alba et al. 2012). *E. coli* was found to be the pathogen most heavily effected by the evolution of CTX-Ms (Canton, Gonzalez-Alba et al. 2012). CTX-M's are named after their strong hydrolytic affinity for cefotaxime (Leflon-Guibout, Jurand et al. 2004). CTX-M's are classified into five groups CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25(Leflon-Guibout, Jurand et al. 2004). The most common CTX-M enzyme is CTX-M-15(Leflon-Guibout, Jurand et al. 2004). What sets the CTX-M family of genes apart from other resistance genes is that its enzymes are non-homogenous (Canton, Gonzalez-Alba et al. 2012). Evidence also suggests that CTX-Ms didn't originate from mutations occurring in previous resistance plasmids, rather they are descendant from chromosomal *bla* genes from the bacteria *Kluyvera* spp that were then incorporated into mobile genetic elements (Rafael Cantón1, 2012). These finding provides strong evidence suggesting that selective pressure from antibiotics caused the evolution of CTX-M (Canton, Gonzalez-Alba et al. 2012).

Methods

Patient Isolates

The patient isolates used in this study were collected from Dignity Health Mercy Medical Center in Merced, California. For each sample we obtained hospital records of: the date of the samples isolation, the age and gender of the patient, the species of the bacteria, the tissue/source of the isolate, and its susceptibility categorized as Resistant, Intermediate, or susceptible for 16 different antibiotics. Those antibiotics were Ampicillin, Ampicillin/Sulbactam, Piperacillin/Tazobactam, Cefazolin, Ceftazidime, Ceftriaxone, Cefepime, Ertapenem, Imipenem, Amikacin, Gentamicin, Tobramycin, Ciprofloxacin, Levofloxacin, Nitrofurantoin and Sulfamethoxazole/Trimetroprim.

Strain repository

Hospital isolates were streaked onto agar plates and incubated for 24 hours at 37° Celsius. After incubation single colony isolates were grown in Luria Broth (LB) and frozen in 10% glycerol at -80ºC.

Cell culture and growth rate assays

Growth rate inoculum were then taken from standing overnight cultures and diluted to a final working concentration of 10^5 cells per mL in Muller Hinton broth. Each patient isolate was tested against 3 different antibiotic combinations, with 6 replicates for each antibiotic and 6 replicates for the control. The cephalosporin antibiotics used were Ceftazidime, Ceftriaxone, Cefepime all at a concentration of 64 ug/mL. Strains were also tested against ampicillin at 32 ug/mL,

ampicillin, sulbactam at 128 ug/mL, piperacillin 128 ug/mL, tazobactam 4 ug/mL.The growth rate assay was performed in a BIOTEK (Model# 267638) spectrophotometer for 22 hours at a temperature of 25.1 C°. After the 22-hour incubation period the O.D. readings were then converted into growth rates using the freely available GrowthRates software package (Hall, Acar et al. 2014).

Statistical analysis of growth rates

Statistical analyses were conducted using R version 3.3.2 [1]. α was set to 0.05 for all analyses. For Growth rate comparisons a Welche's t-tests, the Mann-Whitney U test, and a permutation test were used to compare two growth rate subsets. Welche's t-test assumes that the two growth rate groups being compared are normally distributed while the Mann-Whitney U test is a nonparametric test. The Jarque-Bera Normality Test [2] with $\alpha = 0.05$ showed that less than 10% of the data were not normally distributed. A q-value of 0.05 was selected for the False Discovery Rate controlling procedure [3].

Results

We tested 47 patient isolates against a total of 7 different antibiotics. Sequencing was done on the 47 isolates and their resistance genes were recorded. Multiple resistance genes were found to be present. The results are based upon the growth rate of each isolate in the presence of different antibiotics.

Figure 1 shows that growth rates for *E. coli* isolates correlate well with standard determination of antibiotic susceptibility. Those isolates that are *resistant* and those that are *susceptible* have different mean (average) growth rates for the cephalosporin antibacterials: ceftazidime (CAZ), ceftriaxone (CRO), and cefepime (FEP). *Resistant* isolates have a higher average growth rate than *susceptible* isolates. The difference in growth rate means (averages) is statistically significant for each of the three antibiotics; we can reject growth rate equality between *resistant* and *susceptible* E. coli isolates with a two-tailed t-test at a significance level of 0.05 (or 0.0025). All 47 isolates are resistant to ampicillin, therefore there is no box-plot for the growth rates of isolates that are susceptible to ampicillin. Ceftazidime (CAZ) seems to have the greatest effect.

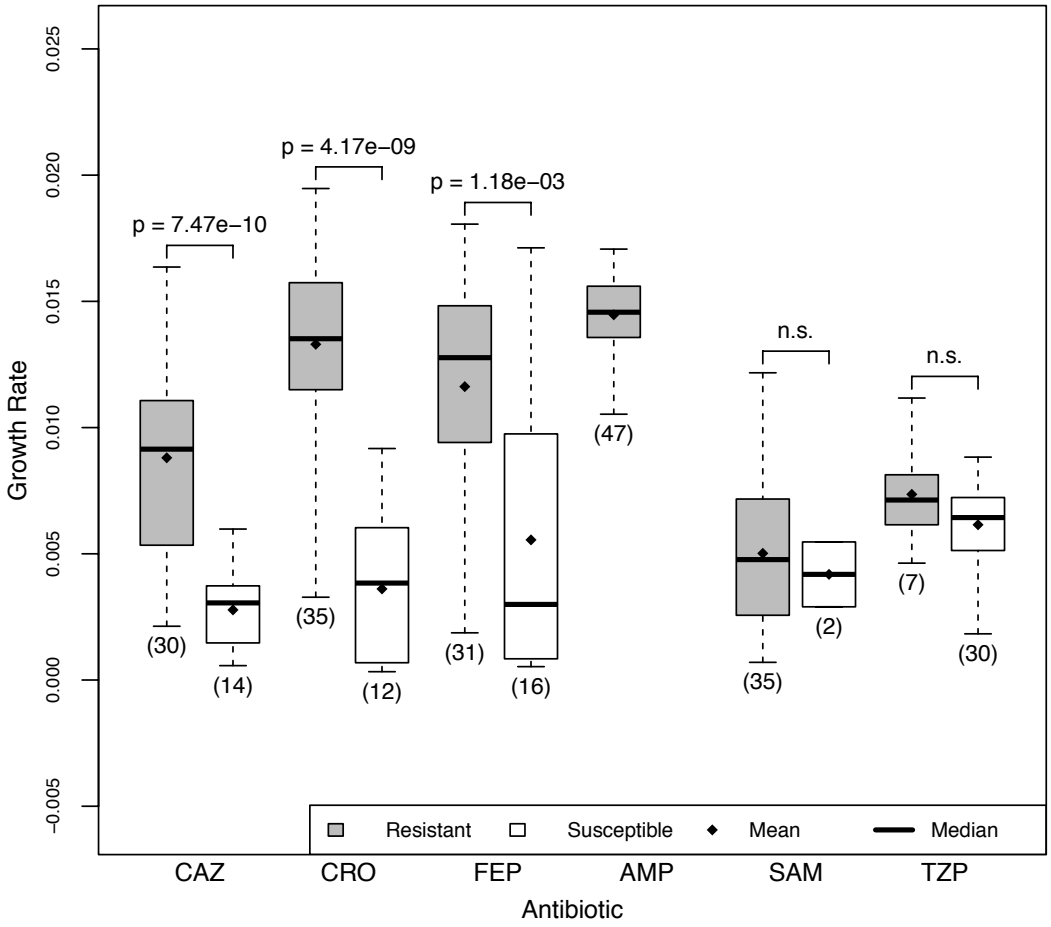

Figure1:
Boxplots represent the growth rates of E. coli isolates in the presence of an antibacterial agent. There are two boxplots per antibiotic, the first (gray) shows the spread of growth rates for E. coli isolates that are *resistant* to the antibiotic, and the second (white) shows the spread of growth rates for E. coli isolates that are *susceptible* to the antibiotic. The arithmetic mean, the median, and p-values (>0.1) of a two-tailed t-test for mean equality between the *resistant* and *susceptible* isolate growth rate *groups* are displayed; p-values greater than or equal to 0.1 are labeled not significant (n.s.). The number of E. coli isolates whose *growth rate* was used to create each boxplot is given in parenthesis. Note: all 47 E. coli isolates are *resistant* to ampicillin.

Sub-caption: Antibacterial agents are abbreviated as follows: CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; AMP, ampicillin; SAM, ampicillin-sulbactam; TZP, piperacillin-tazobactam. Concentrations of antibacterial agents: 64 ug/mL of ceftazidime, ceftriaxone, and cefepime; 32 ug/mL, ampicillin; 16 ug/mL, sulbactam; 128 ug/mL, piperacillin; 4 ug/mL, tazobactam.

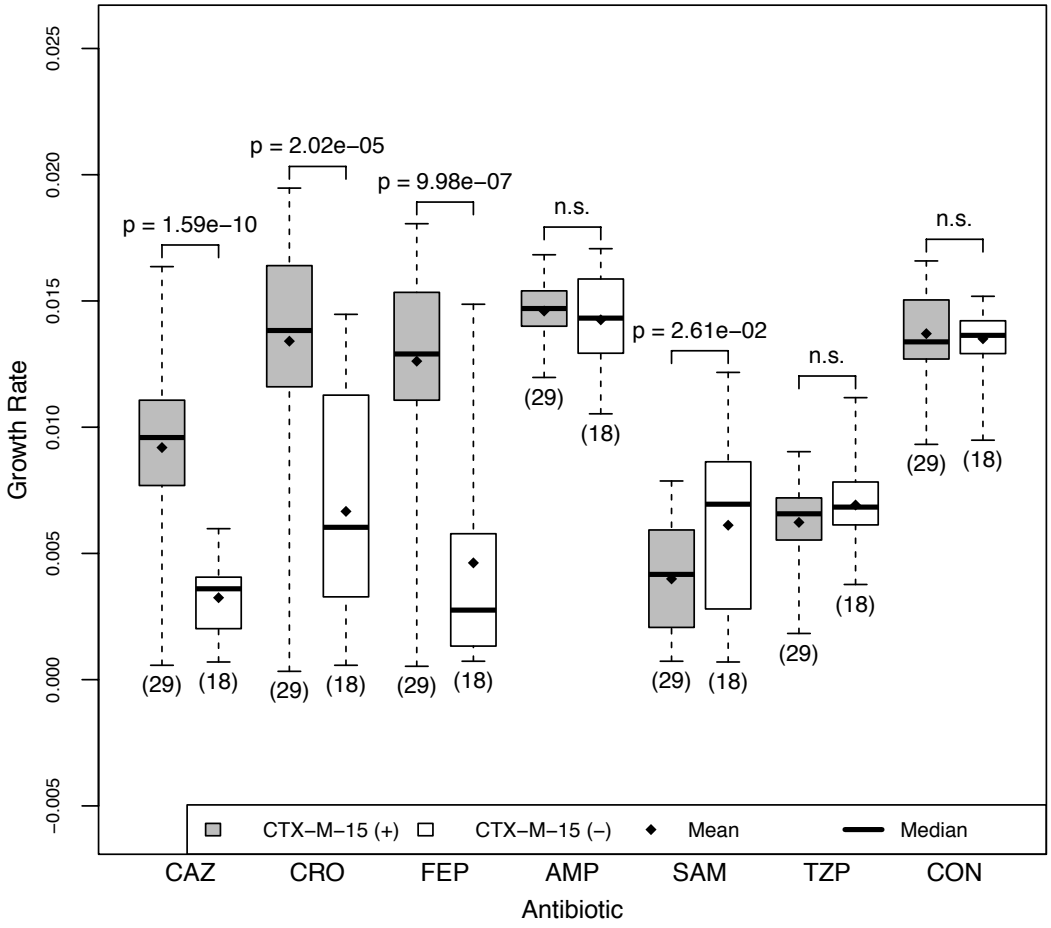

Figure2:
Boxplots represent the growth rates of E. coli isolates in the presence of four antibacterial agents, two antibacterial/inhibitor combinations, and no antibiotic (control). There are two boxplots per antibiotic, the first (gray) shows the spread of growth rates for E. coli isolates that have the blaCTX-M-15 gene (+), and the second (white) shows the spread of growth rates for E. coli isolates that do not have the blaCTX-M-15 gene (-). The arithmetic mean, the median, and p-values (>0.1) of a two-tailed t-test for mean growth rate equality between the isolates that have the CTX-M-15 gene and isolates that do not have the gene are displayed; p-values greater than or equal to 0.1 are labeled not significant (n.s.). The number of E. coli isolates whose growth rates were used to create each boxplot are given in parenthesis.

Sub-caption: Antibacterial agents are abbreviated as follows: CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; AMP, ampicillin; SAM, ampicillin-sulbactam; TZP, piperacillin-tazobactam; CON, control (no antibiotic). Concentrations of antibacterial agents: 64 ug/mL of ceftazidime, ceftriaxone, and cefepime; 32 ug/mL, ampicillin; 16 ug/mL, sulbactam; 128 ug/mL, piperacillin; 4 ug/mL, tazobactam.

In Figure 2 we see that growth rates for *E. coli* isolates that have the CTX-M-15 gene and isolates that do not have the CTX-M-15 gene have different mean (average) growth rates for the cephalosporin antibacterials: ceftazidime (CAZ), ceftriaxone (CRO), and cefepime (FEP). The difference in growth rate means is statistically significant for each of the three antibiotics; we can reject growth rate equality between isolates that have the CTX-M-15 gene (29 isolates) and those that do not (18 isolates) with a two-tailed t-test at a significance level of 0.05 (or 0.0025). The growth rates of all 47 isolates in ampicillin (AMP)

(Both CTX-M-15 + and CTX-M-15 -) are much greater than the growth rates of all isolates in ampicillin-sulbactam (SAM). For the penicillin/inhibitor combination ampicillin-sulbactam (SAM) not having the CTX-M-15 gene is associated with a greater mean growth rate. We can reject mean growth rate equality between isolates that have the CTX-M-15 gene and isolates that do not have the gene with a two-tailed t-test at a *significance level* of 0.05. The differences in mean growth rates is most likely due to the presence of the TEM-1 gene in isolates that do not have CTX-M-15 gene: 19/29 (approx. 65.5%) isolates that have the CTX-M-15 gene do not have the TEM-1 gene while 13/18 (approx. 72.2%) isolates that do not have the CTX-M-15 gene do have the TEM-1 gene; while the CTX-M-15 gene is not associated with resistance to penicillins, the TEM-1 gene is associated with penicillin resistance). However, this difference in mean growth rates is not observed in the single use of ampicillin (AMP). However, there is no statistically significant difference in control growth rates (CON) when compared by the presence and absence of CTX-M-15.

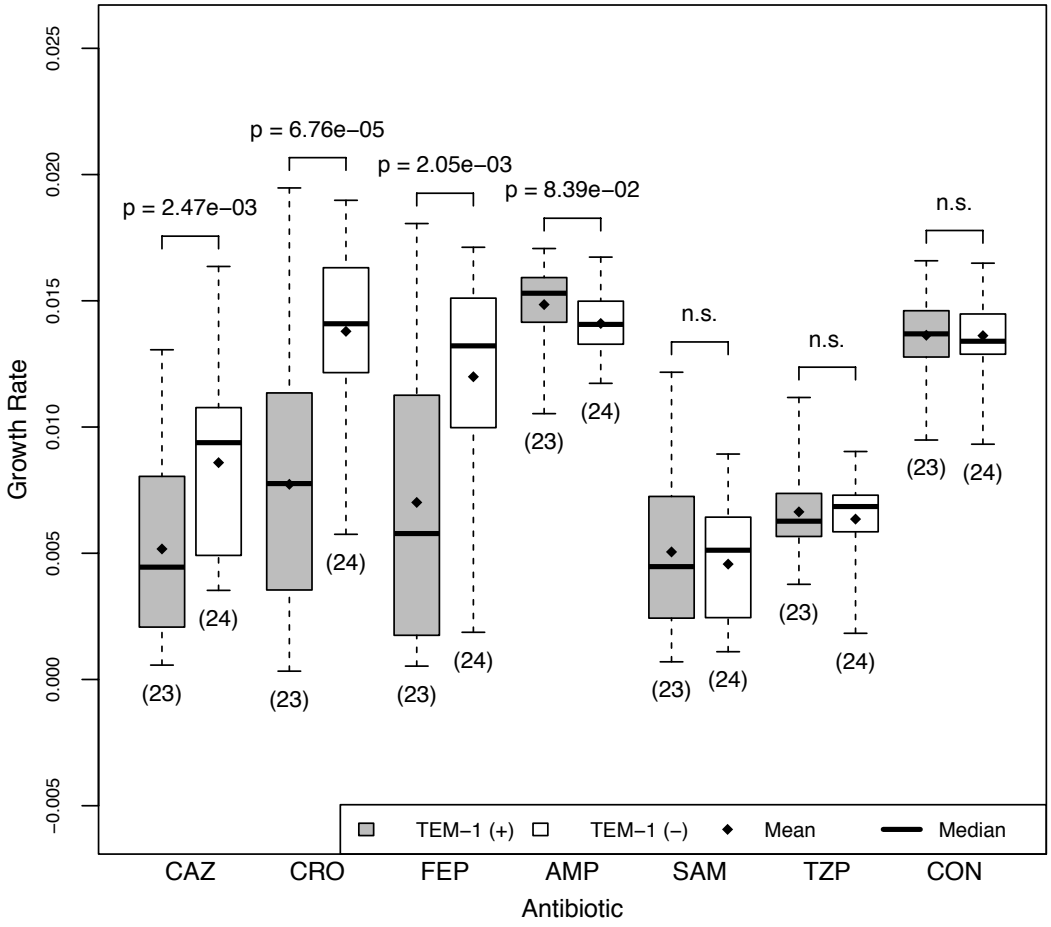

Figure3:
Boxplots represent the growth rates of E. coli isolates in the presence of four antibacterial agents, two antibacterial/inhibitor combinations, and no antibiotic (control). There are two boxplots per antibiotic, the first (gray) shows the spread of growth rates for E. coli isolates that have the blaTEM-1 gene (+), and the second (white) shows the spread of growth rates for E. coli isolates that do not have the blaTEM-1 gene (-). The arithmetic mean, the median, and p-values (>0.1) of a two-tailed t-test for mean growth rate equality between the isolates that have the TEM-1 gene and isolates that do not have the gene are displayed; p-values greater than or equal to 0.1 are labeled not significant (n.s.). The number of E. coli isolates whose growth rate was used to create each boxplot are given in parenthesis.

Sub-caption: Antibacterial agents are abbreviated as follows: CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; AMP, ampicillin; SAM, ampicillin-sulbactam; TZP, piperacillin-tazobactam; CON, control (no antibiotic). Concentrations of antibacterial agents: 64 ug/mL of ceftazidime, ceftriaxone, and cefepime; 32 ug/mL, ampicillin; 16 ug/mL, sulbactam; 128 ug/mL, piperacillin; 4 ug/mL, tazobactam.

Figure 3 shows that for all three cephalosporin antibiotics ceftazidime (CAZ), ceftriaxone (CRO), and cefepime (FEP), the mean growth rate of isolates with the TEM-1 gene is much higher than the mean growth rate of isolates that do not have the TEM-1 gene. This difference is statistically significant (significance level 0.0025), however as can be seen from the summary below, about 79% of isolates that *don't* have the TEM-1 gene have the CTX-M-15 gene, which is associated with cephalosporin resistance. 10/23 (approx. 43.5%) isolates that are TEM (+) have the CTX-M-15 gene.13/23 (approx. 56.5%) isolates that are TEM (+) do not have the CTX-M-15 gene.19/24 (approx. 79.2%) isolates that are TEM (-) have the CTX-M-15 gene.5/24 (approx. 20.8%) isolates that are TEM (-) do not have the CTX-M-15 gene. Note that for ampicillin (AMP), the mean difference in isolate growth rates that have the TEM-1 gene and those that do not are not statistically significant at a significance level of 0.05. However, there is a slight difference where TEM-1 (+) isolates have a slightly higher mean growth rate than isolates that are TEM-1 (-). This was not observed while looking at isolate growth rates by the presence/absence of CTX-M-15. For ampicillin-sulbactam (SAM), there is no difference in growth rates for isolates that are TEM-1 (+) with those that are TEM-1 (-). In Figure 2 there was a difference in mean growth rates when comparing CTX-M-15 (+) isolates and CTX-M-15 (-) isolates in the presence of SAM. This could indicate that CTX-M-15 is interfering with resistance. Isolates that are TEM-1 (+) and TEM-1 (-) have very similar growth rate profiles. Figure 4 show that when we compare the growth rates in each of the antibiotics by the presence and absence of the OXA-1 gene, we do not find a significant difference in the mean growth rates.

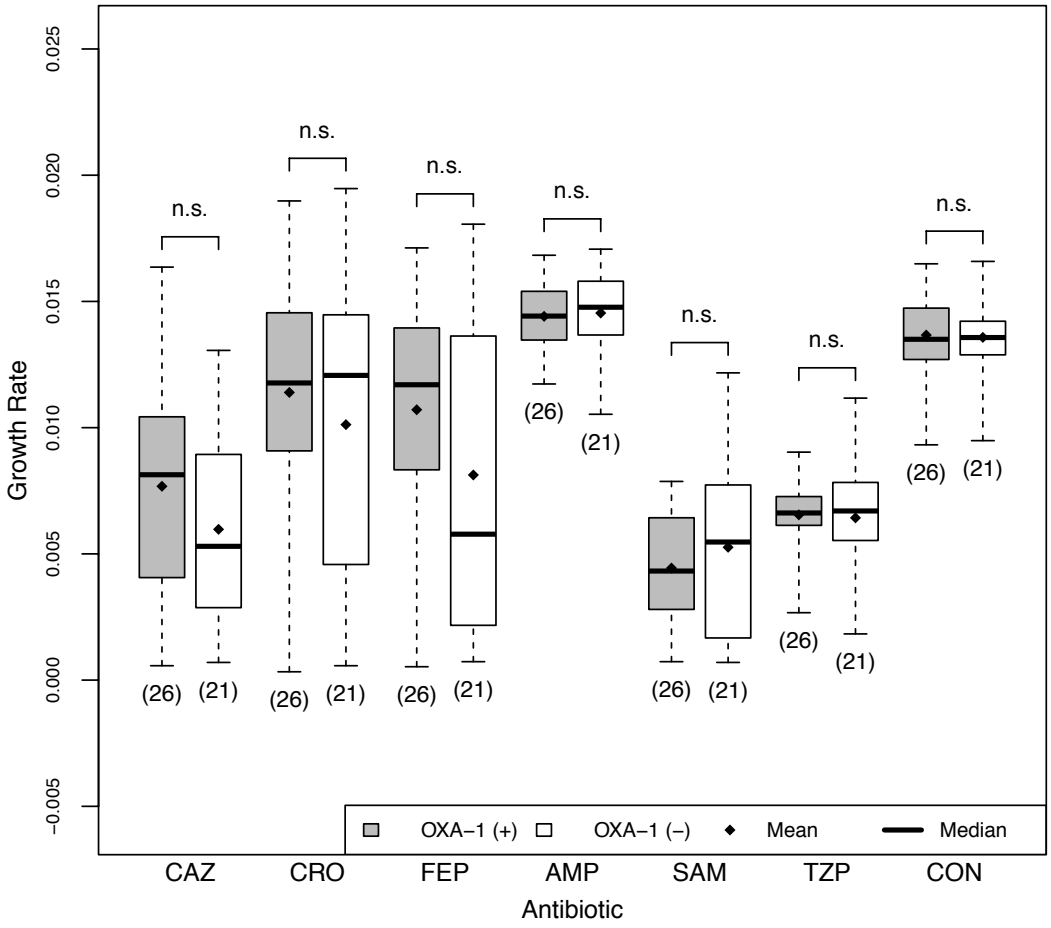

Figure 4:
Boxplots represent the growth rates of E. coli isolates in the presence of four antibacterial agents, two antibacterial/inhibitor combinations, and no antibiotic (control). There are two boxplots per antibiotic, the first (gray) shows the spread of growth rates for E. coli isolates that have the blaOXA-1 gene (+), and the second (white) shows the spread of growth rates for E. coli isolates that do not have the blaOXA-1 gene (-). The arithmetic mean, the median, and p-values (>0.1) of a two-tailed t-test for mean growth rate equality between the isolates that have the OXA-1 gene and isolates that do not have the gene are displayed; p-values greater than or equal to 0.1 are labeled not significant (n.s.). The number of E. coli isolates whose growth rate was used to create each boxplot is given in parenthesis.

Sub-caption: Antibacterial agents are abbreviated as follows: CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; AMP, ampicillin; SAM, ampicillin-sulbactam; TZP, piperacillin-tazobactam; CON, control (no antibiotic). Concentrations of antibacterial agents: 64 ug/mL of ceftazidime, ceftriaxone, and cefepime; 32 ug/mL, ampicillin; 16 ug/mL, sulbactam; 128 ug/mL, piperacillin; 4 ug/mL, tazobactam.

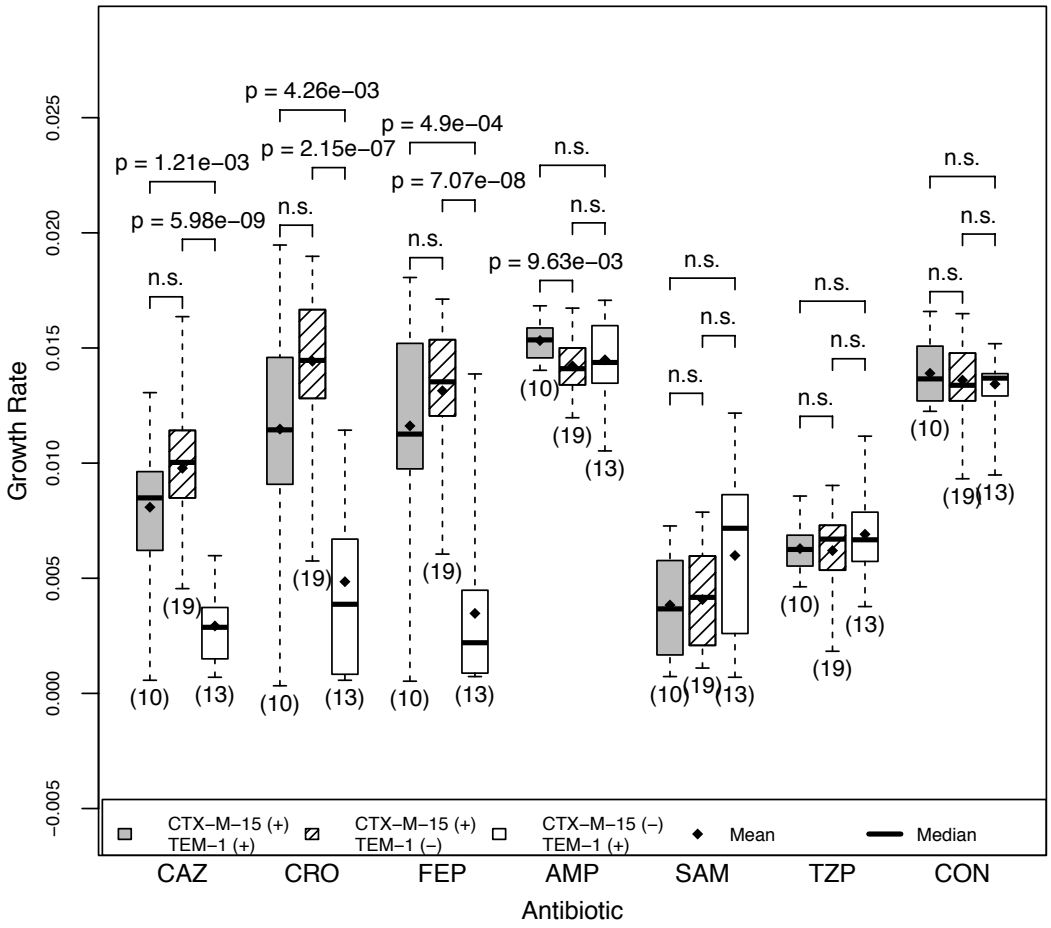

Figure 5:
Boxplots represent the growth rates of E. coli isolates in the presence of four antibacterial agents, two antibacterial/inhibitor combinations, and no antibiotic (control). There are three boxplots per antibiotic, the first boxplot (gray) shows the spread of growth rates for E. coli isolates that have the blaCTX-M-15 gene (+) and the blaTEM-1 gene (+), and the second boxplot (hash marks) shows the spread of growth rates for E. coli isolates that have the blaCTX-M-15 gene (+) but not the blaTEM-1 gene (-), and the third boxplot (white) shows the spread of growth rates for E. coli isolates that do not have the blaCTX-M-15 gene (-) but do have the blaTEM-1 gene. The arithmetic mean, the median, and p-values (>0.1) of a two-tailed t-test for mean growth rate equality are displayed; p-values greater than or equal to 0.1 are labeled not significant (n.s.). The number of E. coli isolates whose growth rate was used to create each boxplot is given in parenthesis.

Sub-caption: Antibacterial agents are abbreviated as follows: CAZ, ceftazidime; CRO, ceftriaxone; FEP, cefepime; AMP, ampicillin; SAM, ampicillin-sulbactam; TZP, piperacillin-tazobactam; CON, control (no antibiotic). Concentrations of antibacterial agents: 64 ug/mL of ceftazidime, ceftriaxone, and cefepime; 32 ug/mL, ampicillin; 16 ug/mL, sulbactam; 128 ug/mL, piperacillin; 4 ug/mL, tazobactam.

Figure 5 shows that for all three cephalosporin antibiotics ceftazidime (CAZ), ceftriaxone (CRO), and cefepime (FEP), among isolates with the CTX-M-15 gene, the presence and absence of the TEM-1 gene does not result in a statistically significant difference in the mean growth rates. For all three cephalosporins, isolates that have the CTX-M-15 gene have a higher growth rate than isolates that do not have the CTX-M-15 gene, independent of the presence of TEM-1. For ampicillin (AMP), isolates that have both CTX-M-15 and TEM-1 genes have a slightly higher mean growth rate than isolates that have the CTX-M-15 gene but not the TEM-1 gene; the difference in growth rates is statistically significant with a significance level of 0.05. This is unexpected but the difference in mean growth rates is so small that it might simply indicate some synergistic effect between CTX-M-15 and TEM-1 for resistance to AMP. Other comparisons did not result in statistically significant differences in mean growth rates. We expected to see a difference in isolate growth rates in the presence of AMP. Those with the TEM-1 gene were expected to have higher mean growth rates than those without.

Conclusions

In this study we tested how patient isolates taken from Dignity Health, Mercy Medical Center respond in the presence of different antibiotics. Bacterial growth rates were determined for each patient isolate in the presence of 7 different antibiotics. Through growth rate assays, we identified that in the presence of ampicillin, isolates that have both CTX-M-15 and TEM-1 genes have a slightly higher mean growth rate than isolates that have the CTX-M-15 gene but not the TEM-1 gene leading us to believe that there may be a synergistic effect between CTX-M15 and TEM-1 for the resistance of ampicillin. It has been well established that nearly 90% of ampicillin resistance in *E. coli* is due to the TEM-1 resistance gene (Cooksey, Swenson et al. 1990). However what is less understood is CTX-M-15's effect on resistance in ampicillin. The CTX-M resistance gene is typically associated with resistance to cephalosporins such as ceftazidime, ceftriaxone, and cefepime (Livermore, Canton et al. 2007). Surprisingly we found that isolates in the presence of cephalosporin showed a decrease in growth rates if they had the TEM-1 resistance gene. This suggests that the presence of TEM-1 has a slightly inhibitory effect on growth in the presence of cephalosporins, which is still not understood at a cellular level.

We also found that in the presence of ampicillin-sulbactam (SAM) there was no difference in growth rates for isolates that were TEM-1 (+) with those that are TEM-1 (-). However there was a difference in mean growth rates when comparing CTX-M-15 (+) isolates and CTX-M-15 (-) isolates in the presence of SAM. Indicating that CTX-M-15 is interfering with resistance for SAM when TEM-1 is present.

Through out the 2000's CTX-M alleles have been replacing TEM and SHV resistance genes throughout Europe, Canada and Asia (Sidjabat, Paterson et al. 2009). In a study done in 2004 in Texas on resistance genes among *E. coli*, CTX-M was also found to be the predominate resistance gene (Sidjabat, Paterson et al. 2009). The inhibitory effect of TEM on growth in the presence of cephalosporins may account for this decline in bacterial populations.

Chapter Three

Discussion

Every year in the United States 2 million people acquire an antibiotic resistant infection and 23,000 people die as a direct result of those infections (Harris, Pineles et al. 2017). Currently antibiotics are prescribed with little understanding of what resistance genes are present within the infecting bacteria. A better understanding of how resistance genes affect the fitness of bacteria is needed in order to effectively combat these antibiotic resistant infections. The resistance genes CTX-M-15, OXA-1 and TEM-1 are well-studied resistance genes however there interactions with specific antibiotics require more research.

Common susceptibility testing methods such as MIC's are time consuming. Recently faster susceptibility testing methods have been devised such as matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). MALDI-TOF is a technique that uses mass spectrometry to monitor resistance. With this method hydrolysis of the β-lactam ring by β-lactamases is visible in the disappearance of the original mass peak. This technique has proven effective for the analysis of the hydrolysis of ampicillin (Sparbier, Schubert et al. 2012).

Another method for identifying resistance genes is the use of DNA microarrays. A DNA microarray is a grouping of DNA fragments attached to a solid surface. Each fragment is known as a probe, which hybridizes or binds with it's target sequence in a sample. In this way a target sequences of interest can be detected. DNA

microarrays can be used to screen for the presence of resistance genes. Probes specific to target resistance genes are bound to a glass plate then a sample is passes over the plate to facilitate hybridization. Next reporter molecules are used to bind to the target sequences that have matched with the probes. The reporter signatures are then recorded which denote which resistance genes were present in the sample (Call, Bakko et al. 2003). This method can produce results where the resistance genes reported do not match with their expected phenotype. This means that bacteria may not express the resistance gene being reporter, other resistance genes may interfere with their functionality or an unknown mechanism may be involved in the disparity between genotype and phenotype. Further study is required for the reliability of this method (Call, Bakko et al. 2003).

Growth rates are a reliable measure of fitness. They calculate the maximum growth by measuring the optical density of that culture over time. A spectrophotometer is used to measure a multi-well assay plate with measurements taken throughout the full growth cycle (Wiser and Lenski 2015). They can be used to predict the outcomes of antibiotic treatments and determine which strains will be the most fit in those treatments. The sensitivity of these assays may lead to novel findings about the nature of antibiotic resistance evolution. Future studies of fitness outcomes using growth rate assays are recommended.

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