

Prevalent Multiple Antibiotic Resistance Phenotypes in Commensal *Escherichia coli*
Isolated from Bovine Feces of the Rocky Mountain Region of Montana

Honors Thesis

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This thesis has been accepted by the Department of the Natural Sciences, qualifying Jacqueline E. Schmidt for Honors candidacy:



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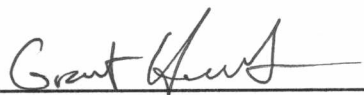
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Dr. Jennifer Geiger, Reader

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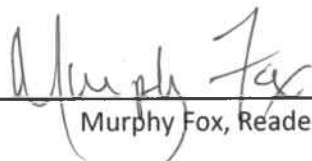
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Abstract

The phenomenon of antibiotic resistance has led to heightened discussion on how these adaptations may have arisen with such universality. Why antibiotic resistance occurs in an unpredictable manner within bacterial populations is poorly understood. It has been observed that resistance to many respective antibiotics is seen within the same organism as part of one phenotype. Each class of antibiotic has a distinct mode of action suggesting that adaptation to one antibiotic would not necessarily illicit resistance to another.

This study evaluates and outlines the environmental contributions to these multiple antibiotic resistance phenotypes present in bovine of the Rocky Mountain region of western Montana. Fecal samples were obtained from eight sites; several sites were sampled on two occasions. *E. coli* isolated from bovine feces were evaluated using an antibiotic susceptibility test to disclose whether they displayed multiple drug resistance and what MDR phenotypes were associated with the environments from which they were collected.

The majority of *E. coli* isolates exhibited resistance to four to five drugs tested. Several sites with no longitudinal likeness displayed resistance more frequently and to greater numbers of antibiotics. In a temporal comparison sites selected for multiple collections expressed resistance phenotypes that varied over time.

Of the respective antibiotics used in this study *erythromycin*, *gentamisin*, *streptomycin*, and *tetracycline* appeared habitually throughout the library. The most

common multiple drug resistance phenotype was *erythromycin-gentamisin-streptomycin* occurring in the total collection with a frequency of 39-66%. Because the mode of action of each of these antibiotics is different, the occurrence of resistance to all three within one organism suggests the presence of a resistance plasmid.

Introduction

Some strains of *Escherichia coli* can contaminate food, impact agricultural economic success, and cause disease and illness in humans and other animal species. Pathogenic properties have been identified in strains such as O157 in North American (Vidovic and Korber, 2006) causing hemorrhagic colitis (bloody diarrhea), hemolytic uremic syndrome (hemolytic anemia and renal failure) and can ultimately lead to thrombotic thrombocytopenic purpura (small blood clotting of blood vessels) (Galland *et al.*, 2001; Paton and Paton, 1998). The invasive nature of *E. coli* is frequently linked to Traveler's Diarrhea diagnoses (Gascón *et al.*, 1993, Karmali, 1989). Likewise, diarrhea is the leading cause of adolescent death in the developing world (Medical Condition News, 2004), killing 2 million children a year (Tansey, 2008; World Health Organization).

Enterohemorrhagic strains of *E. coli* affecting humans have likewise been observed infecting other animal hosts (Zhu *et al.*, 2008; Nataro and Kaper, 1998). Some strains of *E. coli* possess virulence mechanisms, termed Cytotoxic Necrotizing factors, affecting the health of both rabbits (Caprioli *et al.*, 1983) and humans (de Rycke *et al.*, 1990). In avian species, *E. coli* causes colibacillosis and air sacculitis rendering major economic loss in the poultry industry (Diarra *et al.*, 2007; Bass *et al.*, 1999).

These bacteria are highly infectious and challenging to control. Since *E. coli* is highly transmissible through aqueous media, human exposure to fecal contaminated water may cause considerable decline in human health (Fewtrell and Bartram, 2001). Bacteria have a doubling time that is highly efficient and rapid, contributing to their

difficulty to control. Careful soil management is important to prevent the transfer of pathogenic strains to cattle through foods grown in soil rich in pathogenic bacteria (Franz *et al.*, 2005). There are many environments that support such bacterial life and efforts to control these *E. coli* populations have been difficult, possibly a consequence of the defiant and resilient nature of bacteria (Barza, 2002; Summers, 2002; United States Department of Agriculture).

Consumption of antibiotics by humans and antibiotic use in livestock feed have become a common practice, which has mediated increased resistance—the ability of bacteria to resist harmful effects of disruptive agents—in many strains to these drugs (Alexander *et al.*, 2008). For instance, *E. coli* isolated from animals where organic dairy farming is practiced have lower levels of antibiotic resistance than *E. coli* isolated from animals under conventional dairy farming techniques (Walk *et al.*, 2007). Greater resistance coupled with the ability to propagate resistance genes to other populations of *E. coli* via plasmid conjugation ensures high host infection success with little jeopardy of eradication by antimicrobial agents (Bass *et al.*, 1999; Camiolo *et al.*, 1975; Khachatryan *et al.*, 2008; Skurnik *et al.*, 2005; Wang *et al.*, 2003).

The acquisition of antibiotic resistance is not completely understood. Resistance is not exclusively tied to drug exposure. High resistance of *E. coli* isolates was found in Boston residents regardless of exposure to antibiotics (Levy *et al.*, 1988). Multiple antibiotic resistance has been observed in communities with no access to such drugs. In remote communities of Bolivia experiencing very low antibiotic exposure, *E. coli* populations have been identified displaying multiple drug resistance (Pallecchi *et al.*,

2007). Results from a Washington state study on *E. coli* in young cattle without antibiotic exposure display resistance phenotypes emulating those of *E. coli* of parent-cattle hosts receiving the drugs (Khachatryan *et al.*, 2004). This suggests that resistance can be inherited even when selective pressure for antibiotic resistance is absent.

The ability to resist certain combinations of antibiotics is expressed as one phenotype (Bartoloni *et al.*, 2006). These phenotypes are described as multiple drug resistance (MDR) phenotypes or multiple antimicrobial resistance profiles (MAR) because the organism expresses specific antibiotic resistances that appear to be linked due to their observed frequency in certain populations of bacteria. The ecology of antibiotic resistance remains to be understood and may be caused by environmental factors we have yet to detect.

Studies on the changes in MDR phenotypes of bacterial populations challenged with various antimicrobial agents, in various concentrations and environments, demonstrate the flexibility of bacteria to adapt (Anderson *et al.*, 2006; Anderson *et al.*, 2008; Duriez and Topp, 2007; Skurnik *et al.*, 2005). For example, enteric coliform communities in cattle demonstrate greater resistance than their wild counterparts, the American Bison (Anderson *et al.*, 2008). This study advocates exposure as a likely selective pressure for antimicrobial resistance propagation, contradicting results from studies employed in remote settings.

Animal populations with high levels of antibiotic exposure house *E. coli* expressing different MDR phenotypes with different degrees of phenotypic diversity. *E. coli* isolates found in human, cattle, and horse feces displayed high degrees of resistance,

with horse derived isolates exhibiting the greatest diversity (Anderson *et al.*, 2006). *E. coli* from swine farms show MDR phenotypes are unstable over time and change as time passes from the initial fresh sampling to later *E. coli* samples selected from stored manure (Duriez and Topp, 2007), implying the facility of these bacterial populations to tailor their phenotypes to external changes occurring temporally.

Variation in *E. coli* shows spatial changes in MDR phenotypes in particular societies of humans in France and French Guyana (Skurnik *et al.*, 2005), providing that *E. coli* can not only adapt to temporal antibiotic changes but to spatial variations and environments subject to specific conditions as well. In contrast, a single MDR phenotype was found in urinary tract infection patients dominant across the United States (Sahm *et al.*, 2001). It is evident that the mechanisms by which these populations are able to acclimate to the vastness of environmental conditions and antibiotic exposure are complex and still not fully understood.

Due to its prevalence in the environment *E. coli* has been used as an indicator species in soil environments (Rosas *et al.*, 1997) and surface water (McArthur *et al.*, 2000) to detect relative fecal pollution levels. *E. coli* was found in air, water, and soil samples of Mexico City, exposing the human population of the city to contamination containing fecal material (Ezcurra and Mazari-Hiriart, 1996) which may be the cause of numerous episodes of child diarrhea in the city (Torres *et al.*, 1995). Investigation of MDR phenotypes of *E. coli* present in surface water indicates the animal species or populations of animals that may have been contributors of fecal pollution (Wiggins *et al.*, 1999).

The versatility of *E. coli*, among many other bacteria, makes this organism an interesting candidate for study. Its unpredictable behavior in addition to its potential threats and advantages to society encourage the need for a firmer understanding of the mechanisms propagating the survival of these bacteria. This study was conducted to describe the MDR phenotypes prevalent in *E. coli* populations of Rocky Mountain cattle. A greater understanding of the presence of antibiotics or other environmental factors that contribute to the presence of these MDR phenotypes was also sought. Temporal and spatial MDR phenotypic variation of *E. coli* populations was also explored within the study area.

Several hypotheses were examined in this thesis: First, the coliform *E. coli* is highly prevalent as a commensal bacteria in populations of bovine throughout the northern Rocky Mountain region. Correspondingly, an increase in MDR phenotypic variation between collection sites is expected with an increase in distance between sites. Moreover, antibiotic resistance of commensal *E. coli* populations will be influenced by shifts in environment through time. Furthermore, the administration of antibiotics in bovine populations will be reflected in an increased resistance of commensal *E. coli* to such drugs when compared with commensal *E. coli* populations from cattle not receiving the drugs. Ultimately, resistance to respective antibiotics will emerge in association—composing MDR phenotypes, frequently seen throughout the data collected, that are dependent on the various environmental factors discussed earlier.

Materials and Methods

Sample Collection

Eight sites were selected from various regions throughout the Rocky Mountain Range in western Montana (Fig. 1). During the summer of 2007 bovine fecal samples were obtained from ranches near Conrad, Helena, and MacDonald Pass. In January of 2008 samples were collected from ranches near Avon, Helmville, Boulder, Townsend, Wolf Creek, and Three Forks. Subsequent collections were taken during the summer of 2008 from both Conrad and Townsend. Ranches were selected on availability to information concerning history of antibiotic exposure and other environmental influences. Ranches were also selected for location diversity, in an effort to isolate MDR phenotypes adapted to different environmental pressures. Ranch owners responded to questions about previous antibiotic exposure to which their cattle may have been subjected.



Figure 1. Map of collection sites.

Duplicate collection sites from 2007 and 2008 allowed a temporal investigation of MDR phenotypic diversity found in commensal *E. coli* as a derivative of change over time. Several locations were selected for duplicate collections to observe the influence of change in environment through time on MDR phenotypes.

Between 20 and 150 samples were collected from each of the nine sites using sterile cotton swabs for transfer of fecal material into 9% sterile saline solution and labeled by location. Samples were placed on ice for transportation from the site to the lab where they were plated within 12 hours of sampling.

Enrichment, Isolation, and Selection

Fecal samples were vortexed for 1 minute. Then, 0.2 mL of each sample were plated onto two *MacConkey agar* media (EMD Chemical) plates, a medium selective for *E. coli*, one infused with 100mg/L *ampicillin* and the second with 50mg/L *chlorotetracyclin*. This was completed in order to generate two *E. coli* libraries—a pool of isolates that all maintain resistance to a specific antibiotic—in this case *ampicillin* in one library or *chlortetracycline* in the other. Inoculated plates were incubated at 35°C overnight to allow for bacterial growth. Plates were then analyzed for the presence of colonies suspected of being *E. coli*, which appear pink in the *MacConkey agar* media. In 2007, an *Indole* test was used to confirm the identity of *E. coli* which enables bacterial identification through the presence of red pigmentation produced by the cleavage of tryptophan, a component of the media, by an enzyme *tryptophenase* found in *E. coli* (BIORAD). In 2008, colonies of bacteria from selected plates were then transferred to *RAPID E.coli II media*, a chromogenic medium that enables a quick and precise identification of *E. coli* (BIORAD). The *RAPID E. coli II* plates were inoculated from colonies suspected of being *E. coli* and incubated at 35°C overnight. *E. coli* colonies on Rapid *E. coli II* possessed a violet color and were then transferred to nutrient agar slants (EMD Chemical) containing the same antibiotic at the same concentration they were isolated on for preservation and further study. Freshly inoculated slants were incubated overnight at 35°C and then stored at 4°C. Some isolates were lost due to cell death, which may be attributed to change in antibiotic concentration or other unknown factors.

Antibiotic Susceptibility Test

Using a sterile cotton swab *E. coli* was transferred from slants to a nutrient agar (EMD Chemical) plate to create a lawn of bacteria. Seven antibiotic discs: *ampicillin*, 10 µg (Am10), (used in *chlortetracycline* library only); *ciprofloxacin*, 5 µg (CIP5); *chloramphenicol*, 30 µg (C30); *erythromycin*, 15µg (E15); *gentamicin*, 10 µg (Gm10); *kanamycin*, 30 µg (K30); *streptomycin*, 10 µg (S10); and *tetracycline*, 30 µg (Te30), (used in *ampicillin* library only) were placed evenly across the surface of the nutrient agar plates using the standard disk diffusion method (NCCLS). Antibiotics were selected to expose the *E. coli* isolates to representative drugs of different antibiotic classes. Plates were again incubated overnight at 35°C. Isolates with little or no resistance were considered susceptible to an antibiotic if they displayed a clearing zone (or inhibition zone) in the lawn of *E. coli* surrounding that antibiotic disc. Diameter measurements of the inhibition zones were taken for each drug. This method of evaluation enabled measurement of a quantitative value relating to the level of resistance of an isolate to a particular drug. These diameters were recorded in a spread sheet and scored based on standard diameters (Table 1) for *E. coli* strains testing positive or negative for specific antibiotic resistances (Madigan *et al.*, 2003; CDS).

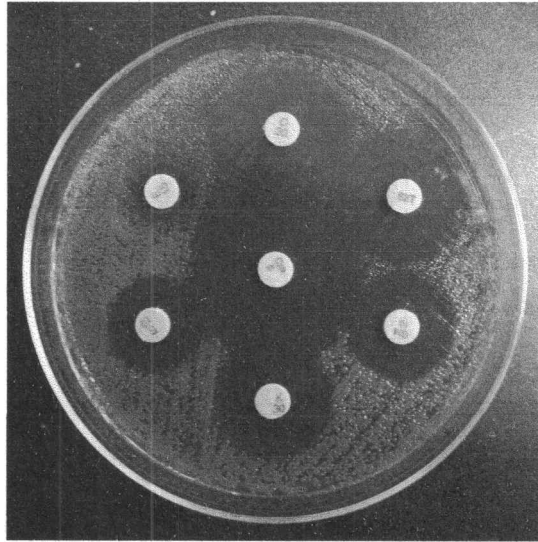


Figure 2. Disk diffusion method with inhibition zones.

Two laboratory strains of *E. coli*—*E. coli* K12 and *E. coli* B— were also tested using the same method. These were used as negative controls for the study due to their long term exposure to laboratory conditions lacking antibiotic exposure.

Table 1. Standard inhibition zones of *E. coli* susceptibility test. Diameters greater than the standard were considered susceptible, those smaller were considered resistant.

Antibiotics	CIP5	C30	E15	Gm10	K30	S10	Te30	Am10
Standard Inhibition Zones (cm)	14	12	13	12	13	11	14	11

Statistical Analysis

The data were evaluated first by calculating the proportion of samples isolated on *ampicillin* or *chlorotetracycline* media. The degree of MDR (the number of antibiotics to which a given isolate demonstrated resistance) was measured and the proportion of the library displaying each degree of MDR was recorded. Subsequently, each location was analyzed for degree of MDR. A G-test was used to test for associations between two different locations and degrees of MDR. Associations with a p-value of 0.05 or less were considered statistically significant, meaning that the two locations were similar with respect to their degree of MDR. The same analysis was applied to locations subject to multiple collections to view changes in degree of MDR over time.

A chi-square test was used to determine strength of associations between pairs of antibiotics with respect to their resistance characteristics. A p-value of 0.05 or less was used to demonstrate that when resistance to one antibiotic was observed, resistance to a second antibiotic was either more or less likely. Some associations between two antibiotics were exempt from statistical analysis because they were observed to be either always associated with each other or never associated with each other (i.e. 100 percent correlation). Ultimately, multiple-variable MDR phenotypes dominant within the two libraries were observed from the most frequent associations within the libraries.

Results

Frequency of E. coli antibiotic resistance

Over the course of the study, 1142 samples were collected. Of those samples 416 or 36% of the total collection were confirmed to contain *E. coli* isolates that exhibited resistance to either *ampicillin* or *chlortetracycline* or both. Another 16% of the samples contained *E. coli* with resistance to only *chlortetracycline* (180 resistant isolates /1142 total samples collected). Of collected samples, 21% were resistant to only *ampicillin* (236/1142). Of the total collection, 43 samples mutually contained isolates with either *ampicillin* resistance or *chlortetracycline* resistance (3.77%).

Table 2. Percent of *E. coli* collected that displayed resistance to *ampicillin*, *chlortetracycline*, or both. Information from Helena and MacDonald Pass was lost.

Location	<i>Ampicillin</i> Library (%)	<i>Chlortetracycline</i> Library (%)	Both (%)
Avon	5%	11%	3.6%
Boulder	0%	0%	0%
Conrad 1	32%	66%	0%
Conrad 2	39%	18%	10%
Helena	33%	?*	?*
Helmville	0%	>1%	0%
MacDonald Pass	7%	?*	?*
Three Forks	5%	15%	1%
Townsend1	17%	42%	11%
Townsend 2	18%	39%	11%
Wolf Creek	0%	1%	0%

*Question marks represent data lost from the library.

During storage at 4°C, 109 *ampicillin* resistant isolates were lost from the 2007 collection and 13 isolates were lost from the *chlortetracycline* library.

Antibiotic Susceptibility

The antibiotic resistance of the *E. coli* strains in the *ampicillin* and *chlortetracycline* libraries was determined by measuring the degree of resistance. The degree of resistance can be defined as the number of drugs to which one isolate has developed resistance. The degree of resistance is later related to the relative frequency of that degree of resistance within the library (Fig. 3). In general, the two libraries were found to have consistent degrees of resistance, with the majority of samples localized around the fourth (27% of the *ampicillin* library, 34% of the *chlortetracycline* library) to fifth (24% of the *ampicillin* library, 29% of the *chlortetracycline* library) degree of resistance.

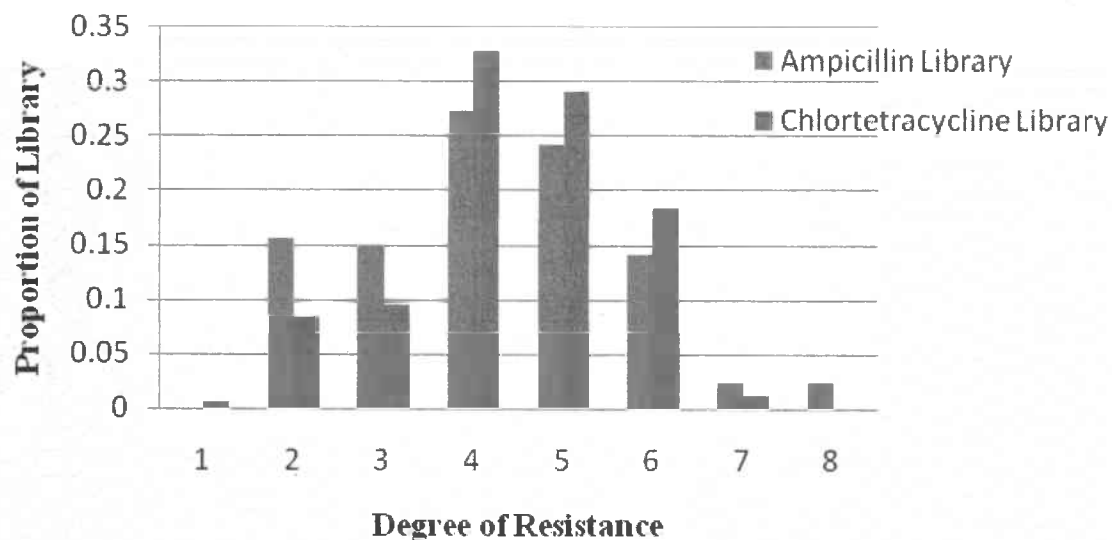


Figure 3. Proportion of total collection with multiple drug resistance. The degree of antibiotic resistance for *ampicillin* and *chlortetracycline* library corresponds to number of antibiotics for which an isolate has attained resistance.

Both libraries displayed high levels of resistance for several individual antibiotics: 78% of the *E. coli* strains in the *ampicillin* library were resistant to *eythromycin*, 76% were resistant to *tetracycline*, and 71% were resistance to *gentamicin* (Fig. 4). In the *chlortetracycline* library, the highest occurrence of resistant *E. coli* strains were held for *eythromycin* (97% of the library), 82% of *E. coli* stains were resistant to *streptomycin*, and 74% were resistant to *gentamicin* (Fig. 4). Little resistance was observed in either library for *ciprofloxacin* (0% of the *chlortetracycline* library, 5% of the *ampicillin* library) or *choramphenicol* (7% of both libraries).

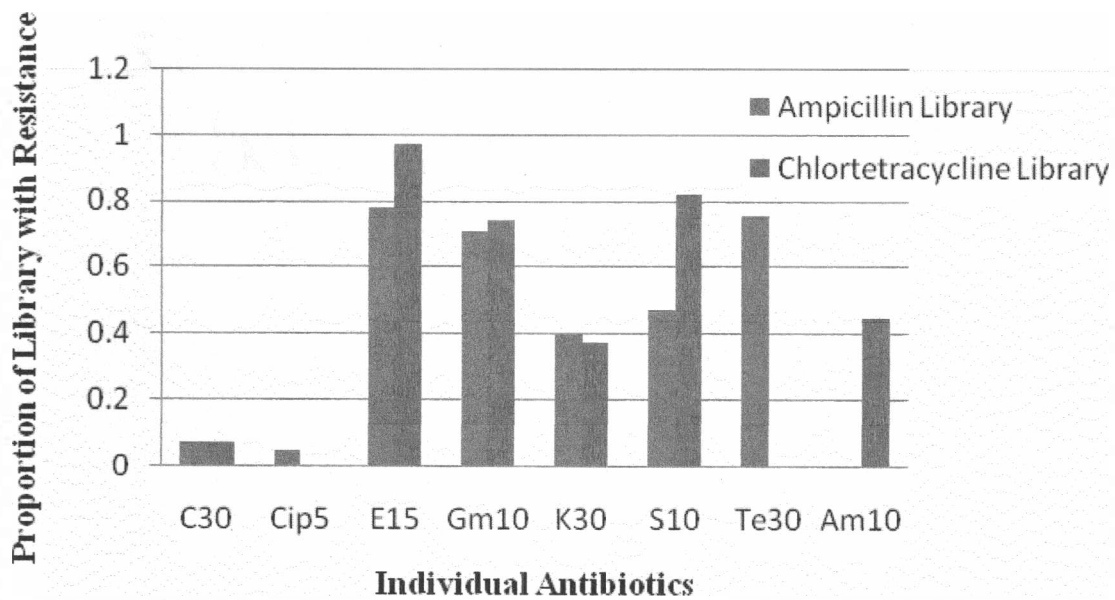


Figure 4. Respective antibiotic resistance. The resistance for respective antibiotics of isolates within the *ampicillin* and *Chlortetracycline* library is expressed as a proportion

Spatial and Temporal Variation

The two libraries were also subjected to spatial variation analysis using a G-test. The majority of locations within the *chlortetracycline* library showed significant variation of antibiotic resistance. Within the *ampicillin* library, about half of the locations sampled varied significantly with respect to antibiotic resistance patterns and degree of resistance. These phenotypic changes were random in nature, some locations in closer proximity displayed greater variability than data sets collected further apart. The G-test confirmed that the diversity in multiple drug resistance among these populations is likely random with respect to geography (Appendices 1 and 2). Although pairwise comparisons

revealed significant associations between some sites, there were no observable geographic patterns (e.g. latitudinal, longitudinal or proximity) to the associations. Sites in close proximity were just as likely to have similar degree of MDR as sites further apart.

The Conrad 2008 data set and both Townsend data sets showed a large degree of variation from the other populations as well as from themselves temporally within the *ampicillin* library. The Conrad 2008 collection displayed substantial differences from the 2007 data set with respect to the degree of resistance (Fig. 5), a trend also observed in Townsend January and summer collections taken in 2008 (Fig. 6).

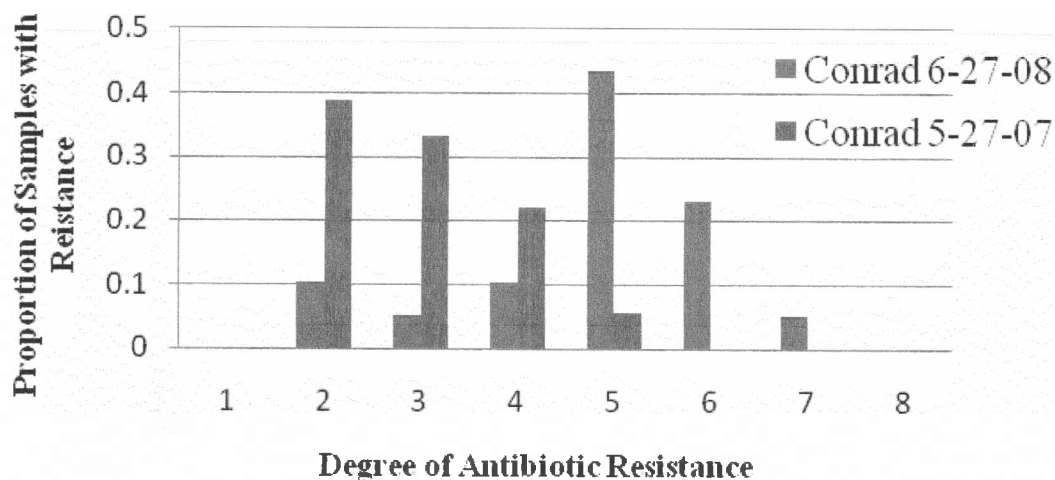


Figure 5. Temporal changes in the *ampicillin* library. Frequency of individual degrees of resistance for isolates selected from Conrad on May 27th of 2007 and again on June 27th of 2008.

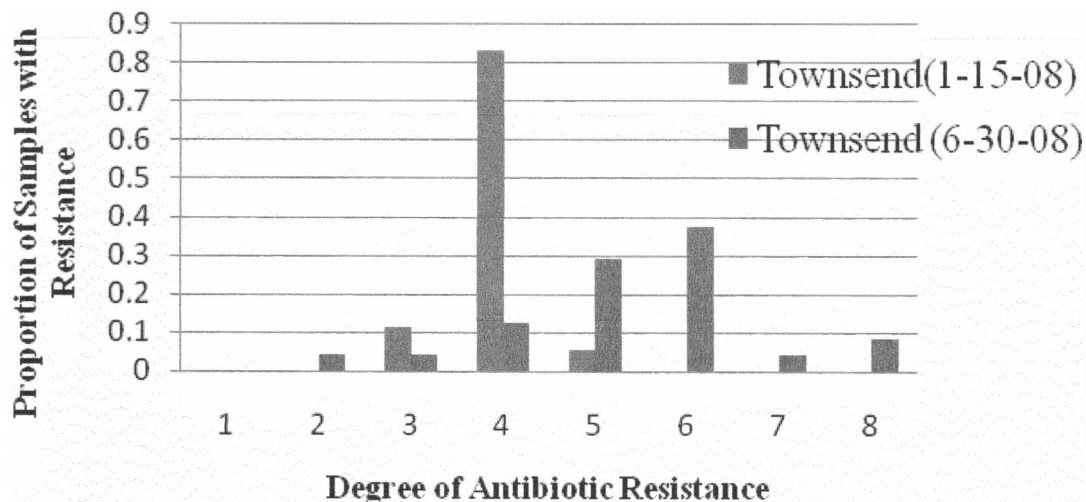


Figure 6. Temporal changes in the *ampicillin* library. Frequency of individual degrees of resistance for isolates selected from Townsend on January 16th of 2008 and June 30th of 2008.

Temporal changes were also observed in the proportion of Townsend and Conrad *E. coli* isolate collections with resistance to the specific antibiotics used. *E. coli* strains from the second Conrad collection (Fig. 7) displayed a greater divergence in MDR patterns from those in the initial collection taken. Initial collections contained *E. coli* strains displaying no resistance to either *chloramphenicol*, *streptomycin*, or *ciprofloxacin*. A large portion of the collection exhibited strains without resistance to *tetracycline* and an intermediate proportion resisted *erythromycin*, *kanamycin*, and *gentamicin*. In contrast, more than 80% of isolates from the subsequent Conrad collection displayed resistance to *erythromycin*, whereas, less than half of isolates from the collection showed resistance to *tetracycline*.

Townsend collections (Fig. 8) did not vary as dramatically as those collected in Conrad. However, Townsend Summer 2008 collections displayed high resistance to both *streptomycin* and *kanamycin*. The January 2008 collection at Townsend had no isolates with resistance to *kanamycin* and less than 10% with resistance to *streptomycin*.

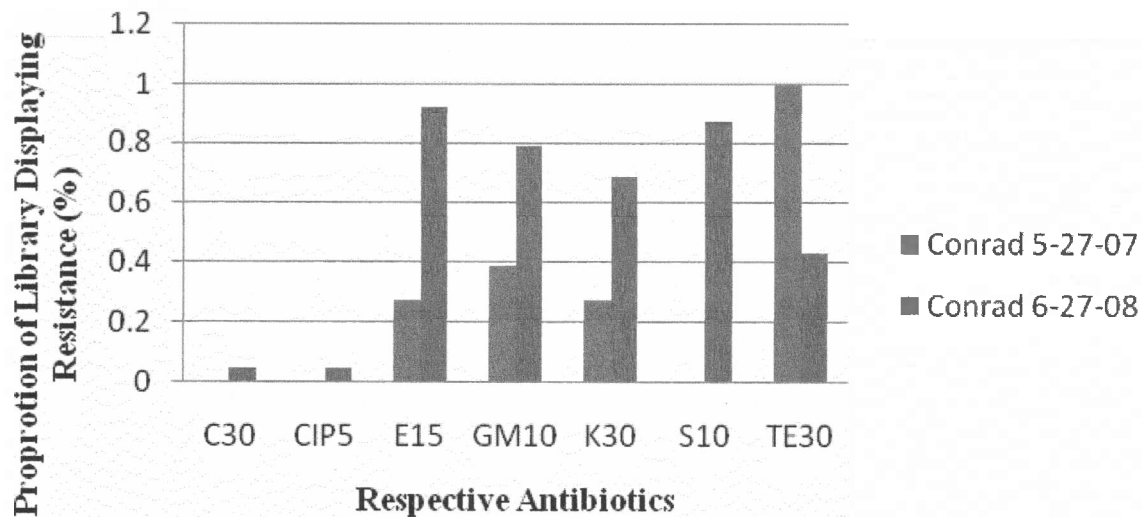


Figure 7. Temporal changes in respective antibiotic resistance on *ampicillin* library. Frequency of resistance for respective antibiotics in isolates collected from Conrad on May 27th of 2007 and again on June 27th of 2008.

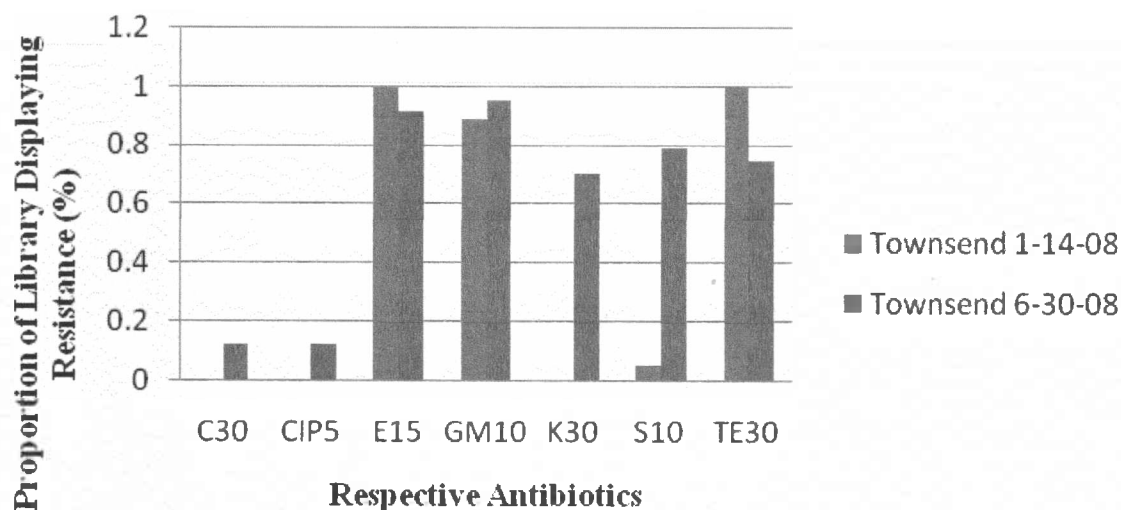


Figure 8. Temporal changes in respective antibiotic resistance on *ampicillin* library. Frequency of resistance for respective antibiotics in isolates collected from Townsend on January 16th of 2008 and June 30th of 2008.

Phenotypes and Antibiotic Associations

Antibiotic resistance patterns can be characterized as a 2-dimensional association between two different drugs analyzed by using the chi-square test. In contrast, MDR phenotypes incorporate resistance to three or more drugs that are always associated with each other (i.e. if resistance is observed in one drug it is observed in all the others) thus precluding the need for statistical analysis. Although the mechanism is unknown, MDR associations suggest that either bacterial populations are exchanging plasmids or they are responding to the presence of multiple drugs in the environment in a concerted fashion.

The data from the chi-square test conducted on antibiotic patterns display three 2-dimensional associations of statistical significance in the *chlortetracycline* library (Appendix 3). The most common 2-dimensional association (an association refers to both antibiotics being present or absent together) was between *streptomycin* and *gentamicin* (p-value < 0.001), present within the *chlortetracycline* library with a frequency of 65%. Associations between *kanamycin* and *streptomycin* and also between *kanamycin* and *gentamicin* were also found in the *chlortetracycline* library, both with a p-value of 0.001 or less.

The most frequent 2-dimensional antibiotic association within the *ampicillin* library (Appendix 4) was between *erythromycin* and *gentamicin* (p-value < 0.001), occurring in 61% of the library. Associations between *kanamycin* and *streptomycin*, *kanamycin* and *erythromycin*, *kanamycin* and *gentamicin*, *kanamycin* and *tetracycline*, *streptomycin* and *erythromycin*, *streptomycin* and *gentamicin*, *chloramphenicol* and *ciprofloxacin*, and *chloramphenicol* and *gentamicin* had a p-value of 0.005 or less in the *ampicillin* library.

The most frequently observed MDR phenotype within the *chlortetracycline* library was *erythromycin-gentamicin-streptomycin* (EGmS), occurring in 66% of the library (Table 3). In the *ampicillin* library it occurred in 39% of isolates.

Table 3. MDR phenotypes and their respective frequency in each library.

Phenotype	<i>Ampicillin</i> Library (%)	<i>Chlortetracycline</i> Library (%)
EGmS	39%	66%
EGmKS	31%	34%
AmCEGmS	24%	31%
AmCEGmKS	17%	17%

Discussion

The prevalence of antibiotic resistant *E. coli* isolates in several of the collections taken during the course of this study, including those from cattle populations administered antibiotics directly, suggest that the increased commercial circulation of antibiotic compounds, regardless of direct exposure, has caused an increase in bacterial communities exhibiting antibiotic resistance—a trend proliferating throughout the microbial world (Guillemot, 1999; Houndt and Ochman, 2000; Hughes and Datta, 1983; Levy, 2002).

Interestingly, study results show that several bovine populations not given antibiotics support *E. coli* communities with occurrences of resistance comparable to those directly subjected to these antimicrobial compounds. Collections obtained from Helena and Townsend, both of which had not received antibiotic supplementation in feed, displayed a high proportion of resistant isolates, similar to the bovine population receiving antibiotics directly through their feed in Conrad. The direct exposure of cattle to antibiotics did not appear to be an influential factor in antibiotic resistance. Conrad was selected for this study based on the direct administration of antibiotics to the cattle herds. With respect to antibiotic resistance, the *E. coli* populations present in this bovine community displayed no increased antibiotic resistance compared to *E. coli* populations from cattle not directly exposed to the drugs. Similar results have been seen in wild and domesticated animals and human populations throughout the world never directly exposed to antibiotics (Bartoloni *et al.*, 2004; Davies, 1996; Pellecchi *et al.*, 2007; Souza *et al.*, 1999). A thorough understanding of the ecology of the genes controlling

antibiotic resistance and factors other than exposure to antibiotics that might control the prevalence of resistance genes circulating in bacterial populations should be developed.

These results may also be the consequence of indirect exposure to other antibiotic resistant strains. In an isolated community of Bolivia, a study was conducted hypothesizing that the genetic heterogeneity of MDR phenotypes in *E. coli* isolates had derived from the infrequent exposure to external MDR donors (Pellecchi *et al.*, 2007). This study shows that even short and rare episodes of exposure permit MDR diversification and spread. Analysis of antibiotics in the environment and fecal contaminants containing *E. coli* exhibiting multiple drug resistance in watersheds near collection sites may provide more information regarding the inheritance of MDR phenotypes prevalent in these Rocky Mountain bovine populations.

The maintenance of antibiotic resistance populations not administered the drugs is unexplained. Khachatryan *et al.* (2006) suggests that expression of acquired resistance genes may not always entail a fitness cost. *E. coli* cells may then be expressing these genes with little or no fitness consequences, even in the absence of these drugs. Concentrations of antibiotics accumulating in the environment may also be the contributor to these persisting resistances. In subsequent studies, soil and watershed samples should be analyzed both for coliform containing similar MDR phenotypes and antibiotic presence.

The use of *ampicillin* and *chlortetracycline* in initial plating was employed to mimic environments in which antibiotic administration was practiced. The high number of fecal samples containing *E. coli* isolates displaying resistance to *ampicillin*,

chlortetracycline, or both reflected the pervasiveness of *ampicillin* and *chlortetracycline* use in animal husbandry practices or some unknown ecological pressure.

In general, the same trends appeared across the libraries for the spatial study, antibiotic associations study, and MDR phenotyping. The majority of isolates localized around the fourth to fifth degree of resistance. Other studies concluded localization occurred around the third degree of resistance, as seen in Bolivian human fecal samples (Bertoloni, 2006). A study on *E. coli* found in urinary tract infection outpatients in Norway found R plasmids harboring three resistance genes (Vorland *et al.*, 1985). Another collection taken from pigs, slaughterers, and pig breeders in Japan suggests 4 or 5 resistance genes can be held on one R plasmid (Saida *et al.*, 1981). This may imply that the number of associated antibiotic resistances is highly variable. The selective pressure of these bacterial populations to adapt R plasmids to the high numbers and concentrations of antibiotics in the environment may enable the dissemination of high multiple drug resistance.

The spatial study demonstrates no significant tie between location and individual antibiotic resistance frequencies. Those locations possessing statistically-significant, nonrandom similarities were not located closer to one another than those locations not demonstrating an association. The 2008 Conrad collection was considered statistically similar to the January 2008 collection of Townsend, while appearing statistically distinct from Avon, located in much closer proximity to Conrad. This may suggest that site specific factors play a greater role in *E. coli* population antibiotic resistance than does spatial separation. Other studies noted greater uniformity in changes across more vast

areas of study. In data generated from human populations analyzed for specific enteric coliform exhibiting antibiotic resistance patterns that varied across social groups and across distance (Skurnik *et al.*, 2005). This may communicate spatial MDR patterns are relevant only at a greater regional scale.

Ultimately, the origin of these MDR patterns must be sought in order to determine the direction of gene flow and from where these populations have derived the genetic heterogeneity attributed to these phenotypes. Houndt and Ochman (2000) hypothesize that large scale increases in background levels of antibiotics historically has lead to an increase in resistance in the species as a whole. These resistance genes inherited from ancestral lines of the bacteria enable the propagation of resistance.

Another possibility lies in the watersheds near the sites at which collections took place. It has been observed that fecal material commonly contaminated by *E. coli* and other coliform populations has been found in rivers and streams circulating through these water systems (Hagedorn *et al.*, 1999; Harwood *et al.*, 2000; Parveen *et al.*, 1997; Shanks *et al.*, 2006). Inevitably, these translocation events may enable gene flow into the populations sampled from some populations previously exposed to antibiotics (populations such as Conrad), leading to the genetic diversification observed across relatively small distances and associations occurring across larger distances.

In addition to the random spatial variation in multiple drug resistance patterns found in the population analyzed in this study, significant change occurred in these patterns temporally. Data from both Townsend and both Conrad collections convey a prominent divergence both in degree of resistance and respective antibiotic resistances

observed in those populations over time, agreeing with a study on alterations present in MDR phenotypes in swine *E. coli* over time (Duriez and Topp, 2007). This departure from degree in resistance and respective antibiotic resistances observed in isolates found in later collections from initial collections of these populations suggest that bacterial communities may be responding to seasonal changes in antibiotic concentrations, or even a propagating increase in antibiotic concentrations in their environments.

Wiuff *et al.* (2005) proposes that bacterial populations subject to antibiotics in bactericidal concentrations will develop phenotypic tolerance to the drugs. Inevitably, these populations will grow persistently in the presence of highly concentrated antibiotics (Wiuff *et al.*, 2005; Balaban *et al.*, 2004; Keren *et al.*, 2004). The decrease in effectiveness of nearly all antimicrobial agents has been observed in clinical settings, as well as in animal husbandry (Barbosa and Levy, 2000). Because the bovine populations of Conrad were subject to direct antibiotic administration, the resistance of *E. coli* to *chlortetracycline* would be expected to be higher in comparison with the *E. coli* found in Townsend cattle not subject to antibiotics directly. Interesting, a higher prevalence was not apparent in the Conrad collection of 2008 from the collection in 2007, but was observed in the Townsend summer 2008 collection from that taken in January of 2008. Because this study was conducted over such a short time, no conclusion can be drawn from these potential trends. A longer investigation of these MDR frequencies at these locations may reveal whether long-term MDR trends are consistent with the data collected during this study.

Inglis *et al.* (2006) describes the temporal effects of antimicrobial agents use on *Campylobacter* species, stating an increased number of isolates occurs displaying resistance to the drugs administered to their beef-cattle hosts. Interestingly, they also found that an additional antibiotic resistance to a drug not administered to the herd was also observed prevalently in one species of *Campylobacter*. In the current study, temporal changes in Conrad with respect to the proportion of isolates with resistance to respective antibiotics occurred, all resistances except to *tetracycline* increased. This trend was not as pronounced in the Townsend temporal study. Interestingly, both Conrad and Townsend displayed a temporal increase in the number of antibiotics to which they showed resistance. Analysis of intestinal lumen from Townsend cattle and of Townsend soil may generate more information regarding why this phenomenon may be occurring. As noted previously, these environments may potentially be hosting high concentrations of antibiotics not previously noted.

Further study could include a more comprehensive exploration of the effects of time on the presence of certain MDR phenotypes, investigating both seasonal changes in MDR phenotypes. Conducting the study of MDR phenotypes over an extended period of time may likewise be beneficial.

The data were also subjected to analysis for the likelihood of nonrandom associations existing between two respective antibiotics. Many associations appeared among *gentamicin*, *kanamycin*, and *streptomycin*. The nonrandom, statistically significant nature of these associations, along with the pervasiveness of these associations within the two libraries, contributes to identifying dominant phenotypes within these

populations. *Erythromycin* occurred commonly throughout the two libraries frequently in association with *gentamicin* and *streptomycin*. Their occurrence was so prevalent that statistical association could not be applied. Commonly observed MDR phenotypes are *streptomycin-tetracycline* (Rosengren, 2008), *ampicillin-streptomycin-tetracycline* (Duriez and Topp, 2007), *streptomycin-tetracycline* (Levy *et al.*, 1988), and *ampicillin-tetracycline-trimethoprim-sulfamethoxazole-chloramphenicol* (Bartoloni *et al.*, 2006; Pallecchi *et al.*, 2007). These phenotypes deviate from the results observed in this study. The diversity of antibiotics used worldwide may contribute to these discrepancies—those used locally and accumulating in the nearby watersheds may vary from those administered globally.

In addition to isolation of *E. coli* on antibiotic containing media, fecal samples collected should be selected on media lacking antibiotics in the future. This will allow recognition of isolates identified as *E. coli* that do not express resistance to antibiotics selected for this study. Perhaps then we could compare these isolates to see the dual absence of some of the antibiotic resistance that always seemed to be present in the library, showing whether a true association is present in isolates between resistance to *chlortetracycline* or *ampicillin* and other antibiotic resistances abundant in the libraries.

The antibiotics selected for this study attack bacteria with such mechanistic diversity that bacteria could not facilitate resistance encompassing the range of antibiotics solely through mutation alone. The presence of high degrees of MDR suggests the existence of an external device enabling this diversification of resistance machinery.

The presence of these MDR phenotypes has likely arisen from the involvement of exchanging resistance plasmids.

Conclusion

The first hypothesis, *E. coli* is highly prevalent as a commensal bacteria in populations of bovine throughout the northern Rocky Mountain region was accepted; the data supported a high frequency of resistance in the collections. However, the proposal that greater distance between sites may contribute to greater variation in MDR phenotypes is neither accepted nor rejected since data remain inconclusive; no particular geographic trends arose in MDR variations. The third hypothesis asserting that antibiotic resistance of commensal *E. coli* populations will be influenced by shifts in environment through time was accepted, collections from sites sampled in duplicate displayed high levels of MDR phenotypic variation across time. The fourth hypothesis that the administration of antibiotics in bovine populations will be reflected in an increased resistance of commensal *E. coli* to such drugs when compared with commensal *E. coli* populations from cattle not receiving the drugs was rejected on the bases that isolates from bovine populations receiving antibiotics closely resembled those without direct drug administration. MDR phenotypes were prevalent throughout the libraries. Thus, the final hypothesis that resistance to respective antibiotics will emerge in association was likewise accepted.

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Appendix 1. Pairwise tests of associations between sites with respect to their MDR levels on the *Chlortetracycline* library.

Test	Chi Square	df	p-Value
Three Forks v Avon	2.55	4	0.636
Three Forks v Conrad	31.05	5	0.001
Three Forks v Townsend 1	0.71	5	0.982
Three Forks v Townsend 2	26.73	5	<0.001
Avon v Conrad	29.24	5	<0.001
Avon v Townsend 1	-0.60	5	1.000
Avon v Townsend 2	24.42	5	<0.001
Conrad v Townsend 1	39.46	6	<0.001
Conrad v Townsend 2	5.70	3	0.127
Townsend 1 v Townsend 2	40.75	6	<0.001

Appendix 2. Pairwise tests of associations between sites with respect to MDR levels on the *Ampicillin* library.

Test	Chi Square	df	p-Value
Three Forks v Avon	1.85	4	0.763
Three Forks v Conrad 1	3.14	3	0.370
Three Forks v Conrad 2	6.78	5	0.237
Three Forks v Helena	6.21	3	0.102
Three Forks v MacDonald Pass	5.13	3	0.163
Three Forks v Townsend 1	9.40	6	0.152
Three Forks v Townsend 2	10.15	3	0.017
Avon v Conrad 1	4.39	4	0.356
Avon v Conrad 2	14.66	6	0.023
Avon v Helena	6.09	4	0.193
Avon v MacDonald Pass	7.64	4	0.106
Avon v Townsend 1	11.05	6	0.074
Avon v Townsend 2	12.66	4	0.013
Conrad 1 v Conrad 2	28.75	5	<0.001
Conrad 1 v Helena	7.10	3	0.069
Conrad 1 v MacDonald Pass	5.10	3	0.165
Conrad 1 v Townsend 1	30.01	6	<0.001
Conrad 1 v Townsend 2	18.59	3	<0.001
Conrad 2 v Helena	21.41	5	<0.001
Conrad 2 v MacDonald Pass	12.28	5	0.031
Conrad 2 v Townsend 1	6.93	6	0.327
Conrad 2 v Townsend 2	38.16	5	<0.001

Helena v MacDonald Pass	9.85	3	0.020
Helena v Townsend 1	19.06	6	0.004
Helena v Townsend 2	3.36	2	0.186
MacDonald Pass v Townsend 1	14.27	6	0.027
MacDonald Pass v Townsend 2	10.65	3	0.014
Townsend 1 v Townsend 2	31.30	6	<0.001

Appendix 3. Pairwise tests of associations between antibiotics on the *Chlortetracycline* library.

Test	Chi Square	df	p-Value
K30 v Am10	0.75	1	0.385
K30 v S10	15.01	1	0.001
K30 v E15	0.01	1	0.915
K30 v Gm10	23.31	1	<0.001
K30 v C30	0.09	1	0.769
Am10 v S10	1.00	1	0.316
Am10 v E15	0.05	1	0.822
Am10 v Gm10	1.49	1	0.221
Am10 v C30	0.14	1	0.713
S10 v E15	0.01	1	0.904
S10 v Gm10	25.68	1	<0.001
S10 v C30	0.01	1	0.903
E15 v Gm10	0.32	1	0.572
E15 v C30	0.39	1	0.528
Gm10 v C30	1.05	1	0.304

Appendix 4. Pairwise tests of associations between antibiotics on the *Ampicillin* library.

Test	Chi Square	df	p-Value
K30 v S10	24.25	1	0.001
K30 v E15	9.77	1	0.002
K30 v Cip5	0.25	1	0.618
K30 v Gm10	16.21	1	<0.001
K30 v C30	2.17	1	0.141
K30 v Te30	8.01	1	0.005
S10 v E15	13.79	1	<0.001
S10 v Gm10	13.02	1	<0.001
S10 v C30	1.71	1	0.192
S10 v Cip5	0.14	1	0.711
S10 v Te30	1.28	1	0.257
E15 v Cip5	0.03	1	0.866
E15 v C30	0.42	1	0.518
E15 v Gm10	21.41	1	<0.001
E15 v T30	0.73	1	0.393
Cip5 v C30	13.11	1	<0.001
Cip5 v Gm10	0.06	1	0.811
Cip5 v Te30	0.01	1	0.992
C30 v Gm10	4.16	1	0.041
C30 v Te30	2.82	1	0.093
Gm10 v Te30	0.41	1	0.521