**Title:**

**Rational Design of Antibiotic Treatment Plans**

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**Abstract:**

**Introduction**

Antibiotic resistance is an inevitable outcome whenever antibiotics are used. There are many reasons for this: 1) As humans (also as eukaryotes), we are vastly outnumbered by bacteria in nearly all measures, including total population size, biomass, genetic diversity, emigration, and immigration [[1](#_ENREF_1)]; 2) bacteria can use horizontal gene transfer to share resistance genes across distantly related species of bacteria, including non-pathogens [[2](#_ENREF_2)]; 3) compared to humans, bacteria have relatively few vulnerable target sites [[3](#_ENREF_3)]; 4) microbes are the sources of nearly all antibiotics that are used by humans [[4](#_ENREF_4)]. Given the overwhelming numbers of bacteria, the limited number of target sites, the numerous ways that they can infect humans, and that they have been exposed to naturally occurring antibiotics for billions of years, resistance to antibiotics used by human populations is unavoidable.

Once resistance is present in a bacterial population, it is exceedingly difficult to remove for several reasons. If any amount of antibiotic is present in the environment, antibiotic resistance genes will confer a large fitness advantage [[5](#_ENREF_5)], and even when antibiotics are not present in an environment, the fitness costs for carrying and expressing resistance genes are small to non-existent [[6](#_ENREF_6)]. In addition to it being difficult to remove antibiotics from the environment [[7](#_ENREF_7)], even if humans were to completely abandon the use of antibiotics, resistance would persist for years [[8](#_ENREF_8)].

Efforts to remove resistance genes from clinical environments by either discontinuing or reducing the use of specific antibiotics for some period of time, either through general reduction of antibiotic consumption or periodic rotations of antibiotics (cycling) have not worked in any reliable or reproducible manner [[9](#_ENREF_9)]; indeed it would have been surprising if they had worked [[10](#_ENREF_10)],[[11](#_ENREF_11)].

Since antibiotic resistance *is* unavoidable, it only makes sense to accept its inevitability and develop methods for mitigating the consequences. One reasonable approach is to rotate the use of antibiotics. This has been implemented in many ways and there are recent studies to model the optimal duration, mixing versus cycling, and how relaxed antibiotic cycles may be and still function as planned [[12](#_ENREF_12),[13](#_ENREF_13)]. However, none of those models have focused on developing a method for designing an optimal succession of antibiotics.

In a previous publication [[14](#_ENREF_14)], we proposed that susceptibility to antibiotics could be restored by rotating consumption of multiple antibiotics that are a) structurally similar, b) inhibit/kill bacteria through the same target site, and c) result in pleiotropic fitness costs that reduce the overall resistance of bacteria to each other. We showed an anecdotal, proof-of-principle example [[14](#_ENREF_14)] of how this might work with a series of β-lactam antibiotics in which some would select for new amino acid substitutions in the TEM β-lactamase and others that would select reversions in TEM ultimately leading back to the wild-type (un-mutated) state.

Our current work seeks to identify β-lactam treatment plans that have the highest probability of returning a population expressing a small number of variant TEM genotypes to the wild type state. The wild type TEM-1, and a handful of its descendants, confer resistance to penicillins alone. However most of the descendants confer resistance to either cephalosporins or penicillins combined with β-lactamase inhibitors (inhibitor resistance), and a few confer resistance to both. Of the 194 clinically identified TEM genotypes that encode unique amino acid sequences [[15](#_ENREF_15)], 174 (89.7%) differ from the wild type TEM-1 by at most four amino acid substitutions (see Table 1). Our choice of a system that includes four amino acid substitutions is based upon an apparent threshold for amino acid substitutions among functional TEM genotypes. The rarity of the co-existence of cephalosporin resistance and inhibitor resistance and the fact that no single substitution confers both phenotypes suggested that sign epistasis (i.e. reversals of substitutions from beneficial to detrimental) exists as the substitutions that contribute to this dual phenotype are combined.

The ability to push an evolved TEM genotype back to the wild type state would limit the range of antibiotics to which it could confer resistance. To embark upon our effort of determining the best way to do this, we decided to create a model system based upon the TEM-50 genotype, which differs from TEM-1 by four amino acid substitutions. All four substitutions by themselves confer clearly defined resistance advantages in the presence of certain antibiotics. Additionally, TEM-50 is one of the few genotypes that simultaneously confers resistance to cephalosporins and inhibitor combined therapies.

**Results**

*From experimental data to mathematical models*

We created all 16 variant genotypes of the four amino acid substitutions found in TEM-50 using site directed mutagenesis (Table 2) and measured the growth rates of 12 replicates of *E.coli* DH5α-E expressing each genotype in the presence of one of fifteen β-lactam antibiotics (Table 3). Each genotype was grown in each antibiotic in 12 replicates. We computed the mean growth rate of those replicates (Table 4) and the variance of each sample, as well as the significance between adjacent genotypes that differed by one amino acid substitution. This was done using one-way ANOVA analysis.

The results are summarized in Figures 1-15, where the arrows in the landscape maps connect pairs of adjacent genotypes. For each comparison of adjacent genotypes, we indicate the one whose expression resulted in the faster growth by directing the arrowhead towards that genotype, and implying that evolution would proceed in that direction if the two genotypes occurred simultaneously in a population. In other words, the node indicated by the arrowhead would increase in frequency and reach fixation in the population, while the other would be lost. Red arrows indicate significance, and black arrows indicate differences that were not statistically significant by ANOVA, but that may still exist if a more sensitive assay was used.

We rank ordered the genotypes (Table 5) in each landscape diagram with a score from 1 to 16, with the genotype promoting the fastest growth receiving a score of “1” and the genotype with the slowest growth a score of “16”. This analysis shows that all genotypes have a score of 5 or better and a score of 13 or worse, in at least one landscape, indicating that there is abundant pleiotropy as antibiotic selective pressures change. That pleiotropy provides a basis for effectively alternating antibiotic to restore the wild type.

Based on the strong patterns of pleiotropy we observed, we reasoned that the choice and the succession of antibiotics were at least as important as other cycling considerations. We formalized our approach to optimal cycling as follows.

We start by considering the 15 antibiotics previously mentioned in Table 3: AMP, AM, CEC, CTX, ZOX, CXM, CRO, AMC, CAZ, CTT, SAM, CPR, CPD, TZP, and FEP. For each of these 15 antibiotics, we select exactly one TEM fitness landscape that exists at a specific concentration of the antibiotic. That landscape is a real tensor whose entries are the growth rates we measured experimentally. Those growth rates depend upon the states of the four functionally important amino acid residues involved in the evolution of TEM-50. The indices correspond to four possible amino acid substitutions and exist in either state 0, corresponding to no substitution at that site, or 1, which corresponds to an amino acid substitution that is involved in the resistance phenotype. We can identify with a vector of length 16 whose coordinates are indexed by .



The resulting 15 vectors, one for each antibiotic, are the rows in Table 4. The tensor contains the growth rates of each genotype as a function of antibiotic . The matrix is a transition matrix whose rows contains the fixation probabilities for all possible transitions in a single antibiotic (eg is the probability that genotype *u* is replaced by genotype *v*). For this reason, a transition matrix has nonnegative entries and its rows sum to 1. The rows and columns of are labeled by , in lexicographical order that is fixed throughout. We require that our transition matrices respect the adjacency structure of the 4-cube, that is, unless and are vectors in that differ in at most one coordinate. For that reason our transition matrices can have at most four entries corresponding to transitions to the immediately adjacent genotypes that add to one. In other words, we reasoned that resistant strains are most likely to be in competition with those that express resistance genotypes that are immediately adjacent (vary by a single amino acid substitution). In our model, each TEM has four genotypes that are adjacent, since there are four amino acid residues under consideration.



Our substitution model is a function that assigns a transition matrix to the fitness landscape for antibiotic . An entry of that matrix is denoted .This represents the fixation probability for genotype transitioning to genotype in the presence of antibiotic . If is a sequence of antibiotics, then the matrix product is .



Our goal is to maximize the matrix entry for all 15 genotypes other than 0000.



For each this requires searching over all  antibiotic sequences of length .



*Finding optimal sequences of antibiotics*

We used two substitution models to determine the optimal (most probable) sequences of β-lactams for returning TEM genotypes back to their wild type state. Briefly, the Correlated Probability Model (CPM) allows probabilities to be based upon the actual growth rates. It is given by applying formula (7) to the growth rates in Table 4. The Equal Probability Model (EPM) assumes that beneficial mutations are equally likely and that only the direction of the arrows in Figures 1-15 is important. This means that the matrix entry is if genotype has outgoing arrows and there is an arrow from to .



A visual summary of the highest probabilities seen in the 15 CPM transition matrices is provided in Figure 16. The CPM provides good estimates

if fitness differences between genotypes are small [[14](#_ENREF_14),[16](#_ENREF_16),[17](#_ENREF_17),[18](#_ENREF_18)]. The EPM has

been used in settings where only rank order (as in Table 5) is available [[19](#_ENREF_19)].

For all sequences of antibiotics of a fixed length (2, 3, 4, 5, and 6), we examined the probability that a given genotype is returned to the wild type state. For every starting genotype, we found we were able to return to the wildtype genotype with a probability between 0.6 and 1.0 when using the CPM model and a probability of 0.375 and 1.0 when using the EPM model. These results are summarized in Tables 6-9 and Figure 17. These results show the number of paths and their probabilities (Tables 6 and 7) and the substitutions of the most probable paths (Tables 8 and 9) for returning to the wild type state from various starting points.

Once returned to the wild type state, we identified cycles that would allow for alternation of antibiotics, and allow for some variation through amino acid substitution, but then rapidly return bacteria to the wild type state (Figure 18). Such cycles were possible for path length of 2, 4, and 6 and the probabilities of those paths were respectively 0.704, 0.617, 0.617. We found that in the most probable cases, the genotype varied by only one amino acid substitution before reverting back to the wild-type state. However, when treatment plans with lower probabilities are considered, we find that more amino acid substitutions in the genotype are allowed.

**Discussion**

In this study, we have developed an experimental approach for measuring pleiotropy and a computational mathematical approach for optimizing antibiotic treatment paths. The experimental approach we developed is rapid and high throughput, and should be applicable to many species of resistant bacteria. The mathematical model we created expresses the problem of antibiotic resistance in general terms, and can therefore be applied to other resistance phenotypes where pleiotropy occurs to identify the antibiotic treatment plans that have the highest probability of reversing the evolution of resistance.

The purpose of this study was to determine whether it is possible to use selective pressures to return TEM-genotypes to the wild type state, as observed in 1963. The methods may also be used to select for any particular genotype within our data set, and can therefore be used generally to select, with reasonable precision, for resistance genotypes that may have existed at any time point up to the present. To emphasize the potential of this approach, we have named our computational software package “Time Machine”.

Once given growth rates of adjacent genotypes, Time Machine returned treatment plans that restored the wild type state as observed in 1963 with probabilities greater than 0.6 when using the CPM model and greater than 3/8 (>0.375) when using EPM. These results suggest that when possible it is desirable to use a CPM model including actual growth rates rather than rough ranking data.

Tables 6 and 7 suggest that the maximum probabilities in each row stagnate after a limited number of steps. This is not always the case. We have constructed a particular example (see supplemental information) of two substitution matrices on a 3-locus system where the maximum probabilities can be increased indefinitely..

These results show that great potential exists for remediation of antibiotic resistance through antibiotic treatment plans when pleiotropic fitness costs are known for an appropriate set of antibiotics. While developed using a model of Gram-negative antibacterial resistance, this approach could also be used for Gram-positive bacteria and HIV treatment plans.

**Methods**

Experimental methods

*Strains and Cultures*

We expressed 16 mutant constructs of the blaTEM gene in plasmid pBR322 from strain DH5-αE. The 16 genotypes differ at all combinations of four amino acid residues and have been previously described [[14](#_ENREF_14)]. We grew them overnight (16 hours) in standing cultures and diluted them to a concentration of 1.9X105 as described elsewhere [[14](#_ENREF_14)].

We transferred 80 µl of each culture to a 384-well plate with one genotype present in each of the 16 rows. The first 12 wells of each row were antibiotic free (controls) and the last 12 wells contained a single antibiotic at an inhibitory, sublethal concentration

After plating, a membrane is placed over the plate and simultaneously incubated/measured in the Eon Microplate Spectrophotometer at a temperature of 25.1°C for 22 hours. This relatively cool (<37º) temperature is used because degradation of the antibiotics is much slower, while the growth rate of the bacteria is still sufficient to capture the complete exponential period of growth over the duration of the experiment. Overall, we have found that a temperature ~25ºC yields more reliable and consistent measurement of growth rates in the presence of antibiotics.

Measurements of cell density (light scattering) at a wavelength of 600 nanometers were automatically collected every 20 minutes after brief agitation to homogenize and oxygenate the culture.

*Growth Rates*

The data obtained from the microplate spectrophotometer is exported to the GrowthRates program to derive the growth rates. In essence, by measuring the optical density at frequent intervals the GrowthRates program can estimate the growth rate, , through a linear regression algorithm fitting the data from the exponential growth phase. Details can be found in [[20](#_ENREF_20)] in the section entitled “The Growth Curve” located on pages 233-4. The output of this program for the data we collected was a list of 15 tensors, each of format. These are the rows in Table 4. So if is a genotype, then is the fitness of genotype in the presence of antibiotic . This fitness is a growth rate, so we are here using the letter for a quantity often denoted by.



One-Way Analysis of Variance (ANOVA) was then used to compare the means of the growth rates obtained, and to determine if there were significant differences between the growth rates of adjacent genotypes.

*Time Machine Programs*

-Derivation of Correlated Probability Model (CPM)

Once the growth rates have been determined under various experimental conditions, the next step is to use them to compute fixation probabilities.

If the (multiplicative) absolute fitnesses and of two neighboring genotypes *u* and *v*, differ by a small quantity then the (additive) relative fitness



can be approximated by





where is the generation time. Using a Taylor series approximation,



.

If  , then



is the probability for  to substitute , where  are the neighbors of  with higher fitness than [[17](#_ENREF_17" \o "Gillespie, 1984 #54)].

-Derivation of Equal Probability Model (EPM):

According to the EPM model, the probabilities are equal for all beneficial mutations, so that one needs the fitness graphs only for computing the probabilities. The matrix entry is if genotype has outgoing arrows and there is an arrow from to .



CPM is accurate if fitness differences between genotypes are small, while EPM

may provide better estimates if fitness differences are substantial. Indeed, if the fitness effects of all available beneficial mutants exceed some threshold, then fixation probabilities are independent of fitness values [[21](#_ENREF_21)]. We applied both CPM and EPM, since no complete theory for substitution probabilities exists. Additionally, comparison of two models is useful in learning how sensitive our results are for variation in substitution probabilities.

*-*Optimal antibiotic sequences and pathways of genotypes

Let denote the transition matrix we derived for the antibiotic labeled . For any sequence of antibiotics, we consider the matrix product . This product is also a transition matrix. Its entry in row *a* and column *b* is the fixation probability of genotype mutating to genotype under the antibiotic sequence . That probability is a sum of products of entries in the individual matrices , with one sum for each possible pathway of genotypes from to . Our optimization algorithm enumerates all antibiotic sequences of length , and it selects all sequences that maximize the entry in row *a* and column *b* of the matrix product. In a subsequent step we then analyze these optimal antibiotic sequences, and for each such sequence, we extract the full list of genotype pathways that contribute.



We implemented this algorithm in the computer algebra software Maple, and we ran it for . The running time of the program is slow because of the exponential growth in the number of sequences. At present we do not know whether an efficient algorithm exists for solving our optimization problem for larger values of .



**Figure Legends**

Figures 1-15

These figures present a visual summary of the adaptive landscape 2x2x2x2 tensors in which each resistance phenotype conferred by each TEM genotype is enumerated. Arrows pointing upward represent addition of a mutation. Arrows pointing downward represent reversions. Red arrows indicate significance between adjacent growth rates as determined by one way ANOVA. Genotypes that confer the most resistance to each antibiotic are shown in red.

Figure 16

Summary of CPM Substitutions with the Highest Probabilities. Each arrow is labeled by the drug or drugs corresponding to the maximal transition probability, taken over all 15 drugs. Each arrow is also labeled by the maximal probability.

From the graph, it is possible to find candidate, un-optomized treatment plans. For example, when starting at genotype 1010 the graph shows that the probability for ending at 0000 is 0.71for the sequence ZOX-TZP (0.71 is the product of the arrow labels). Similarly, when starting at 1111 the probability for ending at 0000 is 0.62 for the sequence CEC-CAZ-TZP-AM. When starting at 0001 the graphs shows that a single drug gives probability at most 0.29, whereas the probability for ending at 0000 for the sequence AMC-CRO-AM (one arrow up, two arrows down) is at least.

This graph can also be used to generate circular paths. For example, from a starting point of 0000, the probability for ending at 0000 is 0.62 for the sequence CEC-SAM-AMP-FEP-CPR-CAZ-TZP-AM (4 substitutions and 4 reversions).

Figure 17

Summary of Optimal 6 Step CPM and EPM Treatment Paths. Black arrows show transitions present in six step paths computed using both the CPM and the EPM. Red arrows signify transitions found only in optimum paths computed using the CPM whereas blue signify transitions only found using the EPM.

Figure 18

Summary of Optimal CPM 2, 4, and 6 Step Antibiotic Cycles. Two step cycles are shown in red. Four and six step cycles are shown in blue. Four and Six Step cycles differ only in the number of steps, but the substitutions used within them and the probabilities are identical.

**Table 1**

|  |  |
| --- | --- |
| Number of amino acid substitutions | Number of TEM genotypes |
| 1 | 53 |
| 2 | 53 |
| 3 | 37 |
| 4 | 31 |
| 5 | 10 |
| 6 | 2 |
| 7 | 2 |
| 8 | 0 |
| 9 | 0 |
| 10 | 1 |
| 11 | 1 |

**Table 2 Variant Genotypes Created, Binary Codes and Names of Genotypes Identified in Clinical Isolates**

|  |  |  |
| --- | --- | --- |
| **Number of Substitutions** | **Binary**  **Genotype**  **Code** | **Genotypes with substitutions found in TEM-50** |
| 0 | 0000 | No substitutions  (TEM-1) |
| 1 | 1000 | M69L  (TEM-33) |
| 1 | 0100 | E104K  (TEM-17) |
| 1 | 0010 | G238S  (TEM-19) |
| 1 | 0001 | N276D  (TEM-84) |
| 2 | 1100 | M69L  E104K  (Not identified) |
| 2 | 1010 | M69L  G238S  (Not identified) |
| 2 | 1001 | M69L  N276D  (TEM-35) |
| 2 | 0110 | E104K  G238S  (TEM-15) |
| 2 | 0101 | E104K  N276D  (Not identified) |
| 2 | 0011 | G238S  N276D  (Not identified) |
| 3 | 1110 | M69L  E104K  G238S  (Not identified) |
| 3 | 1101 | M69L  E104K  N276D  (Not Identified) |
| 3 | 1011 | M69L  G238S  N276D  (Not identified) |
| 3 | 0111 | E104K  G238S  N276D  (Not identified) |
| 4 | 1111 | M69L  E104K  G238S  N276D  (TEM-50) |

**Table 3 Antibiotics used for this study**

|  |  |  |
| --- | --- | --- |
| **Antibiotic** | **FDA approval** | **Antibiotic Group** |
| Ampicillin (AMP) | 1963 | Aminopenicillin |
| Amoxicillin (AM) | 1972 | Aminopenicillin |
| Cefaclor(CEC) | 1979 | Cephalosporin |
| Cefotaxime (CTX) | 1981 | Cephalosporin |
| Ceftizoxime (ZOX) | 1983 | Cephalosporin |
| Cefuroxime (CXM) | 1983 | Cephalosporin |
| Ceftriaxone(CRO) | 1984 | Cephalosporin |
| Amoxicillin + Clavulanic acid (AMC) | 1984 | Penicillin derivative +  β-Lactamase inhibitor |
| Ceftazidime (CAZ) | 1985 | Cephalosporin |
| Cefotetan (CTT) | 1985 | Cephalosporin |
| Ampicillin + Sulbactam (SAM) | 1986 | Penicillin derivative +  β-Lactamase inhibitor |
| Cefprozil (CPR) | 1991 | Cephalosporin |
| Cefpodoxime (CPD) | 1992 | Cephalosporin |
| Pipercillin + Tazobactam (TZP) | 1993 | Penicillin derivative +  β-Lactamase inhibitor |
| Cefepime(FEP) | 1996 | Cephalosporin |

**Table 4 Average Growth Rates ( x 10-3): the rows are the fitness landscapes**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **0000** | **1000** | **0100** | **0010** | **0001** | **1100** | **1010** | **1001** |
| **AMP** | 1.851 | 1.570 | 2.024 | 1.948 | 2.082 | 2.186 | 0.051 | 2.165 |
| **AM** | 1.778 | 1.720 | 1.448 | 2.042 | 1.782 | 1.557 | 1.799 | 2.008 |
| **CEC** | 2.258 | 0.234 | 2.396 | 2.151 | 1.996 | 2.150 | 2.242 | 0.172 |
| **CTX** | 0.160 | 0.185 | 1.653 | 1.936 | 0.085 | 0.225 | 1.969 | 0.140 |
| **ZOX** | 0.993 | 1.106 | 1.698 | 2.069 | 0.805 | 1.116 | 1.894 | 1.171 |
| **CXM** | 1.748 | 0.423 | 2.940 | 2.070 | 1.700 | 2.024 | 1.911 | 1.578 |
| **CRO** | 1.092 | 0.830 | 2.880 | 2.554 | 0.287 | 1.407 | 3.173 | 0.540 |
| **AMC** | 1.435 | 1.417 | 1.672 | 1.061 | 1.573 | 1.377 | 1.538 | 1.351 |
| **CAZ** | 2.134 | 0.288 | 2.042 | 2.618 | 2.656 | 2.630 | 1.604 | 0.576 |
| **CTT** | 2.125 | 3.238 | 3.291 | 2.804 | 1.922 | 0.546 | 2.883 | 2.966 |
| **SAM** | 1.879 | 2.198 | 2.456 | 0.133 | 2.533 | 2.504 | 2.308 | 2.570 |
| **CPR** | 1.743 | 1.553 | 2.018 | 1.763 | 1.662 | 0.223 | 0.165 | 0.256 |
| **CPD** | 0.595 | 0.432 | 1.761 | 2.604 | 0.245 | 0.638 | 2.651 | 0.388 |
| **TZP** | 2.679 | 2.709 | 3.038 | 2.427 | 2.906 | 2.453 | 0.172 | 2.500 |
| **FEP** | 2.590 | 2.067 | 2.440 | 2.393 | 2.572 | 2.735 | 2.957 | 2.446 |
|  | **0110** | **0101** | **0011** | **1110** | **1101** | **1011** | **0111** | **1111** |
| **AMP** | 2.033 | 2.198 | 2.434 | 0.088 | 2.322 | 0.083 | 0.034 | 2.821 |
| **AM** | 1.184 | 1.544 | 1.752 | 1.768 | 2.247 | 2.005 | 0.063 | 2.047 |
| **CEC** | 2.230 | 1.846 | 2.648 | 2.640 | 0.095 | 0.093 | 0.214 | 0.516 |
| **CTX** | 2.295 | 0.138 | 2.348 | 0.119 | 0.092 | 0.203 | 2.269 | 2.412 |
| **ZOX** | 2.138 | 2.010 | 2.683 | 1.103 | 1.105 | 0.681 | 2.688 | 2.591 |
| **CXM** | 2.918 | 2.173 | 1.938 | 1.591 | 1.678 | 2.754 | 3.272 | 2.923 |
| **CRO** | 2.732 | 0.656 | 3.042 | 2.740 | 0.751 | 1.153 | 0.436 | 3.227 |
| **AMC** | 0.073 | 1.625 | 1.457 | 1.307 | 1.914 | 1.590 | 0.068 | 1.728 |
| **CAZ** | 2.924 | 2.756 | 2.688 | 2.893 | 2.677 | 1.378 | 0.251 | 2.563 |
| **CTT** | 3.082 | 2.888 | 0.588 | 3.193 | 3.181 | 0.890 | 3.508 | 2.543 |
| **SAM** | 0.083 | 2.437 | 0.094 | 2.528 | 3.002 | 2.886 | 0.094 | 3.453 |
| **CPR** | 2.042 | 2.050 | 1.785 | 1.811 | 0.239 | 0.221 | 0.218 | 0.288 |
| **CPD** | 2.910 | 1.471 | 3.043 | 0.963 | 0.986 | 1.103 | 3.096 | 3.268 |
| **TZP** | 2.528 | 3.309 | 0.141 | 0.609 | 2.739 | 0.093 | 0.143 | 0.171 |
| **FEP** | 2.652 | 2.808 | 2.832 | 2.796 | 2.863 | 2.633 | 0.611 | 3.203 |

**Table 5 Rank order of genotypes in each antibiotic (derived from Table 4)**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Antibiotic | 0000 | 1000 | 0100 | 0010 | 0001 | 1100 | 1010 | 1001 | 0110 | 0101 | 0011 | 1110 | 1101 | 1011 | 0111 | 1111 |
| AMP | 11 | 12 | 9 | 10 | 7 | 5 | 15 | 6 | 8 | 4 | 2 | 13 | 3 | 14 | 16 | 1 |
| AM | 8 | 11 | 14 | 3 | 7 | 12 | 6 | 4 | 15 | 13 | 10 | 9 | 1 | 5 | 16 | 2 |
| CEC | 4 | 12 | 3 | 7 | 9 | 8 | 5 | 14 | 6 | 10 | 1 | 2 | 15 | 16 | 13 | 11 |
| CTX | 11 | 10 | 7 | 6 | 16 | 8 | 5 | 12 | 3 | 13 | 2 | 14 | 15 | 9 | 4 | 1 |
| ZOX | 14 | 11 | 8 | 5 | 15 | 10 | 7 | 9 | 4 | 6 | 2 | 3 | 12 | 16 | 1 | 3 |
| CXM | 11 | 16 | 2 | 7 | 12 | 8 | 10 | 15 | 4 | 6 | 9 | 14 | 13 | 5 | 1 | 3 |
| CRO | 10 | 11 | 4 | 7 | 16 | 8 | 2 | 14 | 6 | 13 | 3 | 5 | 12 | 9 | 15 | 1 |
| AMC | 9 | 10 | 3 | 14 | 6 | 11 | 7 | 12 | 15 | 4 | 8 | 13 | 1 | 5 | 16 | 2 |
| CAZ | 10 | 15 | 11 | 8 | 6 | 7 | 12 | 14 | 1 | 3 | 4 | 2 | 5 | 13 | 16 | 9 |
| CTT | 12 | 3 | 2 | 10 | 13 | 16 | 9 | 7 | 6 | 8 | 15 | 4 | 5 | 14 | 1 | 11 |
| SAM | 12 | 11 | 8 | 13 | 5 | 7 | 10 | 4 | 16 | 9 | 14 | 6 | 2 | 3 | 15 | 1 |
| CPR | 7 | 9 | 3 | 6 | 8 | 13 | 16 | 11 | 2 | 1 | 5 | 4 | 12 | 14 | 15 | 10 |
| CPD | 13 | 14 | 7 | 6 | 16 | 12 | 5 | 15 | 4 | 8 | 3 | 11 | 10 | 9 | 2 | 1 |
| TZP | 6 | 5 | 2 | 10 | 3 | 9 | 12 | 8 | 7 | 1 | 15 | 11 | 4 | 16 | 14 | 13 |
| FEP | 10 | 15 | 13 | 14 | 11 | 7 | 2 | 12 | 8 | 5 | 4 | 6 | 3 | 9 | 16 | 1 |
| best value | 4 | 3 | 2 | 3 | 3 | 5 | 2 | 4 | 1 | 1 | 1 | 2 | 1 | 3 | 1 | 1 |
| worst value | 14 | 16 | 14 | 14 | 16 | 16 | 15 | 15 | 16 | 13 | 15 | 14 | 15 | 16 | 16 | 13 |

**Table 6. Maximum Probability Using CPM**

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Starting  Genotype | 1 Step | # | 2 Step | # | 3 Step | # | 4  Step | # | 5 Step | # | 6 Step | # |
| 1000 | 1.0 | 1 | 1.0 | 3 | 1.0 | 7 | 1.0 | 15 | 1.0 | 31 | 1.0 | 63 |
| 0100 | 0.617 | 1 | 0.617 | 6 | 0.617 | 36 | 0.617 | 219 | 0.617 | 1360 | 0.617 | 8568 |
| 0010 | 0.715 | 1 | 0.715 | 2 | 0.715 | 3 | 0.715 | 4 | 0.715 | 5 | 0.715 | 6 |
| 0001 | 0.287 | 1 | 0.287 | 1 | 0.592 | 2 | 0.592 | 8 | 0.726 | 2 | 0.726 | 4 |
| 1100 |  |  | 0.617 | 3 | 0.617 | 18 | 0.617 | 108 | 0.617 | 657 | 0.617 | 4110 |
| 1010 |  |  | 0.715 | 1 | 0.715 | 6 | 0.715 | 27 | 0.715 | 112 | 0.715 | 453 |
| 1001 |  |  | 0.559 | 1 | 0.559 | 4 | 0.726 | 1 | 0.726 | 2 | 0.729 | 1 |
| 0110 |  |  | 0.617 | 1 | 0.617 | 10 | 0.617 | 78 | 0.617 | 555 | 0.617 | 3805 |
| 0101 |  |  | 0.592 | 1 | 0.592 | 9 | 0.612 | 1 | 0.612 | 9 | 0.617 | 34 |
| 0011 |  |  | 0.361 | 1 | 0.361 | 9 | 0.586 | 2 | 0.600 | 2 | 0.617 | 8 |
| 1110 |  |  | - |  | 0.617 | 2 | 0.617 | 24 | 0.617 | 215 | 0.617 | 1720 |
| 1101 |  |  | - |  | 0.592 | 2 | 0.592 | 24 | 0.617 | 12 | 0.617 | 252 |
| 1011 |  |  | - |  | 0.532 | 1 | 0.532 | 1 | 0.684 | 1 | 0.690 | 1 |
| 0111 |  |  | - |  | 0.586 | 1 | 0.600 | 1 | 0.617 | 4 | 0.617 | 84 |
| 1111 |  |  | - |  | - |  | 0.617 | 4 | 0.617 | 72 | 0.617 | 906 |

**Table 7. Maximum Probability Using EPM**

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Starting Genotype | 1 Step | # | 2 Step | # | 3 Step | # | 4 Step | # | 5 Step | # | 6 Step | # |
| 1000 | 1.0 | 1 | 1.0 | 3 | 1.0 | 7 | 1.0 | 15 | 1.0 | 31 | 1.0 | 63 |
| 0100 | 1/3 | 1 | 1/3 | 6 | 1/3 | 39 | 3/8 | 1 | 11/24 | 1 | 11/24 | 9 |
| 0010 | 1/2 | 1 | 1/2 | 4 | 1/2 | 6 | 1/2 | 8 | 1/2 | 10 | 1/2 | 12 |
| 0001 | 1/2 | 1 | 1/2 | 1 | 2/3 | 4 | 2/3 | 8 | 2/3 | 14 | 2/3 | 24 |
| 1100 |  |  | 1/3 | 27 | 7/18 | 1 | 7/18 | 1 | 7/18 | 4 | 11/24 | 5 |
| 1010 |  |  | 1/2 | 3 | 1/2 | 19 | 7/12 | 1 | 7/12 | 8 | 169/288 | 1 |
| 1001 |  |  | 2/3 | 2 | 2/3 | 4 | 2/3 | 7 | 2/3 | 12 | 149/216 | 1 |
| 0110 |  |  | 1/3 | 1 | 1/3 | 10 | 1/3 | 81 | 3/8 | 1 | 11/24 | 1 |
| 0101 |  |  | 7/24 | 1 | 3/8 | 1 | 11/24 | 1 | 11/24 | 4 | 25/54 | 1 |
| 0011 |  |  | 1/4 | 4 | 1/4 | 32 | 1/2 | 2 | 1/2 | 18 | 1/2 | 133 |
| 1110 |  |  | - |  | 1/3 | 2 | 1/3 | 24 | 1/3 | 221 | 3/8 | 6 |
| 1101 |  |  | - |  | 7/24 | 2 | 3/8 | 2 | 11/24 | 2 | 11/24 | 14 |
| 1011 |  |  | - |  | 1/3 | 3 | 1/3 | 8 | 7/18 | 1 | 5/12 | 1 |
| 0111 |  |  | - |  | 4/27 | 1 | 19/96 | 8 | 1/3 | 4 | 3/8 | 6 |
| 1111 |  |  | - |  | - |  | 1/3 | 4 | 3/8 | 4 | 11/24 | 4 |

**Table 8. CPM substitutions and antibiotics from optimal 6 step treatment plans (\*Maximum probability for path)**

|  |  |  |  |
| --- | --- | --- | --- |
| **Mutations** | **Drugs associated with substitutions in optimal paths (probability)** | **Reversions** | **Drugs associated with substitutions in optimal paths (probability)** |
| 0000-1000 | CTT(0.38\*) | 1111-1110 | CEC(1.0\*), CAZ(0.74), CTT(0.29), CPR(1.0\*), TZP(0.15) |
| 0000-0100 |  | 1111-1101 | AM(1.0\*), AMC(1.0\*), CAZ(0.26), TZP(0.85) |
| 0000-0010 |  | 1111-1011 |  |
| 0000-0001 |  | 1111-0111 | ZOX(1.0\*), CXM(1.0\*) |
| 1000-1100 |  | 1110-1100 | TZP(0.49\*) |
| 1000-1010 |  | 1110-1010 | AM(0.10), CRO(0.47\*), CPD(0.28), FEP(0.28) |
| 1000-1001 |  | 1110-0110 | CAZ(1.0\*), CPR(1.0\*), CPD(0.33), TZP(0.51) |
| 0100-1100 | SAM(1.0\*) | 1101-1100 |  |
| 0100-0110 | CTX(1.0\*), CPD(1.0\*) | 1101-1001 |  |
| 0100-0101 |  | 1101-0101 |  |
| 0010-1010 | CTT(0.22) | 1011-1010 | TZP(0.30) |
| 0010-0110 |  | 1011-1001 | TZP(0.92\*) |
| 0010-0011 |  | 1011-0011 | TZP(0.18) |
| 0001-1001 | AM(1.0\*), CTT(0.47), SAM(1.0\*) | 0111-0110 |  |
| 0001-0101 |  | 0111-0101 |  |
| 0001-0011 |  | 0111-0011 |  |
| 1100-1110 | CAZ(0.85\*), SAM(0.046), FEP(0.32), | 1100-1000 | CTT(0.25) |
| 1100-1101 | AMP(1.0\*),CAZ(0.15), SAM(0.95), FEP(0.68) | 1100-0100 | CTX(1.0\*), ZOX(1.0\*), CXM(1.0\*) |
| 1010-1110 | CEC(1.0\*), CTT(0.47) | 1010-1000 | CTT(0.53\*), TZP(0.49) |
| 1010-1011 |  | 1010-0010 | ZOX(1.0\*), TZP(0.43) |
| 1001-1101 |  | 1001-1000 | CTX(0.42), CTT(0.56) |
| 1001-1011 | CTX(0.50\*) | 1001-0001 |  |
| 0110-1110 | FEP(1.0\*) | 0110-0100 | CXM(0.58), TZP(1.0\*) |
| 0110-0111 | ZOX(1.0\*), CXM(0.94), CPD(1.0\*) | 0110-0010 |  |
| 0101-1101 | AMP(1.0\*), FEP(1.0\*) | 0101-0100 | CTX(0.42), CXM(0.41), CPD(0.15) |
| 0101-0111 | CTX(0.58), ZOX(1.0\*), CXM(0.59), CPD(0.85) | 0101-0001 |  |
| 0011-1011 | CTT(0.04) | 0011-0010 | CTT(0.33), TZP(0.45) |
| 0011-0111 | ZOX(1.0\*), CPD(1.0\*) | 0011-0001 | CTT(0.20), TZP(0.55) |
| 1110-1111 | AM(0.90), CRO(0.53), SAM(1.0\*), CPD(0.39), FEP(0.72) | 1000-0000 | CPR(1.0\*) |
| 1101-1111 | AMP(1.0\*), SAM(1.0\*), FEP(1.0\*) | 0100-0000 | AM(0.62\*) |
| 1011-1111 | TZP(0.03) | 0010-0000 | TZP(0.71\*) |
| 0111-1111 | CPD(1.0\*) | 0001-0000 | CTT(0.092), CPR(0.14) |

**Table 9. EPM substitutions and antibiotics from optimal 6 step treatment plans**

|  |  |  |  |
| --- | --- | --- | --- |
| **Mutations** | **β-lactams associated with substitutions in optimal paths (probability)** | **Reversions** | **β-lactams associated with substitutions in optimal paths (probability)** |
| 0000-1000 |  | 1111-1110 | CTT(1/3) |
| 0000-0100 |  | 1111-1101 | AM(1.0\*) , AMC(1.0\*) |
| 0000-0010 |  | 1111-1011 |  |
| 0000-0001 |  | 1111-0111 |  |
| 1000-1100 |  | 1110-1100 | TZP(1/2\*) |
| 1000-1010 |  | 1110-1010 |  |
| 1000-1001 |  | 1110-0110 | CAZ(1.0\*), CPR(1.0\*), TZP(1/2) |
| 0100-1100 | SAM(1.0\*) | 1101-1100 |  |
| 0100-0110 |  | 1101-1001 | CPR(1/3\*) |
| 0100-0101 | TZP(1.0\*) | 1101-0101 | CAZ(1.0\*), TZP(1.0\*) |
| 0010-1010 |  | 1011-1010 | CTT(1/3\*) |
| 0010-0110 |  | 1011-1001 | AM(1/2\*), CTT(1/3) |
| 0010-0011 |  | 1011-0011 |  |
| 0001-1001 | AM(1.0\*), SAM(1.0\*) | 0111-0110 |  |
| 0001-0101 | TZP(1.0\*) | 0111-0101 | SAM(1/2\*) |
| 0001-0011 |  | 0111-0011 |  |
| 1100-1110 | CTT(1/4) | 1100-1000 | CTT(1/4), CPR(1/4), TZP(1/3\*) |
| 1100-1101 | AMP(1.0\*), CPR(1/4) | 1100-0100 | CTX(1.0\*), ZOX(1.0\*), CXM(1.0\*) |
| 1010-1110 | CTT(1/2) | 1010-1000 | CTT(1/2\*), TZP(1/3) |
| 1010-1011 |  | 1010-0010 |  |
| 1001-1101 |  | 1001-1000 | CEC(1/2\*), CTX(1/2\*), CTT(1/2\*), CPR(1/2\*), TZP(1/3) |
| 1001-1011 | CTX(1/2\*) | 1001-0001 | CEC(1/2\*), CPR(1/2\*) |
| 0110-1110 | CTT(1/3), | 0110-0100 | TZP(1.0\*) |
| 0110-0111 |  | 0110-0010 |  |
| 0101-1101 | AM(1/2), AMC(1/2) | 0101-0100 | CEC(1/2\*), AMC(1/2\*) |
| 0101-0111 |  | 0101-0001 | AM(1/2\*), CEC(1/2\*) |
| 0011-1011 | AMC(1/2\*) | 0011-0010 |  |
| 0011-0111 |  | 0011-0001 | AMC(1/2\*) |
| 1110-1111 | SAM(1.0\*) | 1000-0000 | CPR(1.0\*) |
| 1101-1111 |  | 0100-0000 | FEP(1/4) |
| 1011-1111 | CTT(1/3) | 0010-0000 | SAM(1/2\*), TZP(1/2\*) |
| 0111-1111 | SAM(1/2), CPD(1.0\*) | 0001-0000 | CEC(1/2\*), CPR(1/3), FEP(1/3) |

**Figure 1 AMP: Ampicillin 256 µg/ml**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:AMP8X:AMP8X256Landscape copy.pdf**

**Figure 2 AM: Amoxicillin 512 µg/mlMacintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:AM:AM512Landscape copy.pdf**

**Figure 3 CEC: Cefaclor 1 µg/ml**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:CEC:CEC1Landscape copy.pdf**

**Figure 4 CTX: Cefotaxime 0.05 µg/ml**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:CTX:CTX0.05Landscape copy.pdf**

**Figure 5 ZOX: Ceftizoxime 0.03 µg/mlMacintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:ZOX:ZOX0.03Landscape copy.pdf**

**Figure 6 CXM: Cefuroxime 1.5 µg/mlMacintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:CXM:CXM1.5Landscape copy.pdf**

**Figure 7 CRO: Ceftriaxone 0.045 µg/mlMacintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:CRO:CRO0.045Landscape copy.pdf**

**Figure 8 AMC: Amoxicillin/Clavulanate 512 µg/ml and 8µg/ml**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:AMC:AMC512.8Landscape.pdf**

**Figure 9 CAZ: Cefazidime 0.1 µg/ml**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:CAZ:CAZ.1Landscape copy.pdf**

**Figure 10 CTT: Cefotetan 0.312 µg/ml**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:CTT:CTT0.0312Landscape copy.pdf**

**Figure 11 SAM: Ampicillin/Sulbactam 8 µg/ml and 8µg/mlMacintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:SAM:SAM8.8Landscape copy.pdf**

**Figure 12 CPR: Cefprozil 100 µg/ml**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:CPR:CPR10Landscape copy.pdf**

**Figure 13 CPD: Cefpodoxime 2 µg/ml**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:CPD:CPD2Landscape.pdf**

**Figure 14 TZP: Pipercillin / Tazobactam 8.12µg/ml and 8 µg.ml**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:TZP:TZP8.128Landscape copy.pdf**

**Figure 15 FEP: Cefepime 0.0156µg/ml**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):Landscapes:FEP:FEP0.0156Landscape.pdf**

**Figure 16: Summary of Highest CPM probabilities**

**Macintosh HD:Users:mirasimac:Dropbox:The Geometry of AR:ManuscriptFigures:NEWMUT.HIGHPROB051214.pdf**

**Macintosh HD:Users:mirasimac:Dropbox:The Geometry of AR:ManuscriptFigures:REVPROB051214.pdf**

**Figure 17. Summary of Optimal Six Step Sequences (EPM and CPM)**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):ManuscriptFigures:6CycleM1M2.pdf**

**Figure 18. Summary of 2, 4, and 6 Step CPM Antibiotic Cycles**

**Macintosh HD:Users:miriambarlow:Dropbox:The Geometry of AR (1):ManuscriptFigures:CyclingOut.pdf**

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