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Concentration-dependent selection of cefotaxime resistant E. coli – A pharmacodynamic model

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Abstract

Bacteria with an increased propensity to develop resistance to antimicrobial drugs has today become an alarming health problem.

When a bacterial population consisting of sensitive and resistant bacteria are treated with antibiotics, the sensitive are inhibited while the resistant population can still reproduce. The developed phenomena, is called selection of resistant bacteria.

The basis of this paper is an in vitro study with two different strains of E.coli with different levels of responsiveness to antibiotics. By varying the dose and elimination rate for an antimicrobial drug, cefotaxime, different lengths of the selective window were obtained. The selective window is the time period where there is a bactericidal effect of the sensitive strain but a growth of the resistant strain. The aim was to study how the selection of the resistant strain depended on the length of the selective window.

In this thesis a pharmacodynamic model for the growth of bacteria is constructed. Parameters in the model were estimated to fit real data by the Maximum-Likelihood method. The purpose was to get an increased understanding of when selection of resistant strains occurs.

The result of the estimated model shows selection of the same strain as real data, which indicates that the model describes the pharmacodynamic factors rather well. However, the deviation from experimental data in relation to the number of bacteria seem to increase with the length of the selective window.

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During the work of this project, we discovered that the wild-type strains sometimes mutated to more resistant bacteria. In the model, the selection of the mutated strain seem to increase with the length of the selective window. An explanation to the increased deviation from experimental data with the length of the window could be that some factors which were assumed to be equal for both the wild-type and the mutated bacteria in reality differ.

Acknowledgements

First of all, I would like to thank my tutor Mikael Andersson, who with great pedagogics and encouragement guided me through the statistical theory of this project. A big thanks to my external tutor Otto Cars, who gave the idea for this project and also supplied me with the medical theory, and whose enthusiasm and motive power forced the project forward. A warm thanks to Sara Olofsson, who provided me with experimental data and without whom this project would never have seen the light of day.

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March 11, 2003

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Chapter 1

Introduction

Antibiotics have been used for more than 50 years to fight infections caused by bacteria. After their discovery, many believed that this was the final cure for infectious diseases. However, within a few years bacteria controlled by antibiotics had developed resistance to these drugs.

Today, antibiotic resistance has become one of the world's most alarming health problems. Although antibiotics have been used for so many years, the knowledge about optimal dosing regimens to optimize efficacy and minimize costs, toxicity and resistance, is still incomplete.

In this paper, we will concentrate on the last mentioned factor, namely the optimal dosing to prevent emergence of resistance.

A statistical model, which hopefully could be used to predict when selection of resistant variants occurs will be constructed.

This introduction provides a more detailed presentation of the background to this thesis, which also explains common medical terminology which will be used. Furthermore, this is followed by a presentation of the purpose and disposition of this paper.

1.1 Background

It is known that different dosing-regimens of antibiotics may vary in selection of resistant bacteria. But the nature of variation with different dosing regimens, is unknown.

The background to this paper is an experimental setting by a group at the Antibiotic Reasearch Unit - Departments of Clinical Bacteriology and Infectious Diseases at Uppsala University [13], who have studied a concentration-dependent selection of cefotaxime resistant strains of E.coli-bacteria with an in vitro kinetic model.

The following subsections will provide some elementary information of the bac-

terium E.coli and the antiinfective drug cefotaxime that were used in the study. Also included is an explanation of what we mean by selection and the terms pharmacodynamics and kinetics.

1.1.1 Cefotaxime resistant E.coli

E.coli, or Escherichia coli, is the most common aerobic bacterium in the normal intestinal flora and the most common bacterium causing infections of the urinary tract. It can also cause other extraintestinal infections.

This group of bacteria can produce different enzymes - β-lactamases - which are able to degrade (to a variable extent) so called β-lactam antibiotics like cefotaxime.

1.1.2 Development and selection of resistant bacteria

Some bacteria are naturally resistant to antibiotics, which could be a result of an missing enzyme. Those who are not naturally resistant to antibiotics, can develop resistance in many ways. There are two principal ways for bacteria to become resistant.

- Chromosomal mutation
- Acquisition of foreign DNA
  - plasmid
  - transformation

The most common resistance-mechanisms are,

- Production of an enzyme that degrades the antibiotic
- Reduced penetration into the bacterial cell
- Increased efflux from bacterial cell
- Changed target

Since antibiotic resistance confers a metabolic cost for the resistant bacteria, these are often inferior to normal bacteria. But when sensitive bacteria are inhibited or killed by antibiotics, the resistant bacteria are selected, and therefore able to spread.

Resistance classification

So, how do we classify bacteria as resistant or not? This is done by so called MIC-determination, (MIC=Minimum Inhibitory Concentration). With these data we can classify bacteria into different categories, due to their responsiveness

to antimicrobial drugs. These categories are "Sensitive", "Resistant" and "Intermediate". Sensitive bacteria have low MIC:s and can successfully be treated with antibiotics. Resistant bacteria represent a category of strains that are not inhibited by usually achievable concentrations of antibiotics. The third category represents an overlapping zone between those classified as either sensitive or resistant.

1.1.3 Pharmacokinetics and Pharmacodynamics

The study was done with an in vitro kinetic model. This kind of construction allows simulations of human pharmacokinetics.

Pharmacokinetics attempts to describe the processes involved in the absorption, distribution and elimination of the drug in the body.

By adjusting the half-life time in the in vitro kinetic model, human pharmacokinetics were able to be mimicked. The question of interest in the study was how the pharmacokinetic and pharmacodynamic factors contribute to the emergence of resistance.

While pharmacokinetics attempts to describe [1] what "the body does to the drug", pharmacodynamics attempts to describe what "the drug does to the body". In this case, pharmacodynamics means how bacteria in the body are affected by different concentrations of antibiotics and by different lapse of concentrations.

1.2 Disposition

This introduction is followed by Chapter 2 describing the experimental methods and the results of the experiments. Chapter 3 describes the statistical modelling and it also includes more detailed information about factors that may eventually affect the model. This is followed by Chapter 4, which provides methods for estimating the parameters in the model. In Chapter 5, a summary and discussion of the results is given. Lastly, the conclusions are presented in Chapter 6.

1.3 Purpose

The question of medical interest was to find out when the selection of resistant bacteria, due to dosing-regimens, occurs. So, the main purpose of this thesis was to propose a statistical model for the pharmacodynamics behind these experiments and to design further experiments to fulfil this purpose. It was also intended to estimate parameters from data, to eventually get a practical use of it, e.g. avoiding dosing regimens that could result in selection and the emergence of resistance.

Chapter 2

Experiment, methods and source data

This chapter will provide a more detailed description of how the experiments were done and present the source data that have been the basis to this paper. It will also explain some of the further experimental designs.

2.1 The in vitro kinetic model

All experiments were done with an in vitro kinetic model with two different E.coli-strains. These strains, TEM-1 and TEM-12 respectively, contain a plasmid with genes that controls the production of the β-lactamase. The MICs of cefotaxime were 0.012 and 0.032 mg/L for TEM-1 and TEM-12 strains, respectively. This means that TEM-12 was more resistant to exposure of cefotaxime than TEM-1. Therefore TEM-1 and TEM-12 will from now on be referred to as the sensitive and the resistant strain, respectively.

The populations were kept in a closed system and exposed to different concentrations and elimination rates of cefotaxime for 24 hours. See figure 2.1 for an illustration of the model, where the bottle to the left shows the closed system with bacteria, and the other contains cefotaxime and nutrition. There is also one bottle, not shown in the figure, containing rest-products.

The system contained 110 ml of solution, which in the original experiment was mixed with 10^5 and 10^3 $bacteria/ml$ respectively, of each strain. The nutritional resources were unlimited.

During the experiment, samples of 1 ml each, were withdrawn at different timepoints. After 24 hours of incubation on agar-plates, the number of colonies per ml were counted to study the development of the populations, after being exposed to antibiotics. The populations were distinguishable by their different colours.

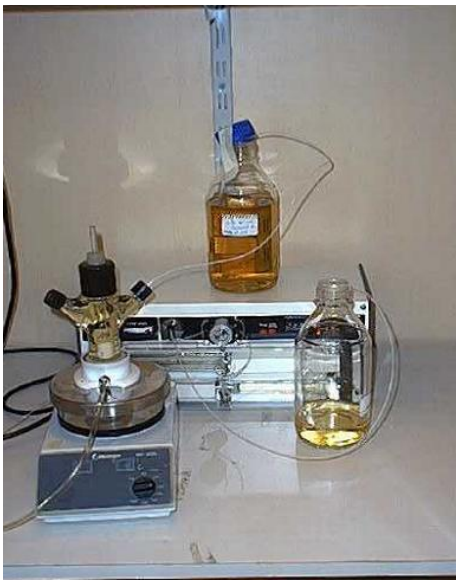

Figure 2.1: Experimental arrangement

2.2 Source data

The source data for this paper were obtained from an experimental setting where the length of the selective window was changed to investigate how this parameter influenced the grade selection of the resistant strain. Selective window, (SW) is the time period where concentration lies above MIC for the sensitive strain and below MIC for the resistant strain. That means that in this window there is continued bactericidal effect of the sensitive strain but a growth of the resistant strain.

The hypothesis was that the selection would be proportional to the length of SW. Later, it will be shown that this was not the case.

The graphs in figure 2.2 shows how the selection varied with the length of SW.

As we can see in the graphs at 24 hours, the resistant strain is the one that is selected for selective windows $1, 2, 4, 8$ hours. For the selective window 12 hours the sensitive strain was selected.

The reason for this was unknown, but could be a result of many factors. So, further experiments had to be designed to investigate possible reasons for this result. The next section will describe some of the experiments designed to investigate this.

2.2.1 Restraints of the data

This method for counting bacteria was not feasible to count numbers below 10.

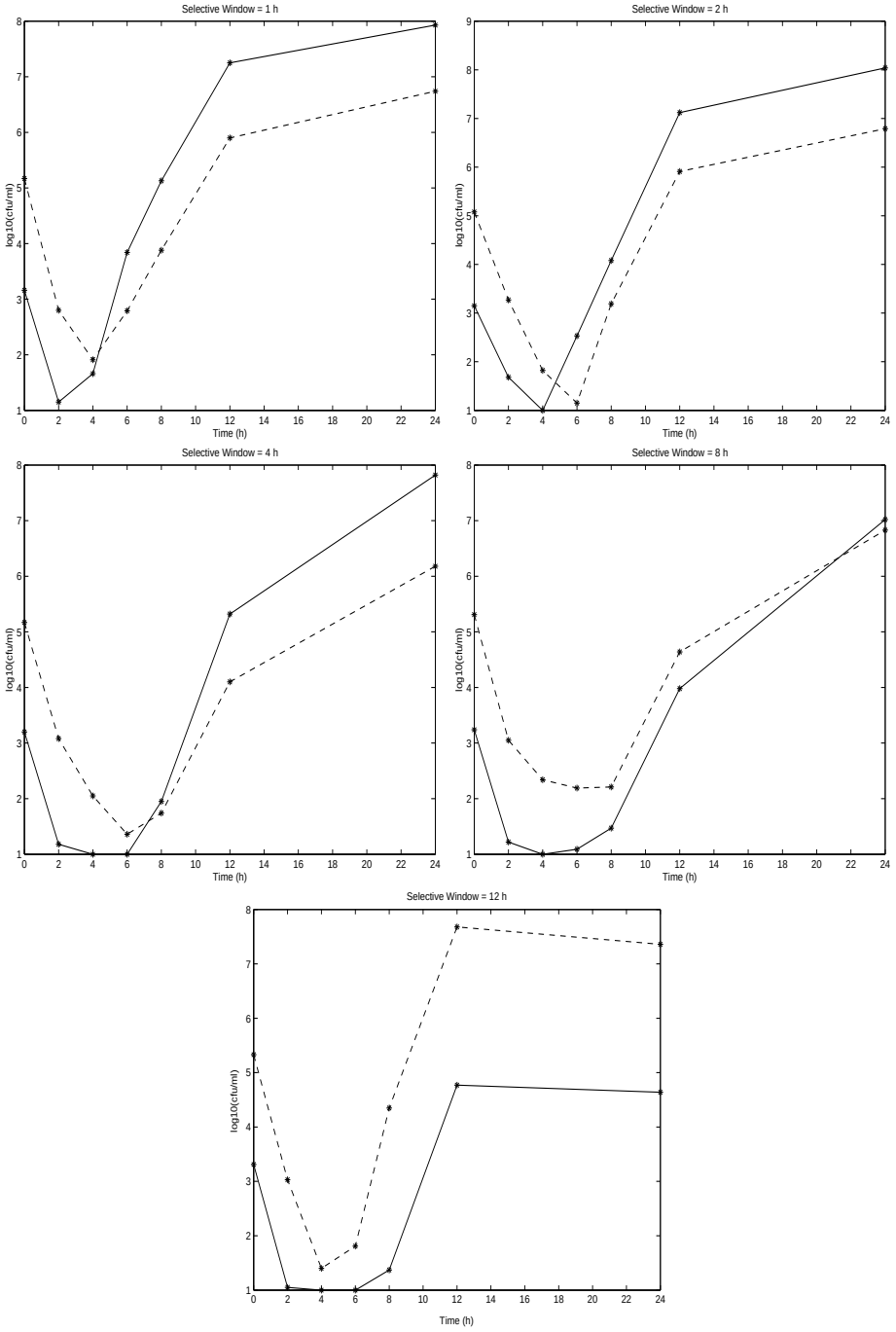

Figure 2.2: Different lengths of Selective Window, - - = Sensitive strain, – = Resistant strain

2.3 Experimental design for more data

Several factors may have been the reason for the result presented in the previous section. To be able to model the relationships between the pharmacokinetic and pharmacodynamic factors, further experiments were done. Some of the results from these experiments which have been of importance for the modelling will be presented in this section.

To investigate whether there were any differences between the strains except from the MIC, experiments with the two strains in separate settings were done under the same conditions. That means, with the same inoculum, half-life time and concentration above MIC. The result showed almost exactly the same growth curve for the both strains. So, the only difference between those two strains seemed to be the MIC.

In two other experiments with the strains in separate settings, where the time above MIC was kept constant, but the concentration and half-life time in the experiments were different, an interesting result was observed. In these two experiments, which are shown in figure 2.3 and 2.4, the time above MIC was 10 hours, which means that we expect the bacteria to start growing at this time-point.

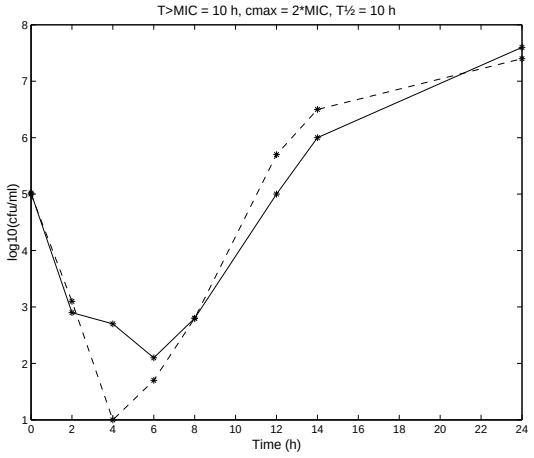

Figure 2.3: Experiment with low concentration and long half-life time, $T > MIC = 10$ h

In figure 2.3, the bacteria start to grow a couple of hours before the concentration has declined to the MIC and in figure 2.4 the bacterial growth seems to be a little delayed.

Mutations were not believed to be a factor for E.coli strains, but because the bacteria in figure 2.3 seem to be less inhibited than normally, an investigation of genetic changes were done.

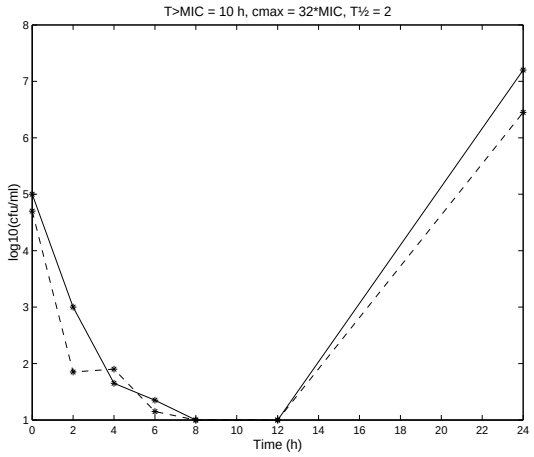

Figure 2.4: Experiment with high concentration and short half-life time, $T > MIC = 10$ h

2.3.1 Investigation of genetic changes

To investigate whether any genetic changes had occurred, MIC was tested during and after the experiments, by a method called E-test.

E-tests

An E-test consists of a plastic indicator with an antibiotic gradient, which is put directly on an indicator plate on which bacterial solution is spread. After 24 hours of incubation, the indicator shows which concentration was the minimum required to inhibit reproduction.

Result

E-test for the experiments in figure 2.3 and 2.4 showed that there was an increased MIC at 24 hours for the experiment in figure 2.4 but not for 2.3. The MIC value for the resistant strain increased from 0.032 mg/L to 0.50 mg/L and for the sensitive strain from 0.012 to 0.19 mg/L.

An interesting question was also whether mutations had occurred during the experiments for the source data. E-test for experiment with "SW=2", showed an increase of MIC for the sensitive strain, but not for the resistant strain, which eventually could be explained by the lower inoculum. The MIC value for the sensitive strain at 6 h was 0.19 mg/L, but at 24 h the MIC was 0.008 mg/L, which is lower than the initial value. This can eventually be explained as a result of a plasmid loss.

How these genetic changes influence the pharmacodynamic will be discussed in the next chapter.

Because we unexpectedly found out that these strains mutated, further inves-

tigations were done to find out whether there where any possible interactions between the strains.

Notation

At the time for writing this report, MIC-determination for the other selective windows of the source experiments were not available.

2.3.2 Investigation of possible interaction

For some kinds of bacteria, transmission of DNA can occur by interaction between two strains. The sensitive strain is the recipient of DNA transmitted by the resistant strain.

This interaction was not believed to be very plausible, but was thoroughly investigated by repeating experiments with both strains together and in separate settings.

Result

There was no remarkable difference between experiments with strains together and separate, which it would have been if there were some kind of interaction. This factor will therefore not be included in the following modelling.

Chapter 3

The Model

Mathematical modelling is an important tool in many branches of science, not only in biology. As mentioned before, we will try to use this tool to explain which and how certain parameters affect the pharmacodynamics in the experiments behind this work.

For the modelling, we have to know more about factors that eventually affect the pharmacodynamics of the strains. This chapter will therefore not only explain the mathematical theory, but also give some medical information. Also a more detailed description about how the experiments were done to investigate whether, and in what way, a factor should be included or excluded in the model.

The factors will be listed one by one and included in the model. Factors such as temperature, which was kept constant during the experiments, will not be taken under consideration.

While the sizes of the bacteria populations are large, the birth or death of one bacterium will not create a significant change in the size of the population. Therefore, we will use differential equations in the construction of the mathematical models.

3.1 Factors of importance

In the previous chapter we described the experimental results of trials to investigate whether a factor should or should not be included in the model. We found that genetic changes sometimes developed. These changes will lead to a different MIC, but other factors of importance will be the same, independent of these changes. The pharmacokinetic and pharmacodynamic factors that will be important for the modelling are listed below.

- Pharmacokinetic factors
  - Initial concentration of antibiotics, c_{max}
  - Half-life time of antibiotics, $T_{1/2}$

- Pharmacodynamic factors
  - Growth rate without antibiotics, λ_{max}
  - Minimal growth rate under pressure of antibiotics, λ_{min}
  - Growth rate under pressure of antibiotics, $\lambda(t)$
  - MIC

3.2 Pure Birth and death

A bacterium can either reproduce by splitting into two new bacteria, or die. So a very simple start with the modelling is to consider a model where λ denotes the difference between the birth and the death rate, that is the relative reproduction rate at which the population change. Then we can calculate

$$\frac{dN(t)}{dt} = \lambda \cdot N(t), \tag{3.1}$$

where $N(t)$ is the population size at time t. The population is representative of bacteria either of the sensitive strain S, or the resistant, R.

The reality is much more complicated, and we will therefore add some elements to the model. This equation will nevertheless be useful in the next chapter when estimating pure maximal and minimal growth rate.

3.3 Antibiotic pressure

So far, we have looked at a model without any antibiotic pressure. Let us see how the model complicates when we include this factor.

Let c_{max} denote the dose of antibiotics introduced in the system at time $t = 0$. The half-life time, $T_{1/2}$, is known and the exponential decrease of antibiotics will therefore be described by the following mathematical expression

$$c(t) = c_{max} \cdot 2^{-\frac{t}{T_{1/2}}}. \tag{3.2}$$

The reproduction-rate λ will now be replaced by $\lambda(t)$, which varies depending on the concentration of antibiotics.

3.4 Postantibiotic Effect and Post MIC Effect

In some of the experiments, we can observe that bacteria do not start growing directly after the time when concentration has declined below the MIC. This persistent suppressive effect of exposure to antibiotics is a phenomenon that we in this paper will call Post MIC Effect (PME).

Normally, [12] PME is defined as a combination of Postantibiotic Effects (PAE) and Postantibiotic sub-MIC Effect (PASME). That means the total time-lag

for bacteria to resume normal growth in a drug-free medium, after exposure to static concentrations of antibiotics above MIC (PAE) and sub-MIC levels (PASME). We will in this paper use the term PME for the persistent effects of exponentially decreasing concentration of antibiotics.

Studies [1] have shown that there is a relationship between the duration of PAE and the area under the concentration curve (AUC) (i.e. concentration times duration of exposure). We will in this paper make the assumption that PME also depends on AUC, but with a reduced effect due to the declining concentration.

3.4.1 Resynthesis of proteins and enzymes

The knowledge of the mechanisms behind the PAE-phenomenon is still incomplete. Many theories have been proposed. One theory, which may be valid for β-lactam antibiotics, is based on the mechanisms of Penicillin Binding Proteins, (PBP). PBP are enzymes needed for synthesis of the bacterial cell wall.

β-lactam antibiotics are bound to these enzymes, and the theory [3] is that PAE could correspond to the time it takes for the bacteria to rebuild new PBP. This theory will serve as the basis for the further modelling of the PME.

Let us assume that the time required for bacteria to resume normal growth rate is dependent on the time it takes for the PBP to resynthesize. A low initial concentration and a short half-life time will result in a shorter PME than for higher values of these factors.

So, let $P(t)$ denote the number of unsaturated PBP at time t and assume that the initial number of these are P_{max} which also is the maximum number of unsaturated PBP. Since P_{max} is unknown and to avoid making any assumptions about this number, we will look at the relative number, $Q(t)$ instead. That means that $Q(t) = \frac{P(t)}{P_{max}}$.

Furthermore, let us assume that bacteria creates new PBP at a constant rate β and that the PBP are saturated proportional to the concentration lapse and a saturation parameter γ. Then, the change in unsaturated PBP will be given by

$$\frac{dQ}{dt} = \beta - \gamma \cdot c(t) \cdot Q(t). \tag{3.3}$$

An illustration of the process of saturation and resynthesis of new PBP is shown in figure 3.1.

We will now assume that the relative growth rate of bacteria is proportionally dependent on the time it takes bacteria to reach a critical number of unsaturated PBP required for bacterial growth. That means,

$$\lambda(t) = \nu \cdot Q(t) + \lambda_{min}. \tag{3.4}$$

The change in number of bacteria can now be written as

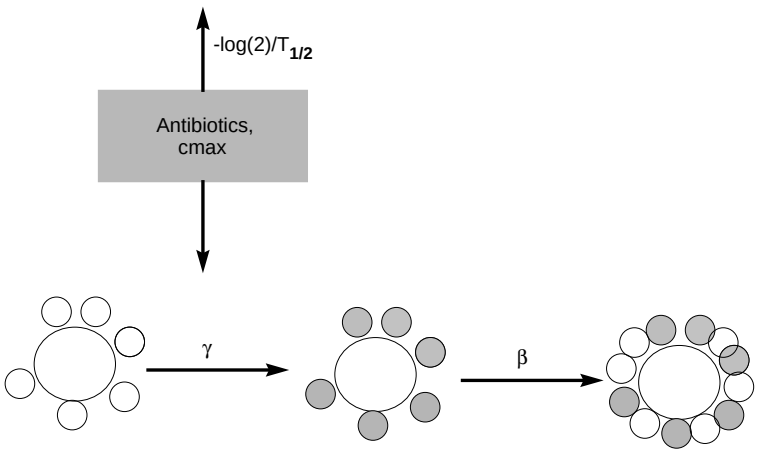

Figure 3.1: Illustration of saturation and resynthesis of PBP

$$\frac{dN}{dt} = \lambda(t) \cdot N(t). \tag{3.5}$$

Equation 3.4 inserted in 3.5 gives

$$\frac{dN}{dt} = (\nu \cdot Q(t) + \lambda_{min}) \cdot N(t). \tag{3.6}$$

This means that the PME will be dependent on how long time it takes for PBP to reach the critical number, $Q_C = \{Q(t_C) : \lambda(t_C) = 0\}$. More precisely, PME in this model, is the difference between time above MIC and the time it takes for PBP to reach the critical number. What we have to think about is that different methods for MIC-determination can give very different results of the MIC-data (almost the double MIC-value with broth cultures compared to the result with E-test), which means that the PME will depend on which method we have used for the MIC-determination.

The graphs in figure 3.2 aims to give a picture of the development of PBP for different concentration-lapses. The first figure shows how PME varies with increasing initial concentrations and a fix half-life time. The second figure shows PME for increasing half-life time and a fixed initial concentration.

As we can see in the figures, PME increases with higher initial concentration of antibiotics and we can also see that we have the same relation between PME and half-life time.

When dosing antibiotics in reality, the aim is to have concentrations above MIC as long as possible. Many experiments in this area, have therefore been done with "time above MIC" as an explanatory variable. The time above MIC is obtained by measuring different combinations of initial concentration and half-life time.

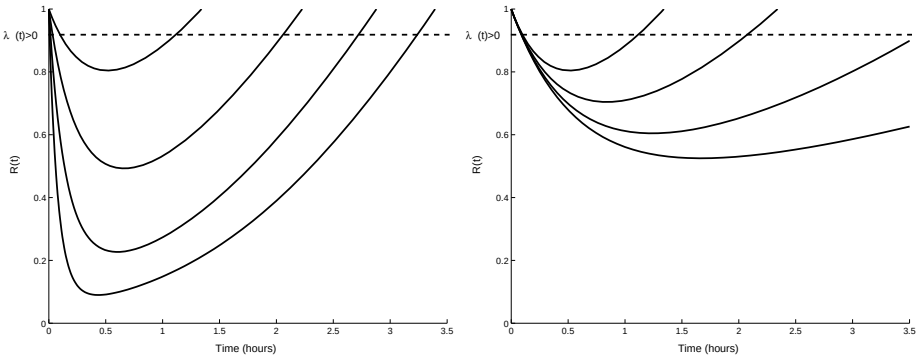

Figure 3.2: PME for different concentration-lapse

Let us look at PME in an example where we increase the time above MIC in three cases. We will look at the three cases when $T > MIC = 2, 6$ and 10 hours. The result of these concentration-lapses is shown in picture 4.1.

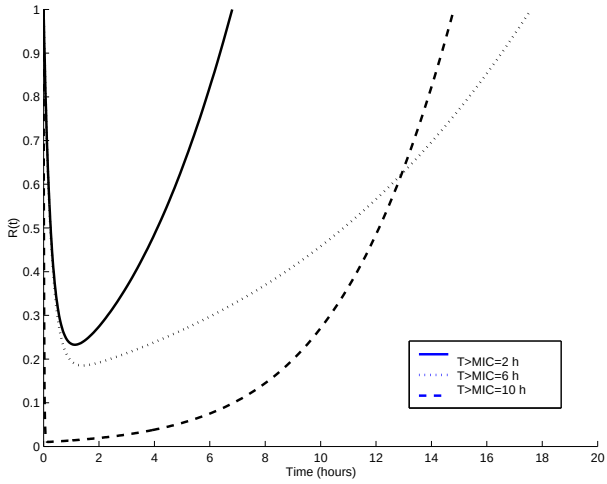

Figure 3.3: PME for different concentration-lapse

The reason for the decreased PME, though a higher time above MIC, is that the initial concentration influences the development of PBP in a different way than half-life time.

3.5 Genetic changes

There are sometimes genetic changes such as mutation and plasmid loss in the bacteria. These were both observed in some of the experiments behind this work. These kind of changes lead to an increase or decrease in MIC, which in turn leads to a different model.

3.5.1 Mutation

Bacteria under exposure of antibiotics, may result in mutations that develop to increase the chances for survival.

In the experiment with selective window 2 hours, which was the only experiment of source data where MIC were determined, a significant increase of MIC was observed. This indicates that mutations had developed. We will therefore include mutation as a factor in the model.

There is no possibility of seeing any morphological difference between the mutated bacteria and the wild-type. This means that the bacterial counts represent the sum of mutated bacteria and the original, if there have been any mutations. If concentrations are within the selective window, we will have an increase of resistant bacteria and a decrease of sensitive bacteria. The development of the total population, that means the number that we are counting, will develop as illustrated in figure 3.4, where the first graph illustrates the development of the sensitive strain in the selective window, the graph to the right illustrates the development of the resistant strain and the last graph shows the sum of these two strains.

As mentioned in the previous chapter, MIC-data for further analysis of mutations were not available at the moment of writing this paper. Without more data, any conclusions whether the mutation-rate is constant or not, cannot be made. We will therefore assume that the mutations follow the simplest model, namely with a constant rate.

So, if we let α denote the mutation-rate, we get the following appearance of the model

$$\frac{dN}{dt} = (\nu \cdot Q_N(t) + \lambda_{min}) \cdot N(t) - \alpha \cdot N(t). \tag{3.7}$$

The mutations can then be described by the following equation,

$$\frac{dM}{dt} = (\nu \cdot Q_M(t) + \lambda_{min}) \cdot M(t) + \alpha \cdot N(t). \tag{3.8}$$

When comparing the expected number with real data, we will look at graphs that represent the sum of the solutions of equation (3.7) and (3.8). That is

$$X(t) = N(t) + M(t). \tag{3.9}$$

3.5.2 Plasmid loss

Antibiotic resistance often confers a biological fitness cost to the bacteria. If the resistance gene is not "needed" by the bacteria, it may be lost. Hence very low concentrations of drugs, may lead to a plasmid loss. This plasmid loss changes the bacteria back to the original variant, without any resistance mechanism.

While the plasmid loss seems to occur when concentrations of antibiotics are extremely low, the effects of a lower MIC are assumed not to make any remarkable

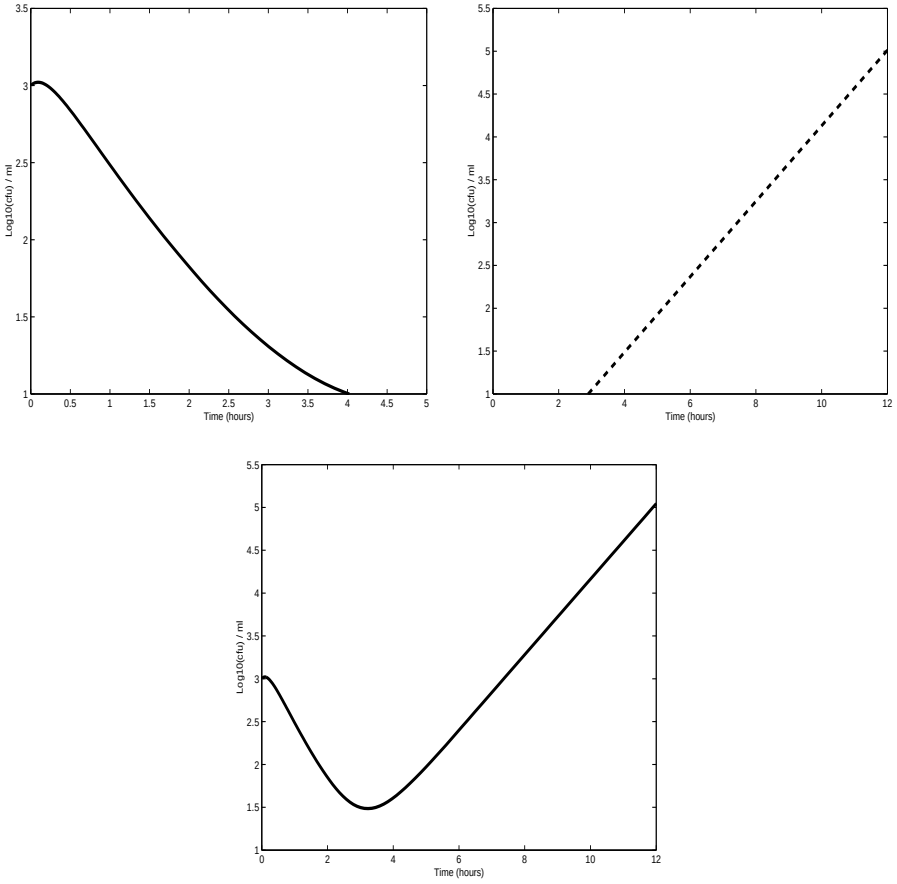

Figure 3.4: Development of the sensitive, resistant and total population respectively

difference in the reproduction-rate, $\lambda(t)$.

Notation

Because the mutated bacteria loses their resistance without any pressure of antibiotics, the maximal and minimal reproduction rate were not able to estimate for these genetic variants. And because we did not see any differences between the wild-type sensitive and resistant strain, we will assume that all genetic variants in this work have the same maximal and minimal reproduction rate.

3.6 Restrictions in the model

In the figures of source data in the previous chapter, we could observe that the bacterial growth seemed to decrease after 12 hours. This could be a result of

limited space in the system.

The modelling will therefore be restricted to only include the first 12 hours, where the bacteria do not seem to be affected by this factor.

3.7 Discussion

The model seems to be adequate in a theoretical perspective. What we do not know, is whether the mutation-rate is constant, or if it is a function of the concentration. Experiments to investigate this were not available at the moment of writing this paper.

There is no explicit solution to the differential equation system. So, a numerical method will be required to solve these equations which also means that we first have to estimate the unknown parameters in the model. The estimating procedure will be described in next chapter.

Chapter 4

Estimating parameters

The model presented in the previous chapter is so far only theoretical. By estimating the parameters in the model, we could get a practical use for it. This chapter will describe the methods and the results of estimation.

4.1 Reduction of parameters

The parameters λ_{min} and λ_{max} respectively, can be estimated separately by data from experimental settings which will be presented in the next section. For the rest we will use Maximum Likelihood estimation. But we will first reduce the number of parameters by the following relations.

When PBP have reached their maximal number, bacteria resumes their normal growth rate, λ_{max}, that means

$$\begin{gathered} Q(t) = 1 \\ \Longleftrightarrow \\ \lambda(t) = \lambda_{max} \\ \Longleftrightarrow \\ \nu = \lambda_{max} - \lambda_{min} \end{gathered} \tag{4.1}$$

Let us furthermore observe which relation we get if we look at a momentary lapse of a static concentration equal to MIC. The number will then decline to a number that we denote Q_{MIC}. When we have reached this number, we have an equilibrium, where we have neither growth of PBP nor of bacteria. We get

$$\begin{gathered} \frac{dQ}{dt} = \beta - \gamma \cdot MIC \cdot Q_{MIC} = 0 \\ \Longleftrightarrow \\ Q_{MIC} = \frac{\beta}{\gamma \cdot MIC} \end{gathered} \tag{4.2}$$

and

$$\lambda(t) = \nu \cdot Q_{MIC} + \lambda_{min} = 0$$
$$\Longleftrightarrow$$
$$Q_{MIC} = -\frac{\lambda_{min}}{\nu}, \tag{4.3}$$

where ν is defined as in equation (4.1).

Now, equation (4.1), (4.2) and (4.3) gives that

$$\frac{\beta}{\gamma \cdot MIC} = -\frac{\lambda_{min}}{\lambda_{max} - \lambda_{min}}.$$

The parameter β is believed to be independent of which strain we look at, in other words independent of MIC. So, solving this equation with respect to γ gives

$$\gamma = -\beta \cdot \frac{\lambda_{max} - \lambda_{min}}{\lambda_{min} \cdot MIC}.$$

After this reduction of the number of parameters, the model can now be described by the following system of differential equations,

$$\begin{cases} \frac{dQ_N}{dt} &= \beta\Big(1 + \frac{\lambda_{max} - \lambda_{min}}{\lambda_{min} \cdot MIC_N} c_{max} 2^{-t/T_{1/2}} Q_N(t)\Big) \\ \frac{dN}{dt} &= ((\lambda_{max} - \lambda_{min})Q_N(t) + \lambda_{min})N(t) - \alpha N(t) \\ \frac{dQ_M}{dt} &= \beta\Big(1 + \frac{\lambda_{max} - \lambda_{min}}{\lambda_{min} \cdot MIC_M} c_{max} 2^{-t/T_{1/2}} Q_M(t)\Big) \\ \frac{dM}{dt} &= ((\lambda_{max} - \lambda_{min})Q_M(t) + \lambda_{min})M(t) + \alpha N(t) \end{cases} \tag{4.4}$$

Note that N and M in the equations will get an index R or S, depending on which of the two wild-type populations we want to study.

4.2 Maximal and minimal growth rate

Estimation of the maximal growth rate, λ_{max} and minimal growth rate, λ_{min}, can easily be done by solving the differential equation (3.1) which yields

$$N(t) = N(0)e^{\lambda \cdot t} \Longrightarrow \hat{\lambda} = \frac{ln\Big(\frac{N(t)}{N(0)}\Big)}{t}$$

Maximal rate is estimated from an experiment done without any exposure to antibiotics. This data is presented in Appendix A, and gives that

$$\hat{\lambda}_{max} = \frac{ln\left(\frac{10^{7.8}}{10^{4.7}}\right)}{4} = 1.8. \tag{4.5}$$

The corresponding calculations for the minimal rate is done with data from an experiment where we have an extremely high initial concentration in the system. This data can also be found in Appendix A. With these data, we get the following estimate,

$$\hat{\lambda}_{min} = \frac{ln\left(\frac{10^{1.0}}{10^{3.0}}\right)}{2} = -2.3. \tag{4.6}$$

These parameter values will be used in the following calculations for estimating the rest of the parameters.

4.3 Mutation rate, resynthesis rate and MIC

In the previous chapter, we defined $X(t)$ in equation (3.9) as a function of the unknown parameters α and β. To estimate these parameters, we need a distribution function for the number of bacteria.

Let $X(t_j)$ for $j = 0, 1, ..., 5$ denote the number of bacteria at the time-points $t_0, t_1, ..., t_5 = [0, 2, 4, 6, 8, 12]$. If each single bacteria in generation zero, X_0, produces new bacteria with a mean β and variance σ^2, the total number off offspring will depend on the size of the generation before. This yields that we can calculate the size of the j th generation by

$$X_j = \sum_{i}^{X_{j-1}} Z_i,$$

where Z_i is the number of offspring to the i th bacteria of generation $j-1$.

If generation zero consisted of one bacteria, the mean of the size of the next generation would have been equal to β, which we denoted as the mean offspring of one bacteria. Furthermore, the variance is by the theory of Branching processes [7], calculated by

$$Var(X_n) \quad = \quad \sigma^2 \beta^{n-1} \left(\frac{\beta^n - 1}{\beta - 1}\right), for \beta \neq 1 \tag{4.7}$$

In our case, we have n generations between time t_j and t_{j-1}. If each bacteria produces offspring with a mean β in each generation, we get that the mean of the number of bacteria at time t_j can be calculated by

$$E[X(t_j) \mid X(t_{j-1})] = X(t_{j-1})\beta^n = \theta(t_j), \tag{4.8}$$

Furthermore, equation 4.8 gives that

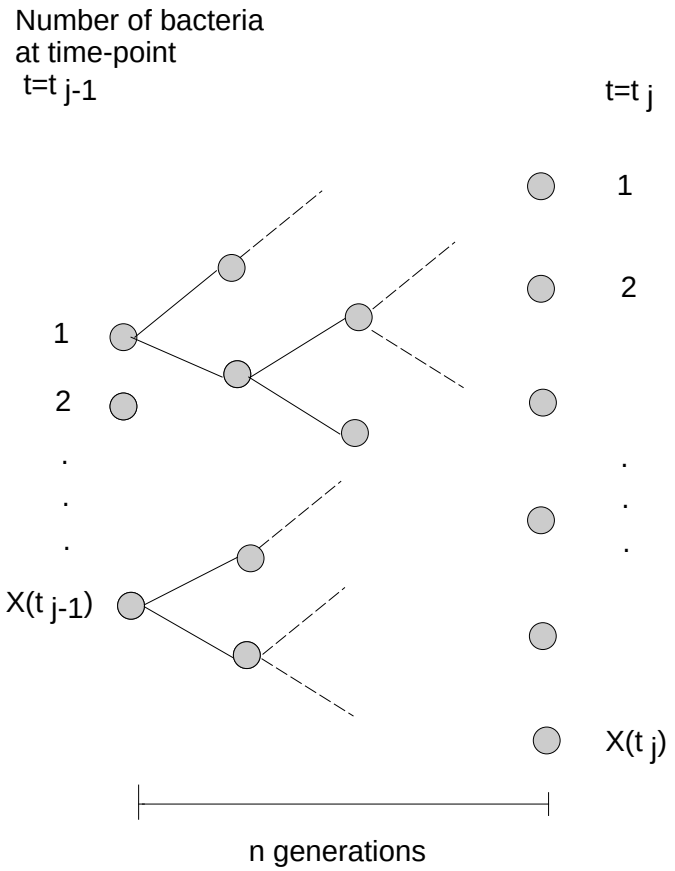

Figure 4.1: Branching process

$$\beta^{n-1} = \frac{\theta(t_j)}{\beta X(t_{j-1})},$$

which inserted in 4.7 yields that the variance in our case is calculated by

$$\begin{aligned} Var(X(t_j) \mid X(t_{j-1})) &= \frac{\theta(t_j)}{X(t_{j-1})}\sigma^2\left(\frac{\beta^n - 1}{\beta(\beta-1)}\right) \\ &\approx \frac{\theta(t_j)^2}{X(t_{j-1})}\sigma^2\left(\frac{\beta^n}{\beta(\beta-1)}\right),\ for\ \beta \neq 1 \end{aligned}$$

Now, set

$$k = \frac{\sigma^2}{\beta(\beta-1)}.$$

Then $X(t_j)$ is approximately Normal distributed with the parameters in

$$X(t_j) \mid X(t_{j-1}) \approx N\left(\theta(t_j), k\frac{\theta(t_j)^2}{X(t_{j-1})}\right) \tag{4.9}$$

In our data-set we have looked at the logarithm of the number, which we also prefer to do now. So after taking the logarithm of the number of bacteria, Gaussian approximation gives that the mean and variance now can be approximated by

$$E[log_{10}X(t_j) \mid X(t_{j-1})] \approx log_{10}\theta(t_j) \tag{4.10}$$

and

$$Var(log_{10}X(t_j) \mid X(t_{j-1})) \approx \frac{k}{log(10)^2 X(t_{j-1})}. \tag{4.11}$$

The parameters can now be estimated by the Maximum-Likelihood method.

4.3.1 Maximum Likelihood

Because data for the number of bacteria below 10 were censored, we have to compensate for this by calculating the Maximum Likelihood function with the distribution function for numbers = 10. This yields that the Maximum-Likelihood function can be calculated as

$$L(\theta(t_j)) \quad = \quad \prod_{i,j} f_{X_i(t_j)}(x_i(t_j);\theta(t_j)) \cdot F_{X_i(t_j)}(10)$$

where

$$\begin{aligned} f_{X_i(t_j)}(x_i(t_j);\theta(t_j)) \quad = \quad & \frac{log(10)}{\sqrt{2\pi k}} \cdot \sqrt{x_i(t_{j-1})} \\ & \cdot exp\left\{-\frac{(log_{10}x_i(t_j) - (log_{10}\theta(t_j))^2 \cdot x_i(t_{j-1}) \cdot log(10)^2}{2}\right\} \end{aligned}$$

and $i = 1, 2, ..., 10$, are the repetitions of the experiment. Here $X(t_j)$ denotes the conditional number.

The log-likelihood is given by

$$\begin{aligned} \ell(\theta(t_j)) \quad &= \quad log(L(\theta(t_j))) \\ &= \quad \sum_{i,j}\left(log\left(\frac{log(10)}{\sqrt{2\pi k}}\right) + \frac{1}{2}log(x_i(t_{j-1})) \right. \\ &\quad \left. - \frac{(log_{10}x_i(t_j) - (log_{10}\theta(t_j))^2 \cdot x_i(t_{j-1}) \cdot log(10)^2}{2}\right) \\ &\quad + log(F_{X_i(t_j)}(10)). \end{aligned} \tag{4.12}$$

What we have now is a maximizing problem. That means, solving the problem with respect to α and β will give us the estimated parameter values.

This problem was solved numerically by letting *Matlab 6.1* solve the differential equations defined by equation (4.4) for different values of the parameters α and β until the values of these parameters that maximizes the log-likelihood was found.

This method gave us the following estimates,

Resistant		**Sensitive**	
Parameter	Estimate	Parameter	Estimate
α_R	$1.42 \cdot 10^{-9}$	α_S	$8.28 \cdot 10^{-10}$
β_R	0.77	β_S	1.00
k_R	4589	k_S	733

As mentioned earlier, the MIC-value is dependent on which method we used for the MIC-determination, why we also let MIC be a parameter in this model. However, letting MIC be a parameter in the model, gave us the following estimates

Resistant		**Sensitive**	
Parameter	Estimate	Parameter	Estimate
MIC_{NR}	0.037	MIC_{NS}	0.0070
MIC_{MR}	0.50	MIC_{MS}	0.19

4.3.2 Error estimates

Let us introduce the vectors $\mathbf{Y} = (\hat{\alpha}, \hat{\beta}, \widehat{MIC}_N, \widehat{MIC}_M)$ and $\mu = (\alpha, \beta, MIC_N, MIC_M)$.

By the general theory of Maximum-Likelihood estimation [9] $\mathbf{Y}$ is now approximate Normal distributed with parameters as follows,

$$\begin{pmatrix} \hat{\alpha} \\ \hat{\beta} \\ \widehat{MIC}_N \\ \widehat{MIC}_M \end{pmatrix} = N\left(\begin{pmatrix} \alpha \\ \beta \\ MIC_N \\ MIC_M \end{pmatrix}, \mathbf{I}^{-1}\right),$$

where $\mathbf{I}$ is the information matrix which is defined by,

$$\mathbf{I} = -\begin{pmatrix} \frac{\partial^2 \ell}{\partial \alpha^2} & \frac{\partial^2 \ell}{\partial \alpha \partial \beta} & \frac{\partial^2 \ell}{\partial \alpha \partial MIC_N} & \frac{\partial^2 \ell}{\partial \alpha \partial MIC_M} \\ \frac{\partial^2 \ell}{\partial \alpha \partial \beta} & \frac{\partial^2 \ell}{\partial \beta^2} & \frac{\partial^2 \ell}{\partial \beta \partial MIC_N} & \frac{\partial^2 \ell}{\partial \beta \partial MIC_M} \\ \frac{\partial^2 \ell}{\partial \alpha \partial MIC_N} & \frac{\partial^2 \ell}{\partial \beta \partial MIC_N} & \frac{\partial^2 \ell}{\partial MIC_N^2} & \frac{\partial^2 \ell}{\partial MIC_N \partial MIC_M} \\ \frac{\partial^2 \ell}{\partial \alpha \partial MIC_M} & \frac{\partial^2 \ell}{\partial \beta \partial MIC_M} & \frac{\partial^2 \ell}{\partial MIC_N \partial MIC_M} & \frac{\partial^2 \ell}{\partial MIC_M^2} \end{pmatrix}.$$

Since we do not have explicit equations for these parameters, we will use the definition of derivation to calculate the elements in the information matrix. That is

$$\begin{aligned}
\frac{\partial \ell}{\partial \beta} &\approx \frac{\ell(\hat{\alpha}, \hat{\beta} + d\beta, MIC_N, MIC_M) - \ell(\hat{\alpha}, \hat{\beta}, MIC_N, MIC_M)}{d\beta} \\
\frac{\partial^2 \ell}{\partial \beta^2} &\approx \frac{\ell(\hat{\alpha}, \hat{\beta} + 2d\beta, MIC_N, MIC_M) - 2\ell(\hat{\alpha}, \hat{\beta} + d\beta, MIC_N, MIC_M)}{(d\beta)^2} \\
&\quad + \frac{\ell(\hat{\alpha}, \hat{\beta}, MIC_N, MIC_M)}{(d\beta)^2}
\end{aligned}$$

and

$$\begin{aligned}
\frac{\partial^2 \ell}{\partial \alpha \beta} &\approx \frac{\ell(\hat{\alpha} + d\alpha, \hat{\beta} + d\hat{\beta}, MIC_N, MIC_M) - \ell(\hat{\alpha} + d\alpha, \hat{\beta}, MIC_N, MIC_M)}{d\alpha d\beta} \\
&\quad + \frac{-\ell(\hat{\alpha}, \hat{\beta} + d\beta, MIC_N, MIC_M) + \ell(\hat{\alpha}, \hat{\beta}, MIC_N, MIC_M)}{d\alpha d\beta}.
\end{aligned}$$

Calculations yield the following covariance matrices for the sensitive and resistant strain, respectively.

$$\mathbf{\Sigma_R} = \mathbf{I_R^{-1}} = \begin{pmatrix} 7.65 \cdot 10^{-29} & -4.44 \cdot 10^{-20} & -3.48 \cdot 10^{-21} & 4.89 \cdot 10^{-20} \\ -4.44 \cdot 10^{-20} & 4.83 \cdot 10^{-5} & -1.27 \cdot 10^{-6} & -1.41 \cdot 10^{-5} \\ -3.48 \cdot 10^{-21} & -1.27 \cdot 10^{-6} & 1.48 \cdot 10^{-7} & -1.17 \cdot 10^{-6} \\ 4.89 \cdot 10^{-20} & -1.41 \cdot 10^{-5} & -1.17 \cdot 10^{-6} & 2.50 \cdot 10^{-5} \end{pmatrix}$$

$$\mathbf{\Sigma_S} = \mathbf{I_S^{-1}} = \begin{pmatrix} 3.12 \cdot 10^{-23} & 3.53 \cdot 10^{-17} & -2.63 \cdot 10^{-16} & -1.86 \cdot 10^{-17} \\ 3.53 \cdot 10^{-17} & 3.80 \cdot 10^{-5} & -2.65 \cdot 10^{-7} & -2.72 \cdot 10^{-8} \\ -2.63 \cdot 10^{-16} & -2.65 \cdot 10^{-7} & 5.02 \cdot 10^{-7} & -1.35 \cdot 10^{-5} \\ -1.86 \cdot 10^{-17} & -2.72 \cdot 10^{-8} & -1.35 \cdot 10^{-5} & 3.67 \cdot 10^{-4} \end{pmatrix}.$$

With these values, we can furthermore calculate the variance for $\theta(\mathbf{Y})$. With *Gaussian approximation* it follows that $\theta(\mathbf{Y})$ can be calculated by

$$\begin{aligned}
Var(\theta(\mathbf{Y})) &= \sum_{i=1}^{3} V(Y_i) \left(\frac{\partial \theta}{\partial Y_i} \right)^2 \Big|_{Y_i = \hat{Y}_i} \\
&\quad + 2 \sum_{i \neq j} Cov(Y_i, Y_j) \left(\frac{\partial \theta}{\partial Y_i} \frac{\partial \theta}{\partial Y_j} \right) \Big|_{Y_{i,j} = \hat{Y}_{i,j}} \\
&\Longrightarrow \\
Var(\theta_R(\mathbf{Y})) &= 726 \\
Var(\theta_S(\mathbf{Y})) &= 4114.
\end{aligned}$$

4.3.3 Confidence interval

Let $\mathbf{X} = \mathbf{\Sigma}^{-1/2}(\mathbf{Y} - \mu)$. Then, (4.3.2) yields [8] that

$$\mathbf{X} \sim N(\mathbf{0}, \mathbf{I}).$$

A confidence interval will now be given by

$$\mathbf{\Sigma}^{-1/2}(\mathbf{Y} - \mu) = \pm\lambda_{\mathbf{0.025}}$$
$$\Longleftrightarrow$$
$$\mu = \mathbf{Y} \pm \mathbf{\Sigma}^{-1/2}\lambda_{\mathbf{0.025}}$$

where $\lambda_{\mathbf{0.025}}$ in this case is the 2.5% quartile of the normal distribution.

The confidence intervals for each parameter are summarized in the tables below.

Resistant strain

Parameter	Estimate	Confidence interval
α_R	$1.42 \cdot 10^{-9}$	$(1.42 \cdot 10^{-9}, 1.42 \cdot 10^{-9})$
β_R	0.77	$(0.76, 0.78)$
MIC_{NR}	0.037	$(0.036, 0.038)$
MIC_{MR}	0.50	$(0.49, 0.51)$

Sensitive strain

Parameter	Estimate	Confidence interval
α_S	$8.19 \cdot 10^{-10}$	$(8.19 \cdot 10^{-10}, 8.37 \cdot 10^{-10})$
β_S	1.00	$(0.99, 1.012)$
MIC_{NS}	0.0070	$(0.0056, 0.0084)$
MIC_{MS}	0.19	$(0.15, 0.23)$

Note that because the covariance matrixes are close to singular, the results may due to numerical problems be a little uncertain.

What we still have to do now, is to analyze whether the model will fill its function, in the question of predicting when the selection of resistance occurs. This will be discussed in the next chapter.

Chapter 5

Results

In this chapter, the results of the pharmacodynamic model with estimated parameters will be presented. The results of the model will be compared with real data and we will check whether the model presented in the previous chapter is adequate for our purposes.

5.1 Prediction

An approximative 95-percent prediction interval for θ can be calculated by

$$\hat{\theta}(t_j) \pm 1.96\sqrt{Var(X(t_j))}.$$

This means that an observation with 95-percent probability will be included within this interval. Figure 5.1 shows the estimated model with predictive interval compared with experimental data.

5.2 Discussion

The parameters α, β and MIC_N in the model was estimated from data from the repeated experiments of SW 2 hours. For this window we can see that real data seem to be well included by the 95-percent predictive interval of the model. But we can also see that we seem to get an increased deviation from real data with increased length of the selective window. In this section possible reasons to this will be discussed.

In graphs in figure 5.1, we have two populations representing the sum of the wild-type bacteria and mutated bacteria, which we called X_R and X_S. Looking at the subpopulations, that means N_R, M_R and N_S, M_S, respectively, of the total populations shows that the part of the total population that consists of mutations increases with the length of the selective window of each experiment in the estimated model.

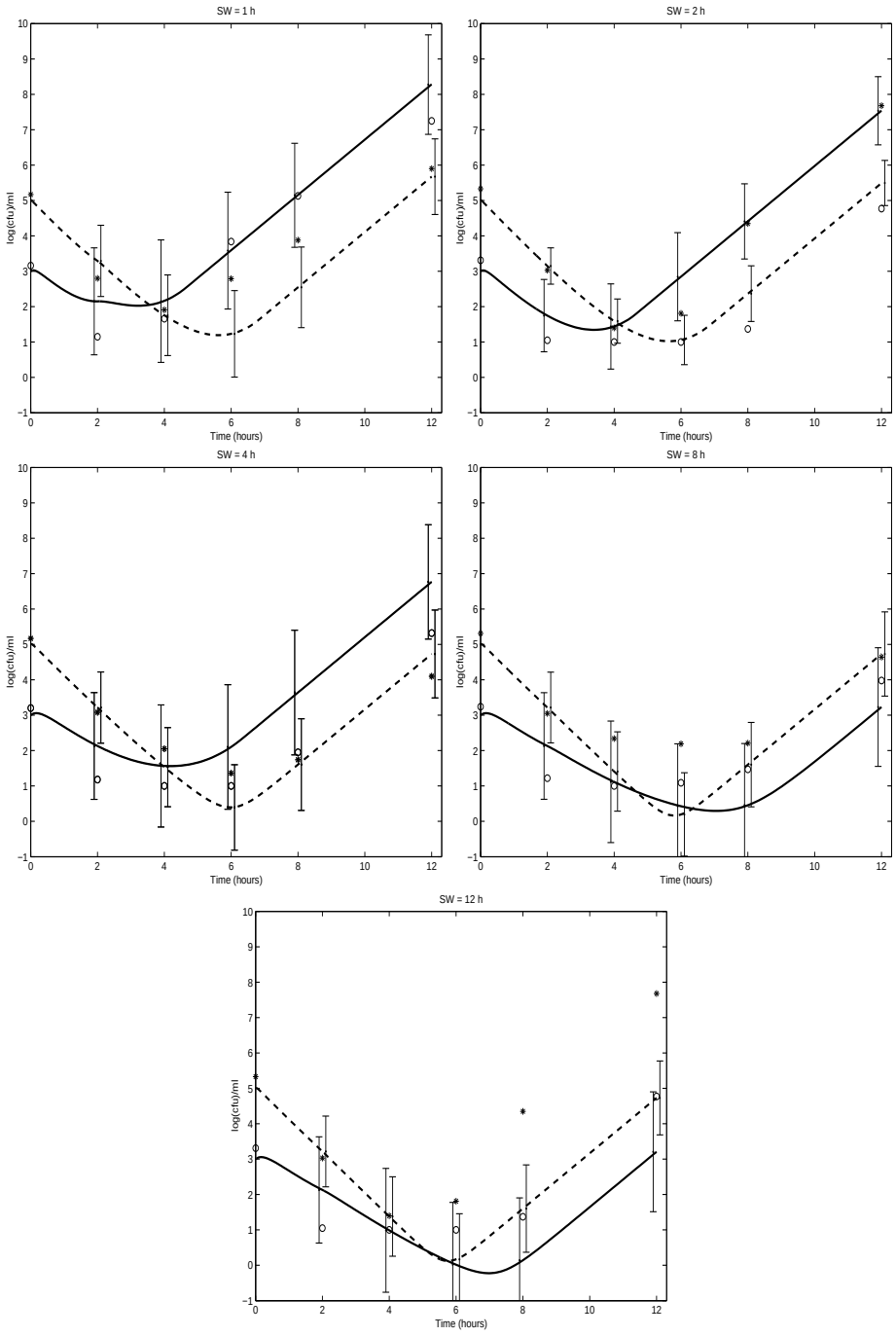

Figure 5.1: Different lengths of Selective Window, Estimated model with confidence interval. - - = Sensitive strain with * = experimental data, – = Resistant strain with o = experimental data

In the first experiment, SW=1 h, the total population in the model consisted of almost only wild-type bacteria and in the last experiment, SW=12 h, the total population was consisting of mutations only.

If we summarize these observations, one possible reason to the increased deviation from real data with increased selective windows could be that factors that were assumed to be equal for N_R, M_R and N_S, M_S, respectively, as a matter of fact differs. For example, wild-type bacteria may have a higher resynthesis rate of PBP than mutated bacteria. One more possible reason could be that the mutation rate, which is constant in the model, in reality depends on the concentration and exposure time of antibiotics.

Also to be mentioned is that it takes a longer time for the mutated bacteria of the wild-type resistant strain to take over the total population than if we compare with the wild-type sensitive strain, which is a result of a lower inoculum. So the inoculum will be an important factor when we look at the selection of the total populations X_R and X_S. In the experiment with SW=4 h, X_R (sum of wild-type resistant and mutated bacteria) is selected. But looking at the subpopulations of X_R and X_S, respectively, would in this model show that the main part of X_R population consists of wild-life bacteria, while the sensitive population X_S consists of mutated bacteria. This means that what we really have, is a selection of the most sensitive strain.

For further investigations, one important thing would be to check by MIC-data whether there really are mutations in the experiments as the estimated model describes. Furthermore investigation of how the wild-type bacteria differs from the mutated bacteria would be of interest.

Chapter 6

Conclusion

Our primary goal in this thesis was to construct a pharmacodynamic model which could explain when selection of the wild-type resistant population occurred. Because we unexpectedly found out that the bacteria in the wild-type populations developed extra resistance by mutating, the question of interest was now to explain when the most resistant population of the four subpopulations were selected.

The conclusion was that the selection of N_S, M_S and N_R, M_R, respectively, increased with the length of the selective window, which could be proved by repeating these experiments and determining MIC-data. So when it comes to the total populations of X_S and X_R the selection in this model will be dependent on how the subpopulations are selected and also on the inoculum of each wild-type strain.

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Appendix A

Data

This appendix provides data not presented in the report, that were used to estimate the parameters.

A.1 Control curve for growth rate without antibiotics

The experiment in figure A.1 was done without any pressure of antibiotics. Because we assumed that bacteria have maximal growth rate without any pressure of antibiotics, this parameter was estimated from data from this experiment.

As we can see in the figure, we have a time-delay for the bacteria to reach normal growth rate, which could be a result of the time it takes for bacteria to "get accustomed" to the new milieu. The maximal growth rate was therefore estimated between 2 and 8 hours.

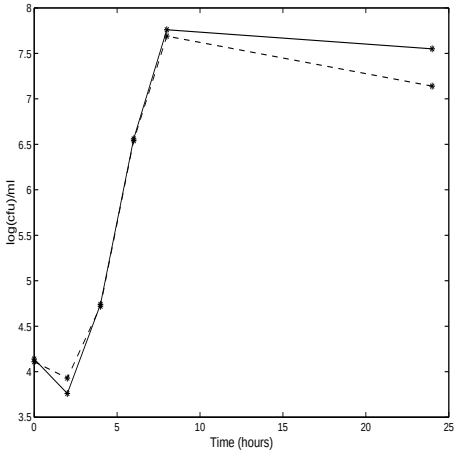

Figure A.1: Control curve for growth rate without antibiotics

A.2 Experiment with high concentration of antibiotics

This experiment was done with extremely high concentrations of antibiotics to be sure of reaching the maximal kill rate of bacteria. The maximal kill rate were estimated with data between 0 and 2 hours.

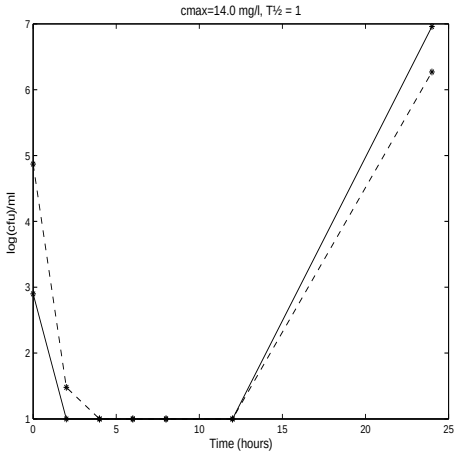

Figure A.2: Experiment with high initial concentration of antibiotics

A.3 Raw data for Maximum-Likelihood estimates

The Maximum-Likelihood estimates in Chapter 4 were estimated with data from 10 repetitions of the experiment with Selective Window = 2 hours, which are presented in the table below. The numbers are presented in log_{10}-scale.

Trial nr i	Strain	t	0	2	4	6	8	12	24
1	S		5.07	2.74	2.26	1.60	3.87	5.16	6.67
	R		3.17	1	1	2.41	3.86	6.11	7.71
2	S		4.94	2.92	1.85	1	2.83	5.77	6.40
	R		3.01	1.40	1	2.89	4.09	7.22	8.12
3	S		5.17	3.61	1.70	1	3.12	6.45	7.00
	R		3.17	2	1	2.32	4.31	7.82	8.30
4	S		5.35	2.86	2.54	1.74	2.04	5.22	7.01
	R		3.29	1	1	2.32	3.27	6.60	8.04
5	S		5.35	2.58	2.54	1.48	2.34	5.76	7.22
	R		3.21	1	1.30	1.85	2.89	7.09	7.99
6	S		5.26	2.79	2.56	1.48	2.40	5.03	7.26
	R		3.17	1	1.30	1.95	3.52	6.47	8.34
7	S		5.30	2.82	2.59	2.00	2.77	5.42	7.73
	R		3.14	1	1	1.60	3.14	6.30	7.96
8	S		5.31	2.88	2.27	1.48	2.60	5.64	6.32
	R		3.10	1.30	1.18	3.63	5.09	7.54	8.09
9	S		5.30	2.85	1.54	1.48	3.28	5.48	6.15
	R		3.09	1	1	3.61	5.36	7.64	8.05
10	S		5.14	3.82	1.48	1	2.93	6.24	7.07
	R		3.26	2.32	1	2.48	4.04	7.31	8.02