Tuberculosis: the hidden disease

Introduction

Excluding HIV/AIDS, Tuberculosis (TB) is responsible for more deaths worldwide than any other infectious agent, with approximately 1.4 million global mortalities in 2011 (WHO, 2012). Although TB mortality rate declined by 41% between 1995 and 2011 and a decline in the number of individuals becoming ill due to the infection has been seen (WHO, 2012), TB continues to be a huge health problem globally - not only because of the increasing number of patients which are co-infected with HIV/AIDS but also because of the rising number of cases of antibiotic resistant TB infections.

The current recommended treatment course for TB is a six month regimen of antibiotics, commonly involving isoniazid and rifampicin (WHO, 2009). There are a number of second line drugs, including fluoroquinolones, also available. A combination of drugs is used in order to reduce the risk of antibiotic resistance emerging.

In 2010 approximately 650,000 multidrug-resistant (MDR) TB cases were reported worldwide (out of 8.8 million cases discovered worldwide (WHO, 2011)), with MDR being defined as resistance to rifampicin and isoniazid. Of these individuals, only 46,000 patients were placed on MDR TB treatment regimens despite there being an estimated 150,000 fatalities a year being caused by MDR TB (WHO, 2011a). This is mainly due to there being no simple way to detect cases of MDR TB or to monitor treatment failures, with the diagnostic procedures most commonly used being incredibly outdated and having low sensitivity (Comas and Gagneux, 2009).

Unfortunately extensively drug-resistant (XDR) TB, in which the pathogen has developed resistance to even second-line treatments, has been noted worldwide with a prevalence of about 9% and is essentially untreatable (Ahmad & Mokaddas, 2009) – further than this Total Drug-Resistant TB cases (which appear to have resistance to every available antibiotic) have also been discovered (Velayati *et al.*, 2009).

TB resistance to antibiotics is well documented (Chhabra *et al.*, 2012: Banerjee. *et al.* 2008) and is often found within a short time period of the drug being prescribed for the first time. For example, streptomycin was originally isolated in 1943, and was introduced for widespread use by 1947 (Conroe, 1978) – unfortunately resistance was already being noted by 1948 (Crofton and Mitchison, 1948).

In addition to this rifampicin, the last drug approved for TB treatment whose action mechanism was novel, was approved for clinical usage in 1968 and isoniazid was launched even further back in the early 1950's (Norton and Holland, 2012). In both cases resistance to these front line drugs (WHO, 2009a) was observed within the first few years of them being prescribed, with rifampicin resistance being found in 1969 (Manten and Van Wijngaarden, 1969) and resistance to isoniazid being noted by 1957 (Fox *et al.*, 1957).

It has been shown that the primary method by which strains of TB become resistant to an antibiotic is a sudden mutation in the DNA sequence of the strain in response to induced selective pressure by the drug (Somoskovi *et al.,* 2001: Zhang and Yew, 2009). It is often thought that a 'fitness cost' is imposed on bacteria with antibiotic resistance, such as a lowered transmissibility or virulence (Borrell and Gagneux, 2009); however it has been demonstrated that some of these mutations do not result in reduced fitness as might be hoped (Billington *et al.*, 1999: van Soolingen *et al.*, 2000) and that even in cases where there is a cost, reversion to a susceptible state is not guaranteed (Andersson, 2006: Maisnier-Patin and Andersson, 2004: Björkman *et al.*, 2000: Gillespie, 2001). Clearly therefore the development of new

antibiotics is of considerable importance, especially considering the problems with bacillus Calmette-Guerin vaccine (Brandt *et al.*, 2002: Brosch *et al.*, 2007: Rodrigues *et al.*, 1993) and the fact that there are no new vaccines available for widespread use on the horizon.

The genome of the H37Rv strain of *Mycobacterium tuberculosis* (*M. tb*), the pathogen species responsible for the majority of TB cases, has been entirely sequenced (Cole *et al.*, 1998) and as such the DNA sequence for all possible novel treatment targets is available for exploration, making this a sensible, but difficult, route of exploration for new mechanisms of drug action, in order to avoid promoting cross-resistance to already existing antibiotics (Coxon *et al.*, 2012).

A previous study exploring the susceptibility to three commonly used antibiotics of a library of *M. tb* transposon mutants (see appendix II) found that changes to four specific genes of H37Rv (the laboratory strain used in all studies) meant that the isolates had a reduced susceptibility to at least one antibiotic. Whilst this reduction did not result in a high level of resistance (the relevant minimum inhibitory concentrations (MIC) (see appendix II) were increased by between 0.5 and 0.75 µg/mL in each case as compared to a MIC of 1.0 µg/mL for wild type strains (da Fonseca, 2012)), the mutations increased the relative fitness of each strain with respect to the parent wild type strain.

These low level mutations could provide the bacteria with sufficient advantage to survive becoming more resistant, making them of great interest and as such have considerable significance in terms of antibiotic treatments. The overall aim of this project therefore is to use a mathematical model to investigate the impact on the level of resistance (i.e. the proportion of bacteria that are resistant to the antibiotic) in a population of bacteria when these low level mutants are accounted for, as opposed to a population which does not include them, and to see how this impact varies according to changes of the mutation rate of the low level resistance mutations.

Background

TB is an airborne disease whose transmission is facilitated by the inhalation by an individual of particles called droplet nuclei, which are expelled from those already infected by coughing which contain the bacillus *M. tb*. The most important risk factor is an impaired immune system, which is most commonly the result of HIV infection (WHO, 2011: Lawn and Zumla, 2011)

Although only 30% of those exposed to the droplet nuclei become infected according to a skin test (Jereb *et al.*, 2003), and of those infected between just 5 and 10% will actually develop active TB in the 2 years after infection (Lin and Flynn, 2010), the fact that latent infection by the pathogen can last for decades can cause real problems as active TB could potentially develop at any point over a long period of time (Wayne and Sohaskey, 2001: Norton and Holland, 2012).

During latent infection the bacillus is not contagious and remains metabolically silent. The infection resides in patient lesions and often rifampicin is the only front-line drug that can be used effectively (Zhang, 2004).

The current recommended treatment regime for TB is Direct Observation Therapy, Short course (DOTS), which requires trained health practitioners to observe infected individuals during their treatment regimens to help prevent patients from dropping out (WHO, 2009: Raviglione and Uplekar, 2006). Previously, drop out rates had been significant because of the length of treatment (6 months) and this was

presumed to be a major factor in allowing antibiotic resistance to develop (Frieden and Sbarbaro, 2007).

The regimen suggested by the British Thoracic Society (BTS, 2005) involves daily doses of at least three drugs (rifampicin, isoniazid and pyrazinamide) for 2 months before reducing this down to isoniazid and rifampicin at least twice a week for the next 4 months. The cocktail of drugs is used to help reduce the risk of antibiotic resistance developing, whilst the long treatment duration is the result of the lowered efficacy of the drugs *in vivo* (Kjellsson *et al.*, 2012) and the difficulties of eliminating the bacillus once it has entered its dormant state (Hu *et al.*, 2003).

The three antibiotics focused on in this project are ciprofloxacin, rifampicin and isoniazid. All three work in somewhat different ways to combat TB (and other pathogens) - ciprofloxacin interferes with DNA gyrase, which plays a crucial role in DNA synthesis, and so prevents DNA and proteins from being synthesized (Sanders, 1988). Rifampicin instead inhibits RNA synthesis by blocking DNA-dependent RNA polymerase, thus preventing DNA-dependent RNA from being created (Mitchison, 1985). Finally isoniazid prevents the bacteria from producing mycolic acid and thus interferes with the pathogens cell wall (Rawat *et al.*, 2003).

Antibiotic resistance has been defined as any reduction in the sensitivity to a drug by a pathogen strain that is significantly different to a wild type isolate strain that has not been treated (Mitchison, 1962), which allows for a broad interpretation of what it means to be resistant.

Resistance mutations in *M. tb* tend to occur in a step-wise manner, as mutations to the DNA sequence of the bacterium due to inadequate antibiotic treatment confer resistance (Shenoi and Friedland, 2009). Non-compliance to treatment is not sufficient alone for the development of resistance however, as pharmokinetic variability has been hypothesized as being essential to the evolution of MDR TB (Srivastava *et al.*, 2011). Horizontal gene transfer need not be considered as a method of developing resistance because there are no plasmids associated with *M. tb* and there are no reports of the transfer of genomic DNA (Zainuddin and Dale, 1990).

Fitness is often measured as some function of the growth rate of the organism, and the relative fitness of a drug-resistant strain of a pathogen compared to a susceptible strain can be of considerable use when investigating whether a mutation for resistance is likely to be able to compete in a population containing susceptible pathogens (Borrell and Gagneux, 2009: Andersson and Hughes, 2007: Pope *et al.*, 2010). One method of defining the fitness cost of developing resistance to an antibiotic is the reduction in growth rate of the organism, which is the result of the impact of the resistance mutations on the organisms function. This cost has the potential to allow susceptible strains to outcompete resistant ones in the absence of antibiotic pressure.

Therefore resistant pathogens could revert back to a susceptible phenotype: this reversion been noted in *M. tb* (Richardson *et al.*, 2009) but mutations that confer resistance with little or no cost have also been found (van Soolingen *et al.*, 2000: Hazbón *et al.*, 2006). On top of this, secondary mutations which balance the potential cost of resistant mutations or co-selection between the mutated resistant gene and another gene essential for the organisms survival are also possibilities that would each reduce the probability of reversion (Andersson, 2006: Andersson and Levin, 2008: Enne *et al.*, 2004).

A relatively small number of mutations are responsible for most of the phenotypic resistance seen: for example at least 95% of pathogen isolates with

resistance to rifampicin have a mutated *rpoB* gene, specifically in the rifmapicin resistance-determining region (RDR) (Ramaswamy & Musser, 1998). Worryingly, the vast majority of rifampicin resistant isolates are also resistant to other antibiotics (O'Riordan *et al.*, 2008).

Almost half of all mutations which lead to isoniazid resistance are occur in the katG gene at codon 315 (Cade *et* al., 2009: Guo *et al.*, 2006), and more than one hundred different types of katG mutation that confer some level of resistance have been found, including some with seemingly no fitness cost (van Doorn *et al.*, 2006).

Unfortunately, although isoniazid is the suggested first treatment for TB (Rieder, 2002: Sirgil *et al.* 2002), isoniazid resistance is the most common type of resistance reported (Pablos-Mendez *et al.*, 1998). Individuals infected with isoniazid resistant strains of the disease are much less likely to be treated successfully and the risk of further antibiotic resistance developing is increased (Menzies *et al.*, 2009: Menzies *et al.*, 2009a).

Finally, for ciprofloxacin (a fluoroquinolone) resistance is usually the result of mutations in the quinolone RDR between codons 88 and 94 in the gyrA gene (Drlica, 1999; Takiff *et al.*, 1994), or possibly the production of MfpA, a protein which can copy DNA and so bind to and inhibit DNA gyrase (Khrapunov and Brenowitz, 2011). There has been some debate over the usefulness of fluoroquinolones in TB treatment, with various studies reaching differing conclusions (Yew *et al.*, 2000: Takiff and Guerrero, 2011: Kennedy *et al.*, 1996).

David (1970) demonstrated that antibiotic resistance in wild-type strains of *M. tb* was very uncommon. Modelling done by Colijn *et al.* (2011) however suggested a much higher probability of resistance to multiple drugs occurring before treatment with values given ranging between 10^{-5} and 10^{-4}. Mutation rates for isoniazid resistance *in vitro* are of the order of $2–3 \times 10^{-8}$ mutations per generation for every bacterium (Nachega and Chaisson, 2003: Bergval *et al.*, 2009). Rifampicin mutation rates of resistance are estimated to be about 9.8×10^{-9} mutations/cell division being given (Bergval *et al.*, 2009). One in every 10^{8} bacterium are resistant to rifampicin (Blanchard, 1996), whilst isoniazid resistance is several orders of magnitude more common with one in every 10^{6} bacterium being resistant (Musser, 1995). For ciprofloxacin a mutation rate of resistance on the order of 10^{-8} mutations per generation per bacterium (Gumbo *et al.*, 2005) is estimated, and this very high mutation rate has led to resistance to the fluoroquinolone spreading quickly. It is further assumed that for a single *M. tb* bacterium the probability of mutating such that the bacillus becomes resistant to more than one drug is equal to the multiplicand of the mutation rates of resistance for each separate antibiotic i.e. in the case of isoniazid and rifampicin one out of every $10^{8} \times 10^{6}$, or 10^{14}, bacilli would be resistant.

The much higher rate of reporting of isoniazid rather than rifampicin resistant isolates after accounting for their relative mutation rates has been investigated (Bergval *et al.*, 2009) and it seems that the lower mutation rate of rifampicin is not significant enough to explain the disparity between the two values and *in vitro* experiments are not accurately reflecting the manner in which isoniazid resistance develops *in vivo* – this is relevant with regards to this project as the strains with mutated genes have only been cultured *in vitro*.

There has been extensive and varied mathematical modeling done with regards to TB, including modeling HIV and TB co-infection (Ramkissoon *et al.*, 2012: Kirschner, 1999), TB epidemics (Aparicio and Castillo-Chavez, 2009) and

investigations into how latent TB acts in its environment (Patel *et al*., 2011). There are also models exploring populations infected with multiple, separate TB strains which can become latent, and the effects this can have on treatment regimens (Sergeev *et al.*, 2011: Colijn *et al.*, 2009).

Animal models have provided a significant proportion of the available knowledge of not only the immunological response to, but also the pathogenesis of, TB (Apt and Kramnik, 2009: Gupta and Katoch, 2005: Orme, 2003), though there has been criticism that these models are not generalizable to humans (Baldwin *et al.*, 1998).

There are few models unfortunately on the evolution of resistance in *M. tb*, which is possibly the result of difficulties in accurately modeling such a complex process with incomplete parameter values, for example with regards to different growth and mortality rates. In particular a large number of varied values for the relative fitness of resistant *M. tb* strains have been calculated depending on the resistance type observed, which can lead to complications whilst modeling. These parameter values are most often not calculated in vivo, which can further complicate matters.

Experimental data

In order to investigate possible antibiotic targets and/or sections of the genome associated with drug resistance, a library of 217 transposon-tagged mutants of H37Rv *M. tb* was created (da Fonseca, 2012). These isolates were tested for any changes in antibiotic susceptibility to three different concentrations (0.5, 1 and 2 times the MIC for each antibiotic) of the three drugs mentioned above. Isolates which contained genes that had a transposon in that caused a change in susceptibility were screened. The mutagenesis technique utilized in the experiment did not allow for investigations into the role of genes necessary for in *vitro* development and as such their potential effect on drug resistance was not studied.

Of the 217 transposon mutants involved in the experiment, 28 were found to have a different antibiotic susceptibility than that of the parent strain. 21 of these demonstrated a reduced susceptibility to treatment, with the remaining 7 all displaying hypersusceptibility (see appendix II). Only 4 of the 21 hyposusceptible (see appendix II) strains were found to have reduced susceptibility to a single antibiotic, potentially indicating that the resistance mechanisms involved were broad ranging. This was especially the case for ciprofloxacin and rifampicin resistance: out of the 18 mutants for which the MIC of ciprofloxacin was not sufficient to inhibit growth, 14 were also similarly unaffected by the rifampicin MIC.

Mutants found with some level of resistance did not tend to survive conditions with twice the concentration of the relevant antibiotics MIC, which implies that the resistance was not of a high level.

Four specific mutants with reduced susceptibility were considered of particular interest because the location of the gene disruption was close to a genomic area that is thought to be linked to virulence, and it is these four mutants that will be the focus of the modeling done in this project. The relative fitness (see appendix II) of these strains and the antibiotic to which susceptibility was reduced are given in Table 1. The mutation rates for the mutated genes are unfortunately not known, and neither is the mutation rate of higher level antibiotic resistance in these mutant strains.

Mutant	Mutated gene	Altered susceptibility phenotype	MIC rifampicin[1] (µg/mL)	MIC ciprofloxacin[2] (µg/mL)	Fitness[3]
20B10	Rv3879c	CIP+RIF	1.75	1.5	1.10
12G6	Rv3888c	CIP	-	1.5	1.02
2B10	Rv3891c	RIF	1.75	-	1.19
7A2	Rv3896c	CIP	-	1.5	1.20

Table 1 – *List of genes that were chosen for further study and the relevant phenotype, antibiotic MIC and fitness. Table, including annotation 3, taken from da Fonseca, 2012.*

1. The MIC of the parent strain is 1 µg/mL.

2. The MIC of the parent strain is 1 µg/mL.

3. A relative fitness of 1 indicates that the mutant has no fitness cost, whereas a ratio greater than or less than 1 indicates increased or decreased fitness, respectively.

Methods

A differential equation model was set up (see figure 1) with three main sub-categories: susceptible *M. tb*, denoted S, those *M. tb* with a mutation with resulted in reduced susceptibility, denoted P, and those *M. tb* which have a mutation conferring a high level of resistance to the antibiotic, denoted R.

Three different versions of the model were run, though all three models were run for the same length of time. Firstly, a model including only fully susceptible and fully resistant bacteria was first run so that later comparisons could be drawn between the level of resistance in this population and one which contains mutants with the four mutated genes of interest. Then a model including bacteria with reduced susceptibility was simulated.

Finally a model solely investigating the potential impact of different initial population sizes of bacteria with reduced susceptibility on the overall level of resistance was run.

The first two versions of the model simulate the impact of a single drug administration of 1 times the MIC of an antibiotic (either ciprofloxacin or rifampicin because the mutated genes of interest resulted in altered susceptibility phenotypes to one or both of these drugs) at day 4 of an *in vitro* experiment in a *M. tb* population containing either some or all of the relevant subgroups noted above. This drug concentration was chosen as it would only affect the growth of the completely susceptible bacteria and it was administered only once to simulate the impact of treatment without over-inhibiting susceptible bacteria growth. The growth rate of the bacteria was otherwise left unchecked to simulate growing cultures in an unlimited culture. *In vitro* conditions were simulated as the vast majority of parameter values were calculated *in vitro* rather than *in vivo*.

The antibiotic was assumed to have an inhibitory effect on the bacteria population for 5 days (based on values for the postantibiotic effect duration in the literature (Gumbo *et al.*, 2007)), after which the susceptible population was allowed to

grow freely. Reversion of bacteria with any level of resistance to a completely susceptible state was not included in this model.

The initial susceptible population size was assumed to be 1,000 (the number of bacteria used in the earlier experiment, with the population sizes of any other category of bacteria being 0.

In this last model there was no simulated antibiotic administration (i.e. no inhibition of susceptible bacteria population growth). The overall initial population size was once again 1,000, and there were no fully resistant bacteria initially, but the number of susceptible bacteria and those with reduced susceptibility was varied so that the impact of different initial population sizes could be investigated.

Considering that the main aim of the model is to investigate the level of resistance in the population, the proportion of resistant bacteria at the end point of the model (as defined in Figure 1) and the effect on this of varying the mutation rate for the mutated genes was the main outcome of interest.

Figure 1 *– Parts a), b) and c) show the different sets of differential equations used to construct the first, second and third models respectively. Part d) shows the equation for the proportion of bacteria with some level of resistance in population.*

a)

$$\frac{dS}{dt} = Fs\,B\,S - (D + \varepsilon)\,S - \gamma\,S$$

$$\frac{dR}{dt} = \gamma\,S + Fr\,B\,R - D\,R$$

b)

$$\frac{dS}{dt} = Fs\,B\,S - (D + \varepsilon)\,S - (\lambda + \gamma)\,S$$

$$\frac{dP}{dt} = \lambda\,S + Fp\,B\,P - D\,P - \beta\,P$$

$$\frac{dR}{dt} = \gamma\,S + \beta\,P + Fr\,B\,R - D\,R$$

c)

$$\frac{dS}{dt} = Fs\,B\,S - D\,S - (\lambda + \gamma)\,S$$

$$\frac{dP}{dt} = \lambda\,S + Fp\,B\,P - D\,P - \beta\,P$$

$$\frac{dR}{dt} = \gamma\,S + \beta\,P + Fr\,B\,R - D\,R$$

d)

$$propPR = \frac{(P + R)}{(S + P + R)}$$

A table of the parameter definitions and values used is given in Appendix III. All values where known were taken from the literature and are assumed to be accurate for *M. tb in vitro*. The model was run using the software package Berkeley Madonna.

Parameter values that were found included the growth rate and mortality rate (which were taken from standard life cycles for the pathogen), the length of the postantibiotic effect, the mutation rates for full resistance and the increased mortality rate of bacteria due to the antibiotic. The relative fitness of susceptible bacteria was set at one to provide a reference, and the fitness of the bacteria with reduced susceptibility was also known (see Table 1). The fitness values for resistant bacteria were widespread but a single value was assumed for simplicity. The growth and death rates of all three types of bacteria was assumed to be the same with the relative fitness values providing the distinctions.

As mentioned before, the mutation rates for the mutated genes of interest were not known so a range of values between 1×10^{-8} and 1×10^{-6} was chosen to be used after considering that the mutation rates of full resistance mutations for antibiotics tends to be of the order 10^{-8} and that the mutant strains will have a more significant impact if their mutation rate is lower than that of full resistance mutations.

The models were run for both rifampicin and ciprofloxacin and no significant differences were observed between the two sets of results in terms of the proportion of resistance observed in the population: thus only the results for the rifampicin model are presented here to avoid repetition.

Results

In the first model, which includes only fully susceptible and fully resistant bacteria, the level of resistance in a population only reached a level greater than 1% if in the initial population there was at least one bacteria that was resistant to the antibiotic (in which case the proportion of resistant bacteria in the population tended inexorably towards 1); otherwise the level of resistance remained very small.

When the second model (which includes a population of bacteria with reduced susceptibility) was run, a significant change in the overall level of resistance in the population was seen. No matter which value for the mutation rate of low level resistance was used, by the end point of the model the population contained more bacteria that had a reduced susceptibility or were resistant than were entirely susceptible to drugs, regardless of the initial population sizes. This is clearly displayed in figure 2, and is the result of the increased fitness of the bacteria with reduced susceptibility as compared to the totally susceptible strain.

Following on from this, figure 3 illustrates how the proportion of the population that has some level of resistance changes over time. No matter what value for the mutation rate was used, the proportion resistant increases dramatically at the fourth time point when the antibiotic was administered. Whilst this increase slows after the ninth time point when the drug no longer has an inhibitory effect, there is still a general increase in the overall level of resistance all the way until the end of the simulation where the proportion resistant finally plateaus close to a value of 1.

In the final version of the model, all three types of bacteria were allowed to

grow without constraint. Figure 4 shows the effect of varying the initial population size of P on the final proportion of the population that is resistant: as the initial size increases the proportion of the population found to be resistant increases, with the key condition being that the initial population size of P is greater than 0. The mutation rate for the important mutated genes was varied over a range of values and in each case the proportion of the population that was resistant tended towards to 1 provided that the initial population size of the reduced susceptibility bacteria was not zero.
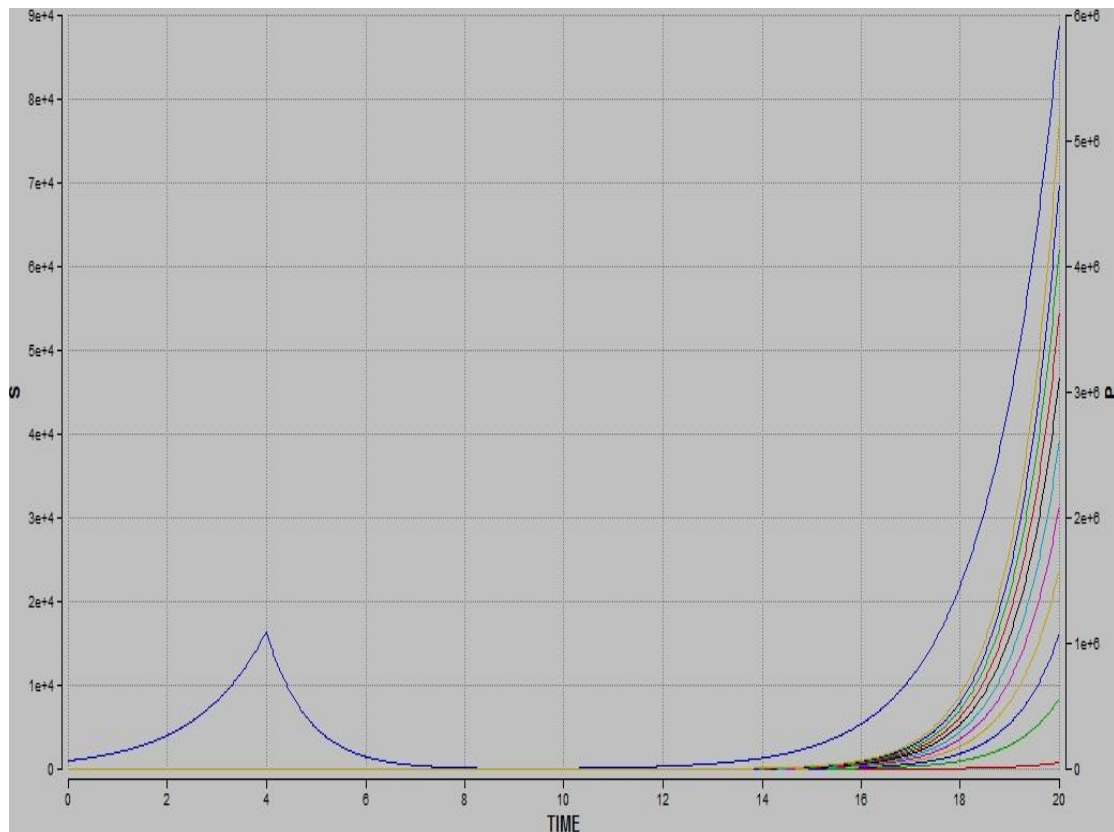

Figure 2 *– Graph showing the change of the susceptible (S, with the scale on the left axis) and reduced susceptibility (P, with the scale on the right axis) population sizes in terms of the number of individual bacteria vs time. The blue line with a sharp peak at time = 4 represents the susceptible population (scale on the left axis) and all other lines represent the various populations with reduced susceptibility (scale on the right axis) dependent on the value of the mutation rate of resistance of the mutated gene being considered.*
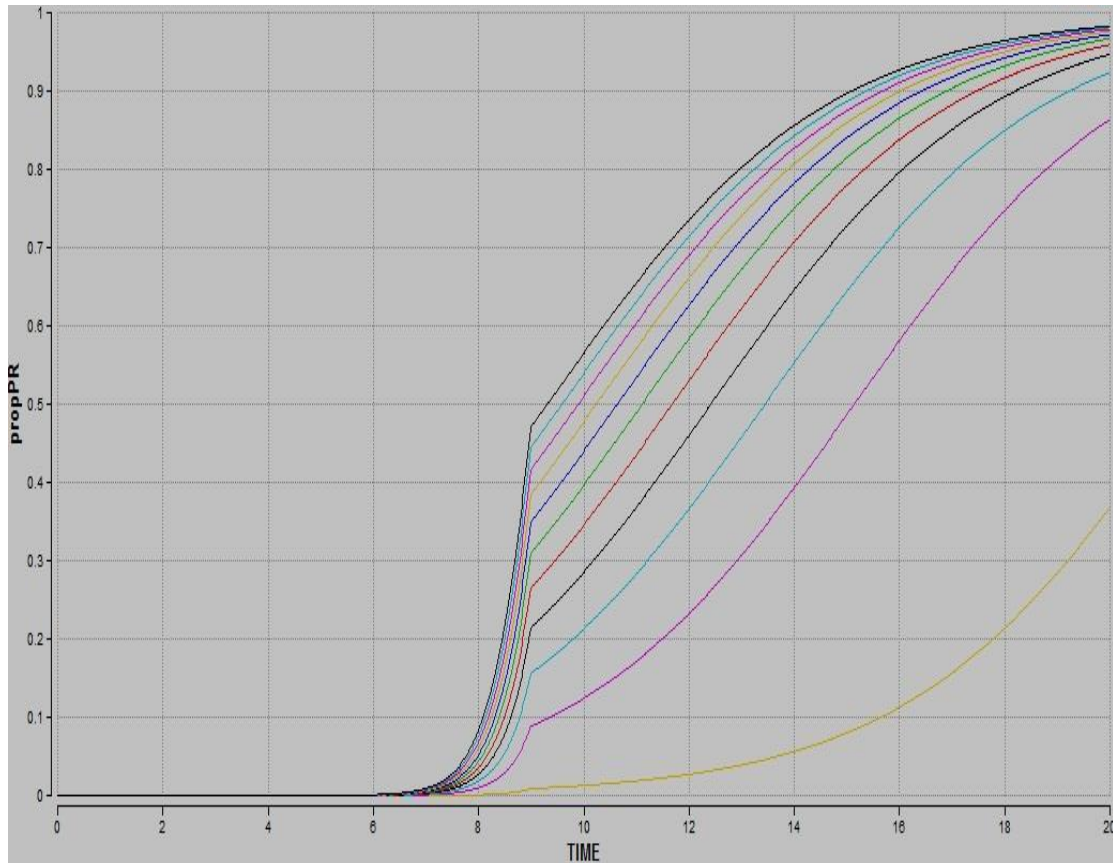
Figure 3 – *Graph showing the change in the proportion of the population that has either reduced susceptibility or full resistance to antibiotics (propPR, which is defined in figure 1) over time, with each separate line representing the different proportions observed for the different mutation rates of resistance value chosen.*
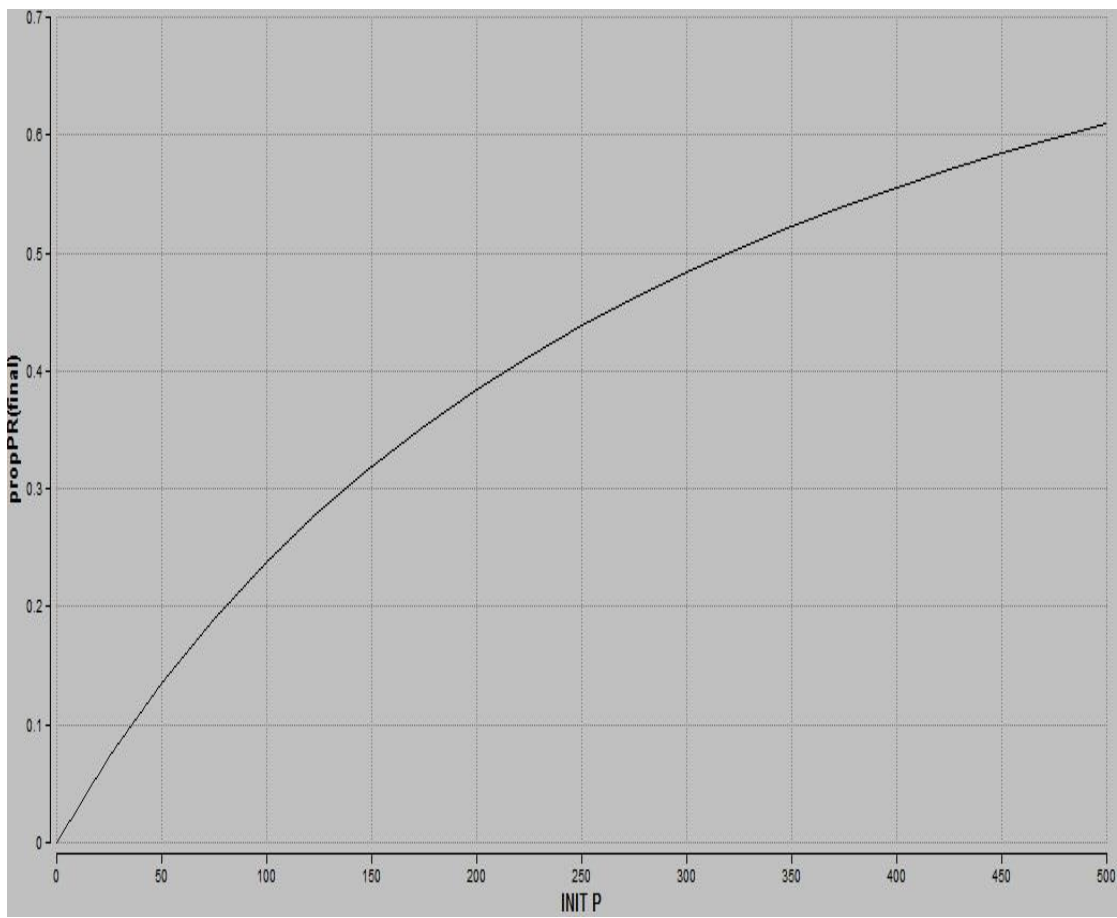
Figure 4 – *Graph showing how the proportion of the population that has some level of reduced antibiotic susceptibility (propPR(final)) changes depending on the initial size of the population with reduced susceptibility (INIT P) chosen. The value of the initial reduced population size given is absolute and based on an overall initial population size of 1000 bacteria.*

Discussion and model criticism

The focus of the project was to investigate the potential changes to resistance level caused by a bacterial sub-population with reduced susceptibility and in all of the models simulated which included a category of reduced susceptibility bacteria an overall increase in the level of resistance was observed compared to simulations without this category. It was also no longer necessary for the initial population to have some resistant bacteria for the resistance level to be considerable when an antibiotic was administered. In addition to this the third model variant highlighted how when even a fraction of the population has a reduced susceptibility the final overall level of resistance can be large.

If this is not just the case *in vitro* it could have an large impact on the diagnosis and treatment MDR TB because the mutated genes seemed to confer not only a reduced antibiotic susceptibility phenotype but also an increase in the relative fitness of the bacteria. It is therefore important to conduct further investigations into these mutated strains in order to calculate the rates of mutation for the four mutant strains focused on, not least because some of these mutated genes had not been considered previously as conferring some measure of antibiotic resistance and the presence of these mutations may increase the likelihood of developing high level resistance in the future.

The level of resistance in the first model ran was small when the initial resistant population size was set to zero because of the resistance fitness cost included in the model. When the initial resistant population was greater than zero the level of resistance in the population was not negligible because the pathogen growth rate is a significant factor in determining final population size as would be expected.

Although the model did produce some interesting results, there are clearly significant flaws with it. A significant assumption about the relative fitness of resistant bacteria that was made was that this value remained constant over time and that no compensatory mutations occurred that would change this. The relative fitness of the bacteria with reduced susceptibility was also assumed to be constant even though in the four genes from which these fitness values were taken there were a range of values.

The mutation rate of the mutated genes of interest is not known; therefore a range of approximate values had to be used which could reduce the models accuracy. In addition to this it was assumed that the mutation rate of full resistance was the same in both completely susceptible bacteria and those with reduced susceptibility, although this may be justified because the mutations are considered random events and may not be linked to previous mutations in this case. Reversion of resistant bacteria to a susceptible state was not included in the model because whilst it has been found to occur it was felt that the probability of reversion would be so small as to have a negligible effect on the outcome of the model.

The model itself was deterministic, and therefore a future goal could be to greatly improve the model by adding stochastic elements to it so that it was more realistic: this is particularly true of resistance mutation events.

As all the values used in the model were calculated from *in vitro* experiments, it may not be reasonable to extend the results to cover *in vivo* situations as well, especially considering the well documented issues with ensuring that sufficient concentrations of the antibiotic administered actually affect the pathogen, meaning that even if increases in population levels of resistance are predicted for *in vitro*

situations this may not be the case *in vitro*. In addition to this, because the fitness of the four mutant strains investigated was not calculated *in vivo* it may be the case that these mutants do not have a greater relative fitness in clinical cases which could then render the models conclusions partially invalid.

Whilst the criticism of the parameter values used is valid, it is important to bear in mind the difficulty of finding accurate and reliable values for any model. One of the best features of the model was the large number of parameter values that were available because of the previous experiments done. This can only have helped to improve the accuracy of the model and the results.

Conclusions

The model formulated in this project provided results which suggest that the overall level of resistance is higher in populations which contain bacteria with a reduced susceptibility to antibiotics than those without this group: this was perhaps to be expected because bacteria with reduced susceptibility will be more likely to survive in the presence of an antibiotic and therefore potentially acquire a mutation for high level resistance. This could have significant clinical consequences in terms of TB treatment regimens because screening for these mutations will become important in order to combat MDR TB.

It is therefore of great importance that further studies of these mutant strains with reduced susceptibility are undertaken and proportion of these bacteria that are found in clinical cases should be calculated. Further adaptations to the model presented could create a more realistic simulation but it is also vital that specific parameter values, especially with regards to mutation rates, are estimated from experiments.

Appendix I – List of abbreviations used

DNA - deoxyribonucleic acid

DOTS - Directly Observed Treatment, Short Course

MDR – multidrug-resistant

MIC – minimum inhibitory concentration

M. tb – Mycobacterium tuberculosis

TB – tuberculosis

WHO - World Health Organization

XDR - extensively drug-resistant

Appendix II – Definitions of some terms used

Fitness is defined here as the difference between the log phase doubling times of a wild type parent strain and the transposon mutant.

Hypersusceptibility is defined here as having an increased susceptibility to an antibiotic when compared to the expected wild type susceptibility i.e. a lower antibiotic concentration than for a wild type pathogen is required to have the same comparative effect.

Hyposusceptibility is defined here as having a reduced susceptibility to an antibiotic when compared to the expected wild type susceptibility i.e. a greater antibiotic concentration than for a wild type pathogen is required to have the same comparative effect.

MIC is defined here as the smallest antibiotic concentration able to stop visible pathogen growth.

In a *transposon library*, a piece of DNA that can alter its genomic position (called a transposon) is inserted into the genome at set points, which can interfere in the regular operation and function of genes. The transposon must be able to be tracked so that it's effect, if any, can be checked.

Appendix III – Values used in the models (where appropriate)

Parameter/condition symbol	Parameter/condition definition	Parameter/condition value
Fs	Relative fitness of susceptible bacteria	1 (reference)
Fp	Relative fitness of reduced susceptibility bacteria	1.2 (da Fonseca, 2012)
Fr	Relative fitness of resistant bacteria	0.95 (various values for each mutation, some given in Bhatter *et al.*, 2011, in this case a single value based on a single mutation was assumed for simplicity)
B	Birth rate of bacteria	1.9 (estimated from doubling times included in such papers as Straus and Wu, 1980)
D	Mortality rate of bacteria	1.2 (estimated from generation times included in such papers as Wayne, 1977)
ε	Excess mortality rate of bacteria due to antibiotic administration	1.9 (assumed from MIC of antibiotic used)

γ	Mutation rate of resistance for genes conferring high level of resistance for susceptible bacteria	9.8×10^{-9} (Bergval *et al.*, 2009)
β	Mutation rate of resistance for genes conferring high level of resistance for reduced susceptibility bacteria	9.8×10^{-9} (Bergval *et al.*, 2009)
λ	Mutation rate of resistance for genes conferring low level of resistance	Range of values used between 1×10^{-8} and 1×10^{-6}
INIT S	Initial size of susceptible population	1000 (from original experiment)
INIT P	Initial size of population with reduced susceptibility	0
INIT R	Initial size of resistant population	0

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