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Evolutionary Dynamics of Antibiotic Resistance

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ABSTRACT

Evolutionary dynamics is the quantitative description of evolution, in terms of population sizes, mutation rates and selective pressure. Antibiotic resistance is an evolutionary process, therefore strategies to prevent or minimise resistance, or to reverse it, require an understanding of evolutionary dynamics. The field of evolutionary dynamics began in 1943 when Luria and Delbrück described the incidence of phage resistance in bacteria in terms of population size and mutation rate. The genetic changes that cause antibiotic resistance are often point mutations, but other changes, such as gene amplification or gene deletion may be involved. Resistance rates may be much higher than mutation rates, because multiple changes in the bacterial genome may result in resistance. To cure a bacterial infection it is not necessary that an antibiotic eliminate every bacterial cell, simply to reduce the bacterial count to a level that can be controlled by the host immune system, so approaches that activate host immunity, such as vaccines, have a role to play in preventing antibiotic resistance. Evolutionary modelling describes the emergence of antibiotic resistance as a process in which antibiotics provide selective pressure. An effective tactic for minimizing or delaying resistance is the use of combination therapy with non-cross-resistant antibiotics, and evolutionary modelling can compare the likely efficacy of different timings, dose ratios and treatment schedules. Resistance breakers – agents that prevent or reverse antibiotic resistance – are another potential approach. When resistance is caused by activation of bacterial efflux pumps, for example, pump inhibitors can act as resistance breakers. In addition to antibiotic resistance caused by spontaneous genetic changes – vertical gene transmission – horizontal gene transmission (HGT) provides mechanisms by which resistance genes can be transferred into previously sensitive bacterial cells independently of cell division, e.g. by a plasmid. The dynamics of HGT show radical differences from vertically transmitted resistance. When a bacterium can exist in both intracellular and extracellular spaces the dynamics of resistance become more complex, partly because evolution is faster in small environments. From the study of evolutionary dynamics, important conclusions can be drawn: early use of molecular diagnostics can minimise resistance. Used in conjunction with drug resistance databases and artificial intelligence (AI) software to optimise combination design, early use of diagnostics has the potential to reduce the incidence of antibiotic resistance by many orders of magnitude.

> *Evolutionary dynamics has no need of vast abstract spaces, like all the possible viable animals, DNA sequences, sets of proteins or biological laws. Better, as the theoretical biologist Stuart A. Kauffman proposes, to think of evolutionary dynamics as the exploration in time by the biosphere of what can happen next: the "adjacent possible".*
> *Lee Smolin, "Time Reborn" (2014).*

Abbreviations

AI: Artificial Intelligence, AMR: Anti-Microbial Resistance, bact/Ri: bacteria resistant to antibiotic I, bact/Rij: bacteria doubly resistant to antibiotics i and j, DHFR: Dihydrofolate reductase, EDGAT: Evolutionary Dynamics-Guided Adaptive Therapy, GLK: Gross Log cell Kill, HGT: Horizontal Gene Transmission, LPS: Lipopolysaccharide, MDR: Multi-Drug Resistance, MIC: Minimum Inhibitory Concentration, MRSA: methicillin-resistant Staphylococcus aureus, MTD: maximum tolerated dose, PK/PD: Pharmacokinetic/pharmacodynamics, sulfa: sulphonamide, TB: Tuberculosis, TNF or TNFα: Tumour necrosis factor, VGT: Vertical gene transmission.

Keywords

Adaptive therapy, Antibiotic resistance, Cross-resistance, Evolutionary dynamics, Horizontal gene transmission, Resistance blockers.

What is evolutionary dynamics? The subject originated as a quantitative description of evolutionary biology: can we provide a mathematical expression for how populations grow, change into new species, migrate into a new habitat, or become extinct? In particular, evolutionary dynamics describes how populations respond to selective pressures – Darwin's "struggle for existence", Spencer's "survival of the fittest". A comprehensive monograph describing the mathematics of evolutionary dynamics is that of Martin Nowak [1]. In considering the dynamics of antibiotic resistance, the primary selective pressure to be considered is from the presence of antibacterial drugs, and the drugs may kill bacteria (cytotoxicity), or they may block bacterial cell division but without killing bacteria – cytostasis - (so that the inhibited bacteria may resume cell division after the antibiotic has gone away) or they may trigger a state of long-term dormancy.

A note on terminology: it was customary among microbiologists for the term "antibiotic" to be reserved for natural products, while drugs made by chemical synthesis were referred to as "antibacterials". The distinction became blurred after some drugs formerly manufactured by fermentation were successfully produced by chemical synthesis (e.g chloramphenicol) and it became common to refer to all antibacterials as "antibiotics" regardless of their method of production. I shall use the term "antibiotic" in this latter sense.

Bacterial growth curves

In the early stages of growth, whether in the test tube or the human body, bacterial reproduction is often close to exponential. Exponential growth is defined as growth at a rate proportional to the current size of the population:

$dN/dt = kN$ equation 1

where N is the population size at time t and k, the growth rate, is defined as $\log_e 2$/doubling time (in whatever units we are using for time).

Then $N_t = N_0 \times e^{kt}$ equation 2

If the potential doubling time of a bacterium is 20 minutes = 0.33 h, then k= 0.693/0.33 = 2.1 h^{-1}, so starting with a single bacterial cell, $N_0 = 1$, after 44h of exponential growth, the bacterial population would number $1 \times \exp(2.1 \times 44) = 1.345 \times 10^{40}$. Taking the mass of a typical bacterium as 1×10^{-15} kg, a single bacterial cell could give rise to a bacterial mass of 1.35×10^{25} kg after 44 h, more than twice the mass of the earth (6×10^{24} kg). Clearly rapidly dividing bacteria cannot remain in exponential growth for more than a few hours. Depletion of nutrients, accumulation of waste products, and sheer physical constraints will result in the actual doubling time after a few hours being less than the potential doubling time. In practice, any biological environment, including the body of an infected human or animal, can only support a limited number of bacteria, the carrying capacity. The carrying capacity of an animal body for a bacterial pathogen will depend upon the volume of the body compartments in which the bacteria are growing, and upon nutrient availability. A number of more realistic growth curves have been described. For the purposes of exploring the dynamics of antibiotic resistance, we assume that bacterial growth follows the Gompertz equation:

$dN/dt = kN/c.\ (A - \mathrm{loge}N)$ equation 3

where N and k are as defined for equation 1 and the constant A is a function of the asymptote, the final cell count at which growth levels off. If the final cell count is represented as Ninf (N at time infinity) then:

$A = \mathrm{loge}\ \mathrm{Ninf}$ equation 4

Thus the expression (A – logeN) in equation 3 is a measure of how far the population is, at time t, from its final value. Similarly, the constant c is a measure of how far the final state is from the initial cell number, N0:

$c = A - \mathrm{loge}\ N0$ equation 5

From examination of equation 3 it may be seen that when $N = N_0$ the expression (A – logeN) will be equal to c, so the equation reduces to dN/dt = kN, so at the beginning of the Gompertz curve growth is exponential with the cell doubling time equal to the cell cycle time. Conversely, when loge N = A (the asymptote) then dN/dt = 0 and no further growth occurs.

Figure 1 shows the growth curve for a slowly growing bacterial culture. Growth that is initially exponential reaches a maximum (at the inflexion point) then decelerates, and eventually levels off – in mathematical terms, approaches the asymptote (which is never completely reached). The growth rate declines logarithmically, and the inflexion point is at the midpoint, on a logarithmic scale, between the origin and the asymptote. In the example shown in Figure 1, a single bacterial cell gives rise to a population that reaches 95% of its asymptotic size in about 400 hours.

Another growth curve that is frequently encountered in the literature is the logistic equation

$dN/dt = kN.\ (1 – N/\mathrm{Ninf})$ equation 6

As with the Gompertz equation, growth starts out exponential, then levels off to a plateau. In the logistic equation, the growth rate declines linearly with distance from the origin, whereas with Gompertzian growth, the decline is logarithmic. The logistic thus reaches its maximum slope (growth rate) later than the Gompertz curve.

Many other population growth curves have been described: not all of them reach a fixed asymptote that corresponds to the carrying capacity of the environment. For example, the discrete logistic curve (May 1976) may, depending upon the parameter values, reach an asymptote, or it may predict population counts that oscillate with time (which occurs with predator/prey relationships) or it may become chaotic. These various curves are mathematical abstractions, and must be considered as, at best, approximations to complex biological relationships. For example, when mutations arise, as happens in the development of antibiotic resistance, the resulting growth curve may show abrupt discontinuities. All our subsequent calculations will treat bacterial growth (in the absence of mutations) as Gompertzian.

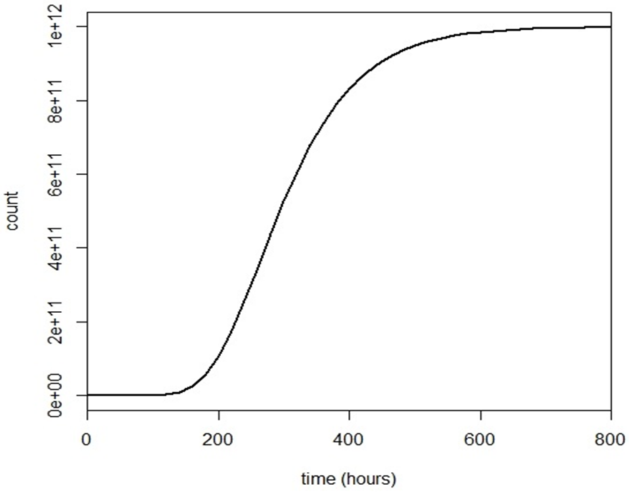

Figure 1: A representative Gompertzian growth curve.

The genetic algorithm

The basis of the theory of evolution proposed in 1859 by Darwin and Wallace was first, that competition for resources (food, territory) coupled to reproduction at a rate such that not all offspring could survive, led to a struggle for survival. Second, what Darwin called "descent with modification" - in modern terminology, genetic heterogeneity - meant that not all individuals were equally fitted to compete for the available resources. Thus those individuals best adapted to their environment ("fittest" in the Darwinian sense) will on average leave a greater number of descendants than other members of the species ("natural selection"). In the case of bacteria growing in a host who has been treated with antibiotics, bacterial variants (mutants) that are unaffected by the antibiotics (or less affected than is the wild-type) will have a reproductive advantage. The antibiotics are said to exert *selective pressure,* and this process is described as *Darwinian selection*. For Darwinian selection to occur, there must be selective pressure. If there is no competition for resources, there will be no selection. When the source of variant bacteria in the presence of antibiotics is genetic mutation, the resulting selection of antibiotic resistance will follow Darwinian dynamics. As we shall see in the discussion (below) in the discussion of horizontal gene transmission, selection can sometimes be non-Darwinian.

Although the word was not in use in 1859, Darwin and Wallace's explanation of natural selection was an algorithm, expressed in plain language. Following the mechanistic description of DNA replication in the 1950s, a number of mathematicians expressed the concept of natural selection in computer language and coined the term genetic algorithm. John Holland, an electrical engineer and computer scientist, realised that the same process that identifies the combination of genes best adapted to a natural habitat could also be used as a general problem-solving technique [2]. A version of his genetic algorithm is shown in Figure 2.

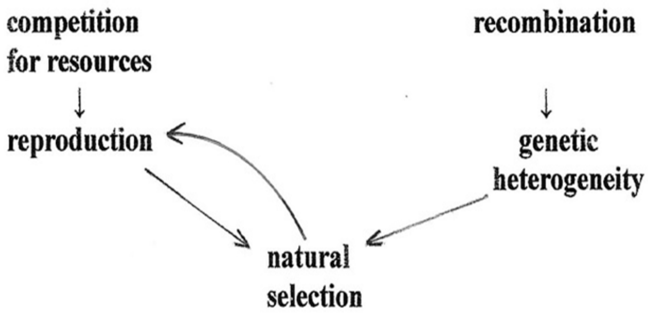

Figure 2: The genetic algorithm.

Holland assumed that the source of variants was recombination, i.e. he modelled combinations of a fixed set of variants, and the term "genetic algorithm" is sometimes reserved for Holland's original assumptions. A broader term, "evolutionary programming" is used to describe algorithms that make different assumptions, such as regarding mutations, rather than recombination, as the source of variation. Recombination is a process that occurs in eukaryotic cell division, and since the subject of the present discussion is bacterial cell division, the source of variation is assumed to be mutation. In other respects, the algorithm used to describe selection of antibiotic resistance is identical to Holland's genetic algorithm. It is an iterative process, involving many repetitions of the process of cell division and selection. In each turn of the cycle, only a fraction of the total population is allowed to progress to the next generation. The decision of which individuals will survive to the next step is made by the *objective function*, described as "natural selection" in Figure 2. The survival of bacterial variants in the presence of antibiotics will be in proportion to their degree of resistance. The selection process is repeated at each cycle of the algorithm, but the enrichment process constitutes a form of positive feedback, so that the score in the objective function increases rapidly from generation to generation.

Mutations may be chemically induced, or caused by exposure to UV or ionizing radiation, but even in the absence of such mutagens there will be a low but finite incidence of spontaneous mutations driven by the fact that the specificity of the base-pairing process in DNA replication has a theoretical limit. The resistance rate, the measured incidence of resistant organisms in a bacterial population, will usually be higher, often much higher, than the mutation rate, because there will potentially be multiple mutations that can contribute to antibiotic resistance.

Antibiotic-resistant mutations, by definition, confer a selective advantage in the presence of the selecting agent, but this advantage comes with a cost: in the absence of the selecting agent, the mutants usually have a slower growth rate, a longer doubling time, than the wild-type. If this were not the case, the mutant would be the wild-type.

The equation of Luria and Delbrück

The study of the evolutionary dynamics in bacteria began in the early 1940s. Luria and Delbrück [3] were studying the biology of bacteriophage ("phage"), viruses that infect and destroy bacteria. They observed that a small proportion of the bacteria (about one in a million) were resistant to phage destruction.

$$\mu = \alpha N(1 - N^{-\alpha-\beta})/ (\alpha + \beta) \qquad \text{equation 7}$$

In equation 7, μ is the number of resistant mutants, α is the mutation rate (the fraction of bacteria that transform from phage sensitivity to resistance per cell division), β is the reversion rate (back-mutation rate), the rate at which resistant bacteria transform back to sensitivity, and N is the number of bacterial cells in the population. Equation 7, called the "fluctuation equation" is derived from the Poisson distribution, the statistical relationship that describes rare, discrete events. It was soon pointed out that a similar relationship also describes resistance to drugs [4].

Antibiotics as selective agents

The evolutionary dynamics of antibiotic-resistance were modelled by combining the equation of Luria and Delbrück with the Gompertz growth curve, and with the cytotoxicity equation of Lloyd [5]. The equations of the model are listed in the supplement, as is the model in the form of a c program (cybermycin.c).

The growth dynamics of bacteria in an infected organism vary according to the bacterial load, its anatomical location, its degree of immunogenicity and the growth parameters (doubling time, quiescent fraction, and cell loss factor). Of the large number of possible scenarios, I have selected three for modelling.

Some bacterial infections proliferate so rapidly that the disease can progress from initial infection to a lethal bacterial load before the innate immune response can be activated, which typically takes 3 – 5 days. Historical accounts of mediaeval plagues tell of individuals who were healthy in the morning being dead within a few hours. Many cases of sepsis appear to progress too fast for the innate immune response to react. Treating such cases requires that an effective antibiotic or combination be administered promptly and antibiotic resistance can make the difference between life and death.

A second category, typified by enterobacterial infections, may involve such a large bacterial load that the innate and adaptive immune responses are overwhelmed. This may be because the gut can contain in excess of 10^{14} bacterial cells, ensuring that antibiotic-resistance mutations (or even double mutants conferring resistance to two unrelated antibiotics) are present from the start of treatment. Where resistance is propagated by horizontal gene transmission the problem is exacerbated by the presence in non-pathogenic bacteria of the intestinal flora of plasmids carrying antibiotic resistance genes, and that these plasmids may cross species barriers.

A third category, typified by tuberculosis, consists of slowly progressing infections, where eventual lethality is the result of cumulative normal tissue damage. Because progression of the disease involves a very large number of bacterial cell divisions the probability of spontaneous antibiotic resistance mutations arising is very high.

Figure 3 summarises a modelling run of treatment of an advanced urinary tract infection, starting with a bacterial load that, in the absence of treatment, would cause lethal kidney toxicity within four days. Daily doses of trimethoprim maintained the count of wild-type bacteria at a tolerable level, but by 8 days the wild-type (sensitive) bacteria were outnumbered by trimethoprim-resistant (R1) mutants which reached a lethal level by 10 days.

Figure 4 shows modelling of a similar infection treated with an alternative antibiotic, nitrofurantoin. Once again, growth of the wild-type bacteria is effectively controlled. A small number of trimethoprim-resistant mutants (bact/R1) appear, but are also controlled by the nitrofurantoin.

However, nitrofurantoin-resistant bacteria (bact/R2) rapidly accumulate and result in treatment failure within 10 days.

Figure 5 illustrates treatment with the combination of simultaneous trimethoprim plus nitrofurantoin, both at their maximum tolerated doses. The combination was curative, within 24h. In this simulation the bacterial count at the start of treatment was assumed to be 1.0 x 10^7 cells/ml. However, if larger inocula were modelled, the result was treatment failure from doubly-resistant (bact/R12) cells dominating after 7 days.

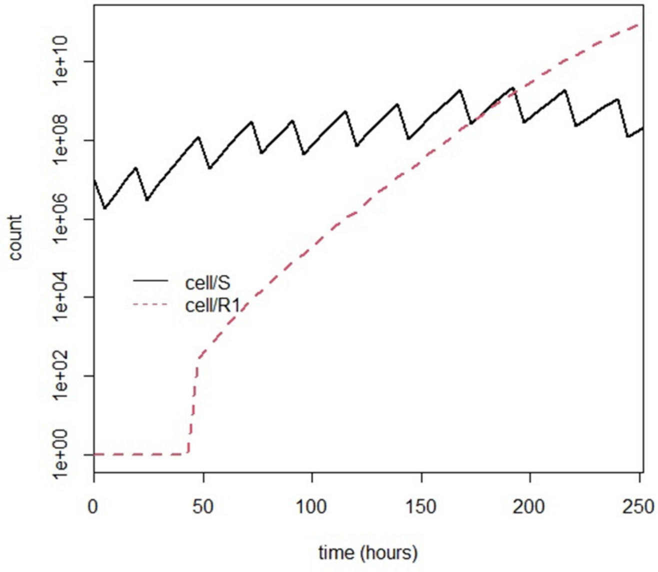

Figure 3: Treatment of an advanced urinary tract/ kidney infection with

trimethoprim, 400 mg daily, as modelled by the cybermycin program

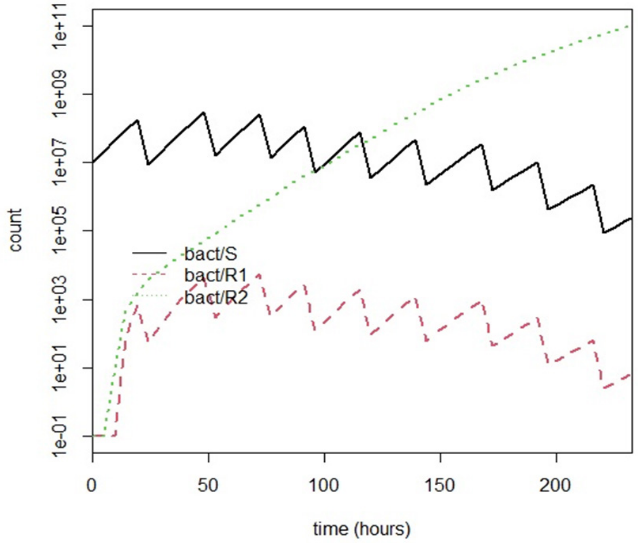

Figure 4: Treatment of an advanced urinary tract/kidney infection with nitrofurantoin, 180 mg daily, as modelled by the cybermycin program

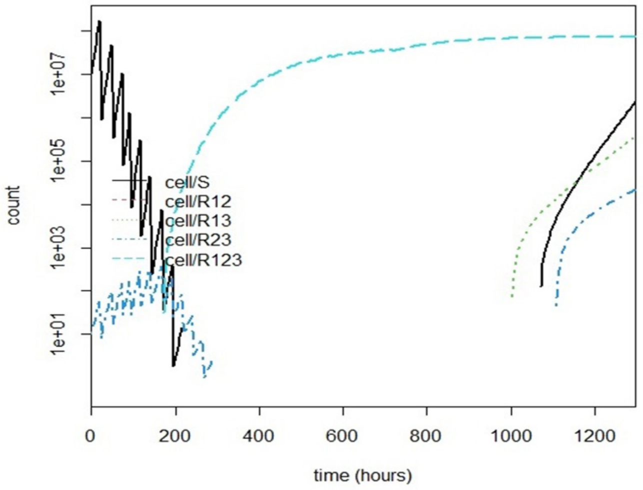

Figure 5: Treatment of an advanced urinary tract/kidney infection with trimethoprim, 400mg plus nitrofurantoin, 180 mg daily, as modelled by the cybermycin program

To summarise conclusions so far: Antibiotic treatment will normally be curative if the bacteria can be eliminated before the appearance of resistant mutants. The probability of resistant cells being present from the start of treatment is a function of the resistance rate (often of the order of 1 x 10^{-6}) and of the bacterial population in the infected organ or tissue.

An example of an infection that is often successfully treated with a single antibiotic is syphilis, which responds (for example) to tetracyclines. Following a course of tetracycline treatment, any surviving spirochaetes in blood or most tissues will be eliminated by the immune system. However, a few bacterial cells will survive in immunological sanctuary tissues, such as nerve cells. These surviving syphilis cells have the potential to emerge from their sanctuary and re-infect the body. The total number of such cells will be low enough that it is unlikely to include any tetracycline-resistant mutants, so the resurgent infection will usually remain tetracycline-sensitive.

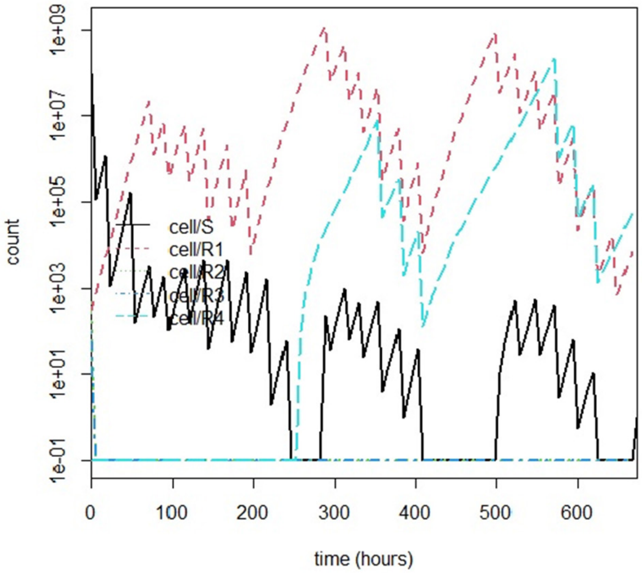

Figure 6: Treatment of an advanced urinary tract/kidney infection with alternating 3-day treatments with trimethoprim, 400mg, nitrofurantoin, 180 mg and sulfamethoxazole, 1600mg, as modelled by the cybermycin program.

When an infection is treated with an antibiotic, if the treatment is subcurative (which will depend upon the bacterial load in relation to the resistance rate) resistance will inevitably arise, and this will become clinically apparent when symptoms of the infection, previously in decline, increase again. At that point, switching to a second, non-cross-resistant, antibiotic will start to eliminate those bacteria resistant to the first antibiotic (bact/R1). Switching antibiotics at an arbitrary time (as in simulations 11 and 12) may be more effective than treatment with a single antibiotic but still suboptimal. For instance, if antibiotic B is less effective than antibiotic A (lower GLK at the MTD), then switching too early will give less kill of the wild-type bacteria (bact/S). Conversely, switching too late will result in a higher population of bact/R1 when treatment with antibiotic B is started, increasing the likelihood of eventual failure from doubly resistant (bact/R12) cells. This dilemma can be avoided by using a diagnostic for bact/R1, and only switching treatment from antibiotic A to antibiotic B when the diagnostic for bact/R1 tests positive. This approach, termed *adaptive therapy*, will be discussed in more detail below.

Some antibacterial agents are *bacteriostatic*, rather than bactericidal, that is, they prevent bacterial cell division but do not kill the bacteria. When the antibiotic is removed, the bacteria will re-enter the cell cycle and continue to proliferate. In principle, if concentrations of a bacteriostatic antibiotic are maintained at a level that completely prevents bacterial growth it should be possible to keep the bacterial count at a tolerated level indefinitely.

In practice, unless the antibiotic is totally non-toxic, long-term control of infection with a bacteriostatic antibiotic may often fail because of cumulative toxicity. Another reason for failure of long-term disease control with bacteriostatic antibiotics (as with bactericidal antibiotics) is the emergence of resistant mutants. While it is believed that most mutations arise from spontaneous errors during DNA synthesis, there is evidence that non-dividing cells can also mutate. Cytosine bases in DNA have a low but finite rate of spontaneous deamination, resulting in uracil, which codes as thymine, representing a point mutation. Both bacteria and eukaryotic cells have evolved mechanisms for removing uracil bases from DNA, which minimises this route of mutation, but such measures may not be 100% efficient. Another type of point mutation that can occur in non-replicating DNA is oxidation of guanine bases at the 8 position. Guanine base-pairs with cytosine, but 8-oxoguanine can base-pair with thymine. Resistance that occurs in static populations will have no immediate effect on disease progression, because in the absence of cell division there can be no selection pressure. However, once cell proliferation recommences, these resistant mutants will again have a growth advantage in presence of the selecting antibiotic.

Cross-resistance and Synthetic Lethality: If two antibiotics have a common mechanism of action, it is likely that mutations that cause resistance to one such antibiotic will also result in resistance to the other (cross-resistance). Cross-resistance may also occur between chemically and mechanistically unrelated antibiotics: for example, antibiotic resistance may be caused by induction of a bacterial efflux pump, a cell membrane protein that removes the antibiotic from the cell. These pumps can have a broad spectrum of activity, so that resistance to one antibiotic by activation of an efflux pump can result in partial or total cross-resistance to other antibiotics that are substrates for the same pump. The opposite phenomenon to cross-resistance is *collateral sensitivity*, where a mutation that makes a bacterial cell less sensitive to one antibiotic can make it more sensitive to a second antibiotic. An example that has been studied mainly in tumour cells, but which also applies to antibacterial therapy, is inhibition of purine nucleotide biosynthesis. Purine nucleotides can be formed from small-molecule precursors (de novo biosynthesis) or from pre-formed purine bases (salvage pathway). Antimetabolite drugs that inhibit purine biosynthesis often themselves require intracellular activation by the salvage pathway, and cells that become resistant to antipurine drugs by deletion of the salvage pathway are then totally dependent upon the de novo pathway, so are then collaterally sensitive to inhibitors of the de novo pathway. This situation is described by geneticists as *synthetic lethality*: either of two mutations or deletions, individually, may be tolerated, but if they occur together the combined effect is lethal.

Resistance to drugs with multiple targets: If a drug has two effective targets, it will behave like a combination of two drugs. For example, if the rate of resistance to inhibition of the first target site is 10^{-6} and the rate of resistance to the second site is 10^{-5}, and if the binding affinities to the two sites are similar, then the probability that a bacterial cell will not respond to such a bifunctional inhibitor will be 10^{-11}. If the binding affinities to the two sites differ, or if one or both of the sites has very tight binding, so that binding to either site results in significant depletion of the free antibiotic concentration the kinetics will be more complex, but still, in general, favourable. However, multi-target agents will still be susceptible to efflux pumps or to resistance that occurs by accelerated metabolic breakdown of the antibiotic.

The Immune System and Antibiotic Resistance

The immune system, like antibiotic therapy, has the ability to kill large numbers of bacterial cells. An understanding of antibacterial immunity is relevant to the antibiotic resistance problem because, in general, the mutations that cause antibiotic resistance do not affect the bacterial cell surface, whereas the bacterial epitopes recognised by the immune system are on the cell surface. As a result, antibiotic-resistant bacteria generally retain their sensitivity to the immune response and to vaccines. In addition, the immune system can kill bacteria regardless of whether they are proliferating

Table 1: Treatment of an advanced urinary tract/kidney infection with trimethprim, nitrofurantoin and sulfamethoxazole, modelled by the cybermycin program. Treatment daily for up to 30 d.

Simulation	Treatment			Survival	Failure from
	Trimethoprim	Nitrofurantoin	Sulfamethoxazole		
1	0	0	0	2.8d	bact/S
2	400	0	0	6.0d	bact/R1
3	0	180	0	8.8d	bact/R2
4	0	0	2000	7.2d	bact/R3
5	400	180	0	4.0d	myelosuppression
6	200	90	0	20.2d	bact/R12
7	200	0	1000	33.2d	bact/R13
8	400	0	2000	cure at 24h	
9	0	90	1000	21.5d	bact/R23
10	200	90	1000	cure at 72h	

In simulation 11, trimethoprim, 400 mg for 6 days followed by nitrofurantoin 180 mg for 6d followed by sulfamethoxazole 1600 mg for 6d. Survival was for 24.8 d with failure from R123. This sequential 3-antibiotic combination was thus less effective than the simultaneous combination of simulation 10.

Simulation 12 compared the effect of alternating 3-day courses of trimethoprim, 400 mg, nitrofurantoin, 180 mg and sulfamethoxazole 1600 mg (Figure 6). Approximate bacteriostasis was maintained for 30 d, then when treatment was stopped after 30d failure at d. 35.9 from bact/R123.

or quiescent. The mammalian immune system consists of a high-capacity/low-affinity system (the innate immune system) coupled to a low-capacity/high-affinity system (the adaptive immune system).

In evolutionary terms, the innate immune system is the earliest. Primitive multicellular organisms, such as coelenterates, contain mobile, phagocytic cells that move freely within the body cavity and engulf and digest cellular debris. These cells are the ancestors of macrophages, which retain the power of phagocytosis but have acquired additional properties, including the ability to secrete cytokines. When mammalian macrophages are activated by bacterial cell wall components (primarily lipopolysaccharide, LPS) they release interleukin 8 (IL-8) which attracts blood neutrophils to the site of infection. Activated macrophages also release tumour necrosis factor alpha (TNFα) which activates tissue neutrophils. Neutrophil activation results in formation of hypochlorite, which is strongly bactericidal. Tissue neutrophils are normally short-lived, entering spontaneous apoptosis after a few hours. However, activated neutrophils up-regulate the anti-apoptotic proteins Mcl-1 and Bcl-X_L, which effectively immortalises them for the duration of the infection. Neutrophil activation is strongly bactericidal but if sustained can be toxic to normal tissues of the host animal or patient. For this reason it will normally turn off after a few days, by a number of mechanisms, including release of the anti-inflammatory cytokine IL-10. In addition to control of innate immunity by negative feedback, positive feedback loops switch on the adaptive immune response, the low-capacity high affinity system that mops up bacteria that may have survived the innate immune response. Unlike the innate immune response, whose main effectors are myeloid cells, the effectors of the adaptive immune response are lymphoid cells. It has two main arms, B lymphocytes and T lymphocytes (there are also non-B, non-T lymphocytes, such as NK cells). B cells secrete antibodies that can destroy extracellular pathogens, and are thus primarily responsible for eliminating bacterial infections. T cells can detect intracellular infection and play the primary role in protection from viral disease. However, some bacterial diseases, including tuberculosis are at least partially intracellular. Activated B and T cells are highly specific to their target pathogens. When adaptive immunity has eliminated a bacterial infection, the activated B cells and T cells undergo apoptosis. However, a small fraction of the activated cells survive as B-memory cells or T-memory cells. These memory cells are protected from apoptosis by expression of anti-apoptotic proteins, but they do not proliferate. In the event of re-infection, or following vaccination, the memory cells are rapidly reactivated and start to proliferate.

Immunotherapeutics are treatments that, rather than directly killing pathogens, act by stimulating the immune system. Clinically, the most important immunotherapeutics are vaccines (which may be either prophylactic or therapeutic) but small-molecule drugs (including many natural products) have been used as macrophage activators, and to a lesser extent as NK-cell activators. daSilva et al. [6] discuss the example of mitoxantrone, which by activating macrophages was able to overcome vancomycin resistance in Enterococcus faecalis. A review of immunotherapeutics is beyond the scope of the present discussion, except to note that they represent a possible strategy for minimizing AMR.

Modelling the immune response

After the central nervous system, the immune system is the most complex organ system of the mammalian body. Like the nervous system, the immune system is an information-processing system. Like the nervous system, the immune system is capable of learning, memory, self/non-self discrimination, and pattern recognition. Because of the great complexity of the immune system, numerous computer models of it have been described. A model, myeloM5, that includes over 40 variables (cell lines, cytokines, and transcription factors was published by Jackson and Radivoyevitch [7]. The cybermycin model includes a simplified version of myeloM5. In this model, cellular immunity kills a constant number of bacterial cells per activated immune cell and humoral immunity kills a constant fraction of bacteria per activated B cell. Unlike the adaptive immune response, the innate immune response is equally active against most bacterial types, so long as they contain LPS in their cell wall. When tissue macrophages are activated by LPS they release TNF. Activation of Abl by TNF then activates the transcription factor STAT5, which in turn increases production of IL-3, GM-CSF and M-CSF. Abl also activates the neutrophil-specific antiapoptotic protein, Mcl-1, resulting in the normally short-lived tissue neutrophils surviving for the duration of the infection. Figure 7 shows the complete elimination of a small number of bacterial cells by the innate immune response. Note that this plot only shows 5 of the 40+ variables tracked by the model.

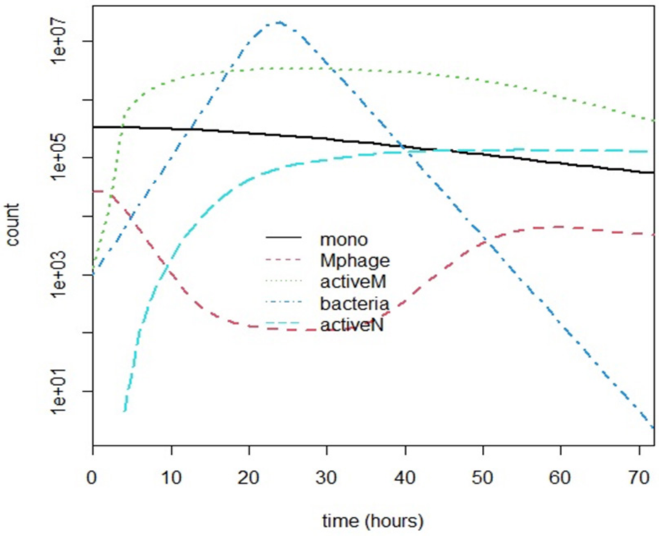

Figure 7: Simulation 7. Activation of the innate immune response by instantaneous infection with 1e+3 bacterial cells as modelled by the cybermycin program.

When the bacterial challenge is larger, the unaided innate immune response is overwhelmed and unable to have much effect on the course of the disease (Figure 8). Figure 9 repeats the simulation of Figure 7 (i.e. bacterial challenge of 1,000 cells) but with a slightly less immunogenic bacterial strain. Up to 100 hours the events of simulation 9 parallel those of simulation 7: macrophages are activated, neutrophils are activated, and the bacterial count peaks at around 24 hours and then declines. The difference is that in simulation 7, the decline in bacterial count continues to zero. By 100 hours the adaptive immune response is triggered, and the infection is cured – whereas in simulation 9 the weaker adaptive immune response is unable to eliminate the small number of cells that survive the innate immune response, and because after 150 hours the innate immune response is switching off these surviving bacteria are able to proliferate and cause relapse.

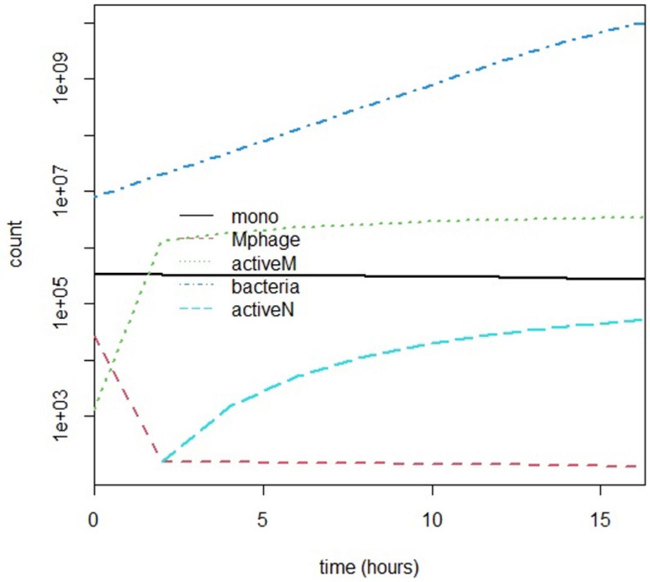

Figure 8: Simulation 8: sub-curative immune response to 8e+6 bacterial cells

In the absence of antibiotics, there are three possible outcomes to infection. Depending upon the degree of immunogenicity of the pathogen and the size of the inoculum, the infection may be completely eliminated, either by the innate immune system alone or by the combined effects of the innate and adaptive immune systems, as in simulation 7. For larger inocula, the bacterial load may reach a lethal level (simulation 8) or for more immunogenic pathogens bacterial proliferation and immune destruction of the bacteria may reach a steady state with the result of chronic infection. In such cases, the more immunogenic the pathogen, the lower the steady-state bacterial count. Because the innate and adaptive immune systems are equally active against antibiotic-sensitive and -resistant cells, antibiotic resistance is particularly dangerous to immune-deprived patients.

The dynamics of extremely virulent infections are such that the bacteria may proliferate to a lethal burden before the innate immune response is triggered (<~6h). Based upon historical accounts, this seems to have been the case with some mediaeval plagues. Immune killing, which requires cell division of B cells or T cells, must be a function of time, so for highly virulent infections the amount of immune function is irrelevant. Even in vaccinated patients, the proliferation of memory cells would be too slow for an effective immune response.

Since this rapid lethality would limit the number of individuals infected, these epidemics would be self-limited. Since these extreme infections are so hard to treat, prophylactic measures including isolation and vaccination must be considered, and perhaps prophylactic drug treatment for high-risk groups such as medical personnel.

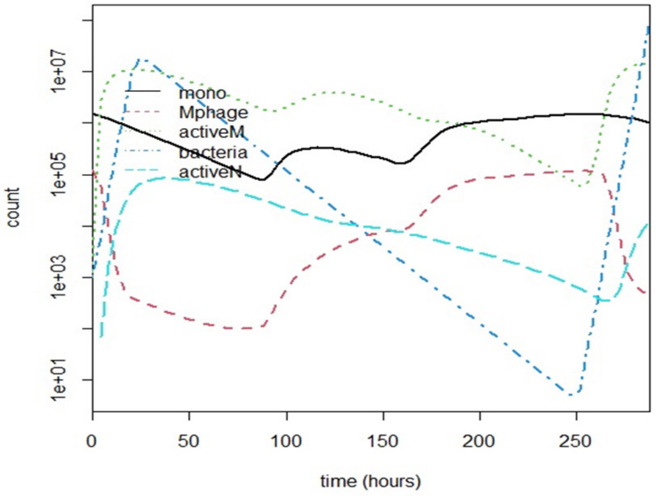

Figure 9: Simulation 9: Recurrence in a less immunogenic infection.

Dynamics of Horizontal Gene Transmission

The genetics of bacteria differ markedly from eukaryote genetics. Most bacteria have a single circular chromosome. As with eukaryotes, replicating bacterial cells copy their DNA once, and only once, in each cell cycle and each daughter cell receives one of the resulting daughter chromosomes. This is termed vertical gene transmission (VGT). Many types of bacteria can obtain DNA by another process, unrelated to cell division: horizontal gene transmission (HGT). Three mechanisms of HGT have been characterised. *Transformation* is the uptake of free DNA from the extracellular medium. It has been studied in vitro, and rate constants measured [8]. Of the DNA fragments that are internalised by bacterial cells, it is estimated that about one in 2,000 are incorporated into the bacterial genome. Transformation is not believed to make a major contribution to antibiotic resistance. *Transduction* is a process in which genes located in a plasmid are transferred from one bacterial cell to another by a phage. Transduction is a significant cause of antibiotic resistance in both gram-positive and gram-negative bacteria. The small plasmids involved in transduction may carry a single antibiotic resistance gene, or occasionally genes for resistance to two antibiotics. In staphylococci, at least, the plasmids involved in transduction are highly strain-specific. *Conjugation* is a form of HGT in which a plasmid is transferred from one bacterial cell to another by direct cell-cell contact, mediated by the cell surface pili. In gram-negative bacteria conjugation is the dominant mode of HGT, and the plasmids involved, known as R factors, often contain multiple antibiotic resistance genes. These plasmids contain genes required for the conjugation process and for plasmid replication. Conjugation can occur between bacterial cells of different species. This can be of great practical importance, for example, in intestinal infections, where large numbers of non-pathogenic bacteria may carry plasmids bearing antibiotic-resistance genes, and pass them on to otherwise antibiotic-sensitive pathogens. Although HGT can occur in eukaryotic cells [9] it is much commoner in bacteria.

The size of plasmids ranges from about 1,000 to one million base pairs; this compares with the typical bacterial genome of 139,000 to 13 million base pairs. The number of plasmids per bacterial cell (the copy number) varies from one to several hundred.

Kinetics of HGT

Conjugation involves cell-cell contact between the plasmid donor cell and the recipient cell. It is thus a second-order process:

vhgt = Ns . Nr . khgt (equation 8)

where Ns and Nr are numbers of drug-sensitive and -resistant cells, respectively, khgt is a second-order rate constant and vhgt is the rate of horizontal gene transfer, cells transfected per unit time [10].

Plasmid loss may occur when a bacterial cell divides and plasmid replication has not kept pace with chromosomal replication, or when chromosomal DNA and plasmid DNA do not segregate together during cell division.

Plasmids represent a cost to their host cell because additional DNA has to be replicated. There is a relationship between plasmid size and cell doubling time [11]. The doubling time for HGT-mediated antibiotic-resistant cells may thus be longer than for antibiotic-sensitive cells. It has thus been suggested that antibiotics that act through inhibition of DNA synthesis (trimethoprim, DNA gyrase inhibitors) may have selective efficacy against plasmid-bearing cells vis-à-vis non-plasmid-bearing cells. This may be true in a kinetic sense, but because of the non-Darwinian dynamics of HGT (discussed below) such antibiotics can only "cure" plasmids if they kill 100% of the plasmid-bearing cells in the population (difficult, but possible in immunocompetent patients).

Implications of HGT for antibiotic resistance

Horizontal transfer of drug resistance genes in bacteria is believed to occur primarily by conjugation [12]. In many bacteria, including gram-negative organisms such as E. coli, Klebsiella, Acintebacter and Pseudomonas, antibiotic resistance has been shown to be both chromosomal and plasmid-mediated. HGT is autocatalytic; from equation 8 it may be seen that the greater the number of Nr, the faster the rate of vhgt. Unlike VGT, HGT is never spontaneous. When Nr is zero vhgt must be zero. It follows that AMR due to HGT can be prevented by strict isolation. For non-zero Nr only one steady state is possible: 100% Nr and zero Ns. HGT is thus non-Darwinian. In Darwinian natural selection, a variant that possesses a growth advantage, however small, must eventually dominate the population. With HGT, plasmid or phage-bearing cells can eventually outgrow non-HGT cells that would otherwise have a growth advantage, e.g. because of a shorter doubling time.

The other generalisation that can be made about HGT is that if two or more antibiotic-resistance genes are carried on a plasmid, bacteria that are resistant to one of those antibiotics will be cross-resistant to the other antibiotics, even when they are chemically or mechanistically unrelated.

Modelling HGT

The background mutation rate in bacteria is estimated at ~1 x 10^{-8} per locus per chromosome replication. It is likely that the rate of horizontal transmission is much higher. The copy number of the plasmids involved in conjugation ranges from 1 to several hundred. The F-plasmid (fertility plasmid) involved in bacterial sexual conjugation has only one copy per cell. Its replication and transfer of the entire *E. coli* genome is estimated to take ~ 100 min. Appearance of resistance by conjugation was modelled as second-order, since it involves cell-cell contact. Loss of plasmids, by contrast, occurs both by cell-cell contact (since the copy number of the donor is decreased) and by plasmid replication not keeping up with cell division, which is first-order.

The cybermycin model of evolutionary dynamics of antibiotic resistance allows for treatment with up to four antibiotics.

Table 2: Treatment of an advanced infection with antibiotic combinations when imipenem resistance is caused by VGT and HGT.

Treatment (mg/day)			**imipenem resistance mode**	**Survival (d)**	**Failure From**
Trimethoprim	nitrofurantoin	imipenem			
0	0	0	VGT	4.9	S
0	0	0	HGT	4.3	R4
150	0	0	VGT	14.2	R1
150	0	0	HGT	14.2	R4
0	90	0	VGT	14	R2
0	90	0	HGT	4.3	R4
0	0	800	VGT	5	R4
0	0	800	HGT	5.7	R4
150	0	800	VGT	>30	
150	0	800	HGT	14.2	
150	90	800	VGT	cure at 3 d	
150	90	800	HGT	14.2	

Resistance may arise to any of them, and in any combination. When resistance is caused by VGT it may be due to pre-existing mutations or to spontaneous mutation during the course of treatment. When HGT was modelled, resistance required that at least one plasmid-bearing bacterial cell was present from the outset of infection. Table 2 shows calculations of predicted effects of treatment with trimethoprim, nitrofurantoin, and imipenem. Resistance to trimethoprim was always caused by HGT. Resistance to imipenem and nitrofurantoin could be the result of VGT or HGT. It was assumed that the plasmid carried genes for resistance to both nitrofurantoin and imipenem.

The untreated infection was predicted to cause death in under 5 days. Treatment with any of the three antibiotics as single agents gave 5 to 10 days of increased survival, regardless of whether resistance was due to VGT or HGT, with treatment failure due to emergence of resistant mutations (or plasmid transfection in the case of HGT). Treatment with the combination of trimethoprim and imipenem resulted in low-level chronic infection for the duration of treatment in the case of VGT. However, in the case of imipenem resistance due to HGT, treatment with the two-drug combination was no more effective than treatment with trimethoprim alone, because the plasmid carried genes for resistance to both nitrofurantoin and imipenem. When resistance was caused by VGT, the 3-antibiotic combination was curative, but once again in plasmid transfected cells the 3-drug combination was no better than trimethoprim alone. When resistance is the result of HGT, reversion from antibiotic-resistance to sensitivity may occur as a result of spontaneous plasmid loss. At the population level, the wild-type (antibiotic-sensitive) cells may outgrow the resistant cells in the absence of selection pressure because the plasmid-bearing resistant cells tend to have longer doubling times, though as noted above, in presence of selection pressure, the non-Darwinian nature of HGT can result in plasmid-bearing cells outgrowing the wild-type even when their doubling time is longer.

Resistance Blockers

Bacteria will become resistant to the simultaneous combination of two non-cross-resistant antibiotics less often than to either antibiotic used as a single agent because two mutations are required to create double resistance, and the frequency of double mutants will be roughly a million-fold lower than that of a single mutation. The dynamics of resistance to a combination of one antibiotic with a blocker of resistance to that antibiotic will be similar to the dynamics of simultaneous resistance to two antibiotics. There are two caveats to this statement: first, since binding of the resistance blocker is assumed to be saturable, its effect will only approach that of a second antibiotic if it is used at a concentration that is close to saturating. Second, if the resistance blocker does not itself have antibacterial activity there will be no selection pressure against the resistance blocker, and in this respect the combination of one antibiotic plus a resistance blocker will be superior to a combination of two antibiotics. An example of this kind is the combination of a beta-lactam antibiotic (e.g. a penicillin) with a beta-lactamase inhibitor (e.g. clavulanic acid). The commonest mechanism of resistance to penicillins is induction of beta-lactamase, which since opening the lactam ring of penicillins abolishes their biological activity results in almost total resistance. Inhibiting the beta-lactamase with clavulanic acid restores the penicillin sensitivity. A combination of amoxicillin and potassium clavulanate is widely used to treat infections of the ear, throat and urinary tract [13].

Another resistance mechanism often found in tuberculosis is removal of the anti-TB drugs from the mycobacterial cells by activation of efflux pumps in the bacterial cell membrane [14]. Since these efflux pumps are susceptible to inhibition by calcium channel blockers, such as verapamil, these agents are being explored as an approach to restoring antibiotic sensitivity in resistant TB. Another approach to blocking antibiotic resistance, when this s caused by cell membrane changes, is though the membrane-disrupting agent HT61 [15-17]. HT61 can sensitise bacteria that have acquired resistance to tobramycin and other antibiotics, but the dynamics of this process are complicated by the fact that HT61 itself has antibacterial activity.

Another intensively studied combination is that of the antifolates trimethoprim, an inhibitor of bacterial dihydrofolate reductase (DHFR) and sulfamethoxazole (which inhibits dihydrofolate synthesis). The combination of an inhibitor of DHFR with an agent that depletes its substrate, dihydrofolate, is a classical instance of a synergistic biochemical sequential blockade. As such, it can reverse trimethoprim resistance which often results from amplification of the DHFR gene. If a bacterial cell, as a result of DHFR gene amplification is seven-fold resistant to trimethoprim, and sulfamethoxazole because of its synergistic biochemical interaction, has restored trimethoprim sensitivity, then the sulfamethoxazole may be said to have reversed the trimethoprim-resistance. However, the dynamics of this combination is complicated by the fact that sulfamethoxazole itself has antibacterial activity, so that trimethoprim and sulfamethoxazole constitute a pair of non-cross-resistant antimicrobial agents,

If the resistance factor of a bacterial mutant, RF_0, is defined as MIC(mutant)/MIC(wild type), and a resistance modifier has binding constant Ki, then a concentration Crm of resistance modifier will give Crm/Ki/(1 + Crm/Ki) fraction of the maximal reversal, RevF, e.g. if the resistance factor (in absence of the resistance modifier) = 40, Crm =30, with Ki of 10, and RevF of 5.0-fold, then the resistance factor in presence of the resistance modifier will be

$$RFcrm = RF_0 / Crm/Ki/(1 + Crm/Ki) * RevF = 40/(30/10/(1+30/10) * 5.0) = 10.67 \qquad \text{equation 9}$$

This relationship can be modelled by the cybermycin program. Figure 10 shows simulated treatment of an advanced kidney infection with trimethoprim, 400 mg/day. After 5 daily treatments the count of the wild type bacteria is reduced to zero, but trimethoprim-resistant (R1) bacteria have emerged and by 9 days have reached a lethal bacterial count.

Figure 11 shows the effect of adding a resistance blocker to the treatment. Emergence of resistant mutants is suppressed, and the regimen was curative after 7 days of treatment.

The assumption that resistance modifier dynamics follow saturation kinetics seems appropriate in the case of efflux pump inhibitors, at least. The other key assumption is that there is no selective pressure against modifiers in the absence of the drugs that they modify.

Using the cybermycin program to predict the incidence of treatment failure suggested, as a first approximation, that a combination of two non-cross-resistant antibiotics plus a compound that blocked resistance to one of them could have the same success rate as a combination of three non-cross-resistant antibiotics. However, this assumes that the resistance blocker is 100% effective, which (for a blocker with saturation kinetics) would only be achieved at infinite concentration.

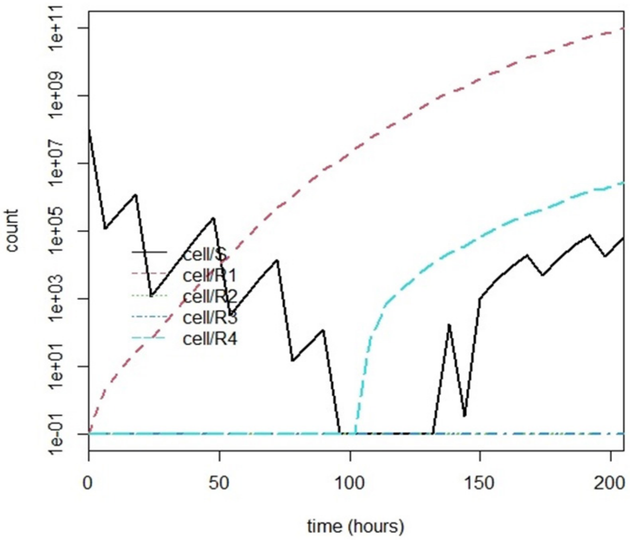

Figure 10: Treatment of an advanced kidney infection with 400mg/d trimethoprim, modelled by the cybermycin program

The effect of a hypothetical blocker of nitrofurantoin resistance on the efficacy of chronic treatment of a urinary tract infection was modelled. This group of simulations showed that nitrofurantoin treatment maintains bacteriostasis for a few days, before being overcome by R2 (nitrofurantoin-resistant) cells which resulted in high-level chronic infection. A moderate dose of a nitrofurantoin resistance blocker decreased the chronic disease burden by >1 log (Figure 12), and a high dose of the blocker was curative (Figure 13).

Mhapankar et al. [18] explored the use of β-lactamase inhibitors, efflux pump inhibitors and membrane permeabilisers in combination, and showed that it was possible to obtain synergistic breaking of resistance to commonly used antibiotics.

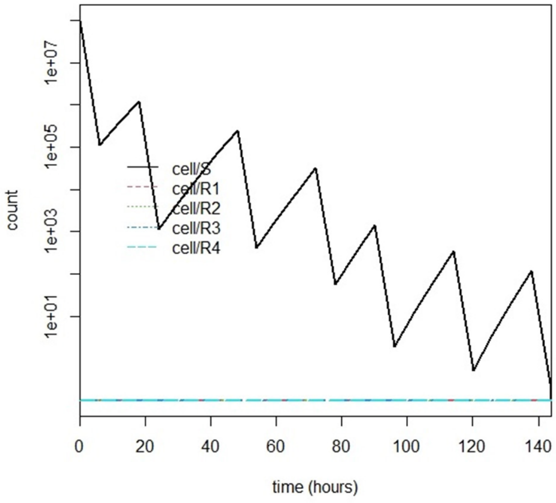

Figure 11: Treatment of an advanced kidney infection with 400mg/d trimethoprim plus a resistance blocker, modelled by the cybermycin program.

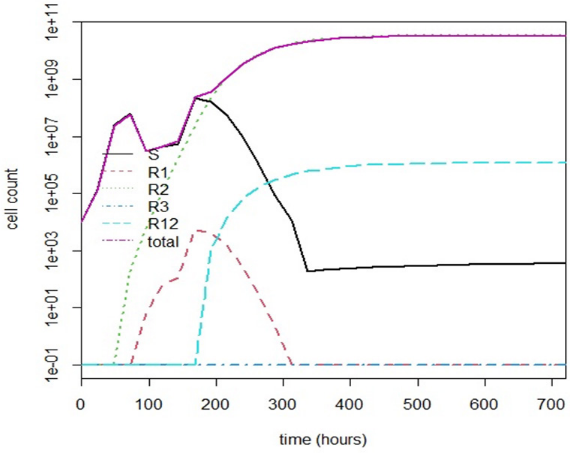

Figure 12: Treatment of a chronic urinary tract infection with 180mg/d nitrofurantoin plus a resistance blocker, modelled by the cybermycin program.

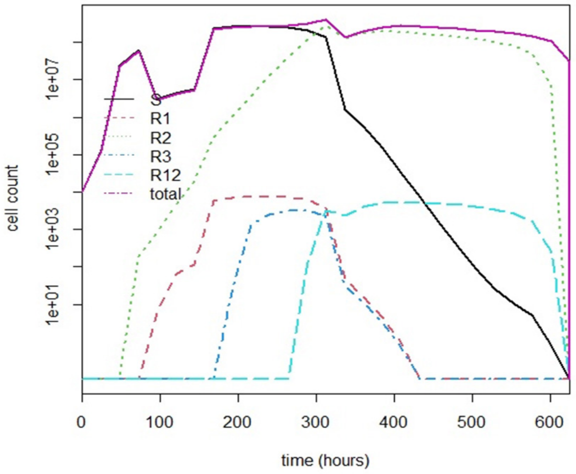

Figure 13: Treatment of a chronic urinary tract infection with 180mg/d nitrofurantoin plus high-dose resistance blocker, modelled by the

cybermycin program.

Table 3: Treatment of advanced kidney infection modelled by the cybermycin program.

Treatment mg/d			survival (d)	Failure from	Other outcome
Trimethoprim	**Nitro-furantoin**	**R1 blocker**			
0	0	0	4.9	S	
150	0	0	11.1	R1	
0	100	0	12.7	R2	
150	80	0	>30		4.09e6 R12 at 30 d
150	100	0	>30		6.88e6 R2 at 30 d. (ss)
150	0	100	>30		7.55e8 S at 30 d. (ss)

ss: steady-state.

Table 3 shows calculated results of treatment of an advanced kidney infection with combinations of trimethoprim, nitrofurantoin and a blocker of trimethoprim resistance. Trimethoprim and nitrofurantoin as single agents both prolonged survival, but in both cases treatment failed from resistant disease. A combination of trimethoprim with low-dose nitrofurantoin resulted in chronic infection with double mutant (bact/R12) bacteria. The combination of trimethoprim with a trimethoprim resistance blocker resulted in chronic infection with trimethoprim-sensitive bacteria (bact/S). Note that failure from bact/S resulted in a higher steady-state bacterial count than failure from bact/R2 or bact/R12, perhaps because bact/S has a shorter doubling time than bact/R2 and bact/R12.

Table 4 shows results of similar calculations, in this case in a more immunogenic strain of bacteria. In this case, the combinations of trimethoprim + low-dose nitrofurantoin and trimethoprim + trimethoprim-resistance blocker, rather than turning an otherwise lethal infection into a chronic condition, were curative.

Table 4: Treatment of advanced kidney infection modelled by the cybermycin program.

Treatment mg/d			survival (d)	Failure from	Other outcome
Trimethoprim	**Nitro-furantoin**	**R1 blocker**			
0	0	0	4.9	S	
150	0	0	11.1	R1	
0	100	0	12.7	R2	
150	80	0			cure at 14 d
150	0	100			cure at 27 d

In summary, as shown by the clinical utility of penicillins in conjunction with beta-lactamase inhibitors, resistance blockers play a useful role in overcoming antibiotic resistance. Combinations of an antibiotic with a resistance blocker have advantages and disadvantages compared with a combination of two non-cross-resistant antibiotics. The chief advantage is that resistance blockers are not subject to selection pressure, so unlike adding a second antibiotic, their efficacy will not be compromised by the emergence of double mutants. A disadvantage is that, whereas a non-cross-resistant second antibiotic will be fully active against bacteria resistant to the first antibiotic, a resistance blocker, which follows saturation binding kinetics, will only be fully active at concentrations much higher than its binding constant. If the resistance blocker has very low toxicity, as with the beta-lactamase inhibitors, in practice this is not a major disadvantage.

The consequences of intracellular infection

Mycobacteria are a family of bacteria whose cell wall contains a waxy outer layer, containing a characteristic lipid, mycolic acid, that makes them highly resistant to immune attack by the host. The two commonest human diseases caused by mycobacteria are tuberculosis (TB) and leprosy. Compared with most bacterial genera, mycobacteria have rather long cellular doubling times, ranging from several hours to a few days; progression of human infections is thus quite slow in comparison with other infectious diseases. Mycobacteria are capable of surviving for long periods in a dormant form, non-proliferating and with very low metabolic activity. After an initial infection, Mycobacterium tuberculosis can remain dormant for many decades, during which time it may cause no symptoms in the host but will still be capable of infecting others.

In population biology, it has been suspected since Darwin's voyage on the “Beagle” that evolutionary change occurs faster in islands than in continental land masses. When a mutation occurs in an individual living either on a small island or on the continent, it will survive, or become extinct, depending on whether it can compete for resources with the existing (wild type) population. However, on an island (or in any small, isolated community) there will be many fewer competitors than in a large interconnected community. A similar principle applies in the form of cellular evolution that leads to antibiotic resistance. Tuberculosis (TB) is a pathogen whose cells can survive either in the pleural fluid, or inside the lung macrophages that may have phagocytosed them. To cure TB, it is necessary to kill or inhibit both the extracellular and intracellular mycobacterial cells. When antibiotic resistance mutations arise, the dynamics of drug response will differ markedly between intracellular and extracellular bacterial populations. Antibiotic resistance in extracellular TB cells will follow the genetic algorithm dynamics described above, and can be described by software such as the cybermycin program (supplement). The mutant cells will be competing with all the other bacteria in the infected organ, e.g. (in the case of TB), the lungs. When a similar mutation occurs in an intracellular bacterial cell, the cell carrying that mutation will only be competing with the other bacterial cells in that particular macrophage. Each of the ten million or so lung macrophages may carry mycobacteria expressing a unique combination of mutations.

Drug resistance in tuberculosis

TB is treated with combinations of up to four antibiotics, and resistance to all four is commonly encountered. In the case of extracellular infections, combinations of four antibiotic resistant mutations can be eliminated by drug combinations, even when horizontal gene transmission (HGT) is involved (HGT does not occur in TB). The disease cannot be described by a genetic algorithm involving multiple mutations in a single population, rather each of the ten million or more macrophages in an infected lung may carry a different combination of mutations, in competition with different numbers of drug-sensitive bacteria. To model each infected macrophage as a genetic algorithm would be computationally intractable, but by making simplifying assumptions, alternative computational approaches can be explored, as will be described below.

In addition to the altered evolutionary dynamics, treatment of intracellular infections is more complex than treating extracellular infection because the drugs must be able to penetrate cell membranes, eliminating in most cases the use of macromolecules or electrically charged small molecules. Intracellular infections are, however, subject to attack by the host's immune system. The innate immune system probably evolved to control both extracellular pathogens (mainly bacteria and fungi) and intracellular pathogens (mainly viruses, but also bacteria such as TB and protozoa such as malaria). When the innate immune system switches off, the adaptive immune system is triggered, and B cells are largely responsible for controlling extracellular pathogens, and T cells for combating intracellular infection. The lung damage that can kill untreated TB patients is the result of oxidative damage resulting from chronic activation of innate immunity, it is an inflammatory response [7].

Streptomycin, a member of the aminoglycoside family of antibiotics, was the first drug shown to have clinical activity against human TB [12]. It is a protein synthesis inhibitor whose selectivity arises from structural differences between bacterial and eukaryotic ribosomes. Streptomycin resistance commonly results from point mutations in the *str A* gene that codes for the streptomycin binding site on the ribosome. Resistance can also result from decreased membrane transport or from increased synthesis by mycobacteria of enzymes that cause structural modification (and resulting inactivation) of the streptomycin molecule. Streptomycin, like other aminoglycosides, is not metabolised by humans, but is excreted unchanged by glomerular filtration. Streptomycin is not orally bioavailable, and is administered by intramuscular injection. Prolonged streptomycin treatment can cause ototoxicity and nephrotoxicity. It is still used clinically in combination for treatment of severe TB, but for early treatment it has been largely replaced by the orally active agents discussed below.

Isoniazide is an analogue of nicotinic acid with selective activity against mycobacteria. This selectivity is attributed to its inhibition of the synthesis of mycolic acid. It is bacteriostatic at low concentrations, and bactericidal at higher concentrations. It is much more active against proliferating cells than against dormant cells. Isoniazide is orally active, and is metabolically inactivated by acetylation. It requires activation by a mycobacterial catalase-peroxidase. For latent TB it can be used as a single agent, but for active disease it is used in combination, often with rifampin, ethambutol and/or pyrazinamide. Its side-effects include elevated liver enzymes in the blood (an indication of liver damage) and peripheral neuropathy, often manifested by tingling and numbness of the fingers.

About 4% of TB patients are resistant to isoniazide, and acquired resistance can occur in previously sensitive infections. In vitro, acquired resistance has been shown to occur with a frequency of about 2 x 10^{-6} per bacterium per cell division. The predominant mechanism of resistance is impaired membrane permeability.

Rifampin (rifampicin) is a selective inhibitor of bacterial RNA polymerases. Resistance to rifampin arises by single-step mutations that produce altered RNA polymerase. It is a very lipophilic molecule, orally bioavailable, and readily penetrates cell membranes, including those of mycobacteria. Following oral dosage, therapeutic concentrations appear in pleural fluid and cerebrospinal fluid within a few hours. For treatment of tuberculosis it is administered daily for at least six months. It is normally used in combination with other antibiotics, but sometimes used as a single agent in treatment of latent TB. The commonest side effect of rifampin is gastrointestinal (nausea, diarrhoea) and it can also be toxic to the liver.

In addition to acquired resistance by genetic mutation, mycobacteria can become tolerant to rifampin by up-regulation of efflux pumps [14].

Ethambutol, like isoniazid, acts by inhibiting mycobacterial cell wall synthesis, through blocking mycolic acid incorporation. Though its mechanism of action is related to that of isoniazid, the two agents are not cross-resistant, so are often used in combination. The current treatment of TB often uses the four-drug combination: isoniazide, rifampin, ethambutol, and streptomycin, though treatment often begins with a three-drug combination. Nevertheless, simultaneous resistance to the combination of the three orally active agents, or even to all four agents, is often seen. The calculations above showed that a simultaneous combination of three non-cross-resistant antibiotics would usually avoid the emergence of resistance. What is different about TB? As discussed below, it is probable that answer lies in the complex evolutionary dynamics resulting from the fact that the TB mycobacteria can survive and proliferate both extracellularly, in the pleural fluid, and intracellularly, including within the pneumocytes. Both intracellular and extracellular mycobacteria can evoke an inflammatory response, which can eventually cause lethal damage to pneumocytes.

Pharmacological sanctuaries

Apart from its effect on resistance rates, the partially intracellular nature of TB infection has pharmacological consequences for its treatment. Streptomycin, in particular, penetrates cell membranes poorly, and is much less effective against intracellular mycobacteria than against extracellular mycobacteria. The mycobacteria causing intrameningeal tuberculosis may be partially protected from water-soluble antibiotics by the blood-brain barrier.

Evolutionary dynamics of drug resistance in tuberculosis

Models that combine evolutionary dynamics with pharmacokinetic/pharmacodynamic (PK-PD) simulations have been described in the literature [19]. The model of Clewe et al. [20] focused on different growth states and their implications for antibiotic susceptibility. Each of the ten million or more tissue macrophages present in the lungs may carry mycobacteria expressing a different combination of antibiotic-resistance mutations, and this complex situation is not amenable to modelling by evolutionary dynamics software such as the cybermycin program described in the supplement.

In evolutionary theory, small, isolated populations whose genotype diverges from that of the main population are sometimes described as examples of the "island effect" - sometimes "Galapagos effect" – and this can be attributed to differences in the competitive environment between the island and the continent. In fact, when a new mutation arises on a small island the isolated mutants will be competing with a much smaller wild-type population than they would on the continent, so their probability of becoming an established variant population is greater, or to put it another way, populations evolve faster on small islands than on larger land masses. The relationship between intracellular mycobacteria in lung macrophages and those in the pleural fluid is somewhat analogous. There is interchange between the intracellular and extracellular mycobacterial populations, but because of the poorly permeable mycobacterial cell wall the intracellular population may be considered as partially isolated – it is not in equilibrium with the extracellular population. Phenotypic change, including drug resistance, arises faster in the intracellular bacterial population than in the pleural fluid. When a mutation arises in the intracellular environment it is only competing with the small number of wild-type bacteria in that particular macrophage, rather than the entire wild-type population in the lungs. Modelling the emergence of antibiotic resistance with the software described in the online supplement showed that in the presence of an antibiotic, resistant variants were selected at the same rate in the intracellular and extracellular environments. The competitive pressures are the same – assuming the intracellular and extracellular antibiotic concentrations are the same. This assumption is likely to be correct for highly lipophilic drugs, such as isoniazid, probably less so for more hydrophilic molecules such as streptomycin. However, in the absence of the antibiotic, with no selective pressure favouring the wild-type, the background proportion of resistant mutants tended to be higher in the intracellular environment than in the extracellular environment. Then, when antibiotic treatment was started subsequently, resistance emerged faster in the macrophages than in the pleural fluid.

The model of tuberculosis used to study the evolutionary dynamics of TB assumes that mycobacteria can exist both in pleural fluid or within cells in the lung. The mycobacteria evoke an inflammatory immune response which leads eventually to the death of pneumocytes. Eventually the lungs are unable to maintain the necessary oxygen tension to sustain life, and the patient dies, either from heart failure or brain damage.

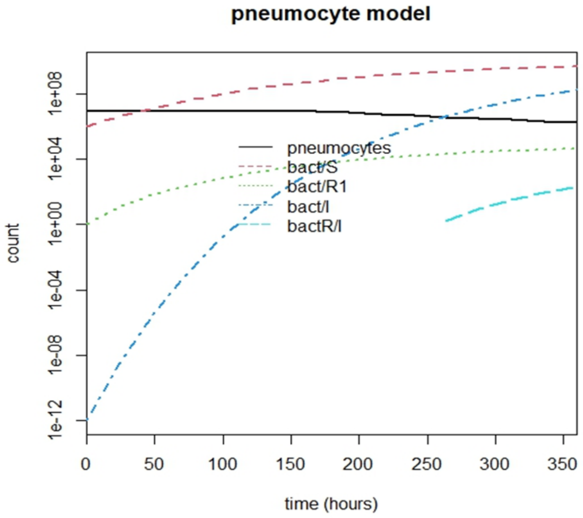

Figure 14: Data of table 5, showing the decline in pneumocyte numbers, and the accumulation of intracellular mycobacteria (bact/I) and the abrupt emergence of antibiotic-resistant intracellular mycobacteria.

Table 5: Modelling terminal stage of an untreated tuberculosis infection.

Time(h)	pneumocytes	bact/S	bact/R1	bactT	Ibact	IbactR	Ratio I/E
0	9.00E+06	1.00E+06	1.00E+00	1.00E+06	0	0.00E+00	0
24	9.04E+06	4.27E+06	1.27E+01	4.27E+06	0	0.00E+00	0
48	9.08E+06	1.45E+07	6.43E+01	1.45E+07	0	0.00E+00	0
72	9.12E+06	4.07E+07	2.25E+02	4.07E+07	0	0.00E+00	0
96	9.15E+06	9.71E+07	6.16E+02	9.71E+07	0	0.00E+00	0
120	9.19E+06	2.02E+08	1.41E+03	2.02E+08	4.85E+00	0.00E+00	0
144	9.22E+06	3.74E+08	2.81E+03	3.74E+08	1.43E+02	0.00E+00	0
168	9.25E+06	6.28E+08	4.97E+03	6.28E+08	2.45E+03	0.00E+00	0
192	7.37E+06	9.72E+08	8.01E+03	9.72E+08	2.66E+04	0.00E+00	0
216	5.93E+06	1.41E+09	1.19E+04	1.41E+09	1.98E+05	0.00E+00	0
240.01	4.82E+06	1.92E+09	1.67E+04	1.92E+09	1.07E+06	0.00E+00	0.001
264.01	3.94E+06	2.49E+09	2.21E+04	2.49E+09	4.39E+06	1.65E+00	0.002
288.01	3.26E+06	3.10E+09	2.80E+04	3.11E+09	1.44E+07	9.43E+00	0.005
312.01	2.71E+06	3.73E+09	3.41E+04	3.77E+09	3.90E+07	3.38E+01	0.01
336.01	2.28E+06	4.36E+09	4.02E+04	4.45E+09	9.00E+07	9.24E+01	0.02
360.01	1.93E+06	4.97E+09	4.63E+04	5.15E+09	1.81E+08	2.09E+02	0.035

Respiratory failure at 15.0 days
Counts are shown as cells/g lung tissue. BactT= total bacterial count (intracellular and extracellular). Ibact = intracellular bacterial count. IbactR = antibiotic-resistant intracellular bacteria

Note that the mutation rates in intracellular mycobacteria are the same as in the mycobacteria in pleural fluid. The reason that the resistant cells appear so rapidly is that a mutant appearing within a pneumocyte or a macrophage only has to compete with the wild-type bacteria in that human cell, whereas a mutant appearing in a mycobacterium in the pleural fluid is competing with the much larger pool of wild-type cells (many orders of magnitude greater) in the whole lung.

Reversion to Drug Sensitivity

Back mutation (reversion) is the situation where a mutant gene undergoes a further mutation that restores the wild-type phenotype. In the context of antibiotic resistance this means that an antibiotic-resistant variant mutates back to antibiotic sensitivity. Of course, in the presence of the selecting antibiotic, the revertants have a selective disadvantage, and are unlikely to survive very long. However, if treatment has been discontinued, any surviving sensitive bacteria, or sensitive bacteria that appear as a result of back mutation, are likely to have a selective advantage in the absence of antibiotic.

Luria and Delbrück M [3] described resistance to bacteriophage, and related the number of resistant bacteria to forward- and back-mutation rates:

$$\mu = \alpha N(1 - N^{-\alpha+\beta}/(\alpha+\beta) \qquad \text{(equation 7)}$$

where μ (mu) is the number of resistant mutants in a bacterial population of total size N cells, α (alpha) is the mutation rate, the fraction of bacteria that transform from phage sensitivity to resistance per cell division, and β (beta) is the reversion rate, the rate at which resistant cells transform back to sensitivity. It was soon realised that the same relationship (often termed the “fluctuation equation”) applied to resistance of bacteria, or viruses, or tumour cells, to drugs.

Mutations are rare, discrete events (they only occur in whole numbers) and equation 7 was derived from the Poisson distribution, the statistical relationship that applies to rare, discrete events.

Resistance rates are usually much higher than mutation rates because there are many mutations that can lead to an enzyme becoming inactive, or less active. Reversion rates are usually lower than resistance rates, because there may be only one amino acid sequence that results in optimal activity. For example, if the activity of a particular enzyme requires a cysteine at the active site, mutations that result in any of the other amino acids in that position could lead to loss of activity, and hence drug resistance, but only a mutation that returns cysteine to that position will reactivate the enzyme and restore drug sensitivity [21]. This argument has assumed that drug resistance resulted from a point mutation. There may be other genetic changes that lead to antibiotic resistance, such as deletions or inversions, but again, though a reverse change is possible (e.g. through mismatch repair) it is likely to be less frequent because, while there are many ways that a chromosomal rearrangement can disrupt enzyme expression or function, there may be only one way of getting it right. Occasionally a *suppressor mutation* may occur: these are mutations that do not restore the wild-type DNA sequence but result in an enzyme whose three-dimensional structure is close enough to the original that it may retain significant activity.

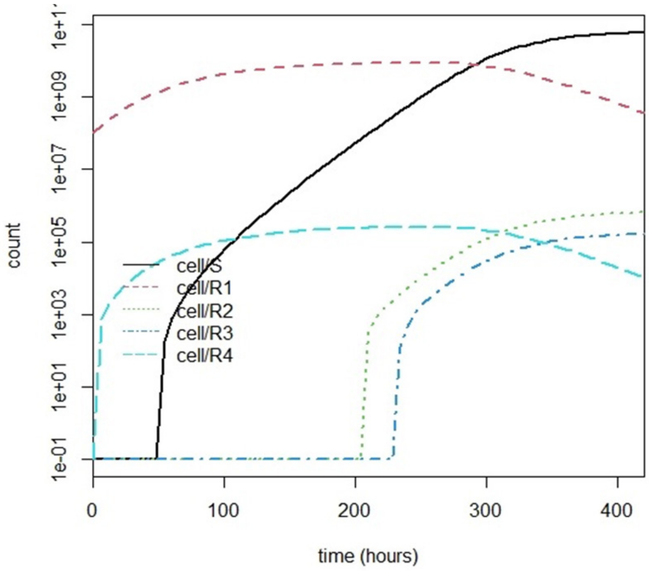

Figure 15: Appearance of antibiotic-sensitive bacteria in a resistant population after cessation of treatment, as simulated by the cybermycin program.

The cybermycin program, described in the supplement, has been used to model the reversion of resistant bacteria back to antibiotic sensitivity. Figure 15 models events after cessation of treatment of a chronic infection. Time zero corresponds to the cessation of treatment with drug 1, when all antibiotic-sensitive bacteria have been eliminated, and all remaining bacteria are bact/R1. Revertants (Bact/S) have reappeared by 48 hr, and are the dominant variant after 14 days. In this simulation the wild-type bacteria (cell/S) were assumed to have a potential doubling time of 1.7 h and the resistant cell/R1 mutants 2.9 h.

Plasmid loss

When drug resistance is the result of horizontal gene transmission, for example by an antibiotic resistance gene carried by a plasmid, reversion to antibiotic sensitivity after the cessation of treatment is much less likely. Plasmid loss can occur, perhaps by unequal segregation of plasmids during cell division, or because plasmid DNA replication for large plasmids may be slower than that of the chromosomal DNA [22]. However, because of the non-Darwinian dynamics of HGT, unless every plasmid-bearing cell is eliminated from the population, the resistant cells will eventually dominate the population, even in the absence of selective pressure, despite aving a growth disadvantage.

Figure 16 shows a simulation of reversion to imipenem sensitivity by plasmid loss following the end of treatment. Sensitive revertants appear within a few hours, but despite having a faster doubling time than the plasmid-bearing cells, the kinetics of HGT are such that by 48h the sensitive cell count has levelled off, while the resistant cells once again dominate the population.

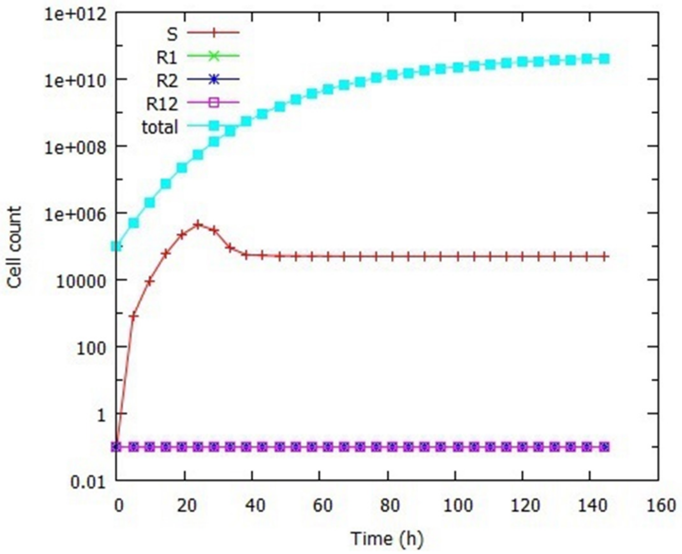

Figure 16: Reappearance of antibiotic-sensitive cells in a culture resistant due to HGT following the end of treatment

The steady-state level of revertants depends on the value of the reversion rate constant, but there is no level at which the revertants can totally dislodge the plasmid-bearing cells. In the absence of plasmid loss, the only steady states that are possible are all sensitive cells, or all resistant cells. High rates of plasmid loss (which may be spontaneous or drug-induced) can result in steady states in which both antibiotic-resistant and -sensitive bacteria coexist, as in Figure 16, but the autocatalytic kinetics of HGT mean that such mixed cultures must always be dominated by plasmid-bearing cells. It has been claimed that some classes of antibacterial drugs, e.g. DNA gyrase inhibitors, can result in "curing" of bacteria, though these claims have been disputed. However, plasmids impose a cost on their host cells, in that a plasmid-bearing cell has to synthesize more DNA during cell division than a cell that has only chromosomal DNA [11]. It follows that drugs that act through inhibition of DNA synthesis (e.g. DNA gyrase inhibitors, or sulfa drugs) will create conditions that favour the survival of plasmid-free bacteria. Drug-induced plasmid curing remains a theoretical possibility, but it will not be easy to achieve.

Design of Antibiotics Combinations

Evolutionary dynamics calculations suggest that the key to overcoming antibiotic resistance is the use of non cross-resistant combinations. The design of combination protocols is a complex, multi-disciplinary science, and the present discussion can only consider those aspects relevant to the antibiotic resistance problem. The disadvantages of multidrug treatment are the possibility of greater toxicity, increased risk of metabolic drug interactions; possibility of poor patient compliance, and of course, cost. The ideal combination will contain the smallest number of antibiotics that prevents the emergence of resistance. How many drugs that will take will depend upon resistance rates and estimated bacterial population sizes. If we assume an average resistance rate of 1 x 10^{-6} and wish to exclude the likelihood of pre-existing mutations at the start of treatment, this suggests that pathogen populations of $<10^6$ cells can be successfully eradicated with single-drug treatment, but that bacterial populations $>10^6$ but $<10^{12}$ cells may require two-agent combination therapy, and populations $>10^{12}$ cells, as may occur in intestinal infections, may require a three-antibiotic combination. Nyhoegen et al. [23] reported that combining a bacteriostatic and a bactericidal drug which could kill non-replicating cells was particularly beneficial. These combinations may not be sufficient to prevent the emergence of resistance if, as with tuberculosis, the pathogen exists partly within cells of the host. If antibiotic resistance is the result of resistance mutations carried on a plasmid (HGT), and the plasmid carries genes for resistance to two or more antibiotics, then even if they are mechanistically unrelated, those antibiotics will be cross-resistant, and the infection must be controlled by up to three antibiotics that are unaffected by the resistance genes on the plasmid.

In addition to being non-cross-resistant, the antibiotics in a combination should have non-overlapping toxicity to the host. Ideally, the agents in a combination should be used at their full therapeutic dose, but if, for example, two myelosuppressive agents are combined, it may be necessary to use one or both of them at a reduced dose, which will compromise efficacy.

A third requirement for drugs in a combination is that they should not be mutually antagonistic. This can happen, for example, when drugs act at different points in the cell cycle. Many antibiotics cause selective inhibition of bacterial protein synthesis, which will result in arrest in G1 phase of the cell cycle. If one of these G1-active antibiotics is combined with an S-phase acting agent, such as trimethoprim, it will cause antagonism, by decreasing the number of bacterial cells entering S phase. It may still be possible to combine two such agents successfully by separating them in time, but this makes compliance more difficult for the patient, and the optimal timing may vary depending upon the cell cycle time of the bacteria.

An opposite, and desirable, effect is when two antibiotics have a synergistic interaction. A classical example of this is the trimethoprim+sulfa combination. Trimethoprim is a potent inhibitor of bacterial dihydrofolate reductase (DHFR), required for RNA and DNA biosynthesis and sulfamethoxazole, and other sulfa drugs, potentiate trimethoprim by inhibiting the synthesis of its substrate, dihydrofolate. A synergistic drug interaction would give no therapeutic gain if it increased the toxicity to the patient as well as the antibacterial effect. The term *therapeutic synergy* implies that the effect is selective: trimethoprim, for example is a potent inhibitor of bacterial DHFR but only a weak inhibitor of mammalian DHFR, while sulfa drugs derive their selectivity from the fact that bacteria synthesize dihydrofolate, while humans obtain it (or its precursor, folic acid) from the diet.

Stein et al. [24] systematically explored three-antibiotic combinations against Klebsiella pneumoniae isolates that expressed high levels of β-lactamase. They were able to identify synergistic two-antibiotic combinations, but adding a third antibiotic did not lead to further synergy.

The design of antibiotic combination protocols is thus a complex multifactorial optimization problem. In addition to minimizing cross-resistance, overlapping toxicity and antagonistic biochemical interactions, it may be necessary to avoid drug interactions at the level of drug clearance or drug metabolism. In practice, it may not always be possible to optimise all these factors simultaneously. It may sometimes make sense to combine drugs with partial cross-resistance. Modelling antibiotic combinations with programs such as cybermycin provides a tool for understanding these complex interactions.

It has been suggested that one way to make antibiotics essentially resistance-proof is to make them bifunctional, i.e. inhibiting two bacterial targets. If, for example, the resistance frequency at each target site was 10^{-6}, the probability of double mutants resistant to both inhibition mechanisms would be 10^{-12}. It is not clear, though, whether such bifunctional inhibitors have any advantages over a combination of two separate inhibitors with different targets. Moreover, certain resistance mechanisms, such as enhanced activity of efflux pumps, would treat a bifunctional inhibitor the same as a conventional single-site inhibitor, in which case the resistance frequency would be as great as for a single-site inhibitor.

Comparisons of sequential administration of two-antibiotic combinations show that resistance to the first antibiotic is more likely to emerge than with simultaneous administration, if the two agents are widely spaced (to minimise toxicity). However, if the agents are alternated frequently, the alternating regimens can be as effective, or nearly as effective, as simultaneous dosing [25-27]. If a biomarker for resistance is available, such as a molecular diagnostic, or an immunodiagnostic specific for the resistant mutant, then *adaptive therapy* becomes possible in which the antibiotic switch can be precisely timed to minimise resistance. Adaptive therapy has been extensively explored in cancer chemotherapy [28], but much less so in antibacterial therapy. It may be a promising way to treat diseases in which antibiotic resistance is particularly frequent, such as tuberculosis. When an infection is being treated with an antibiotic, and a diagnostic for resistance to that antibiotic tests positive, it is, of course, too late to prevent that resistance by switching to a second, non-cross-resistant antibiotic. What switching at that time can accomplish is to minimize the risk of emergence of double mutants resistant to both antibiotics. In the case of tuberculosis it may be necessary to repeat this process three or four times. The benefit of adaptive therapy could be maximised by selecting the next antibiotic in the sequence using a data-driven system that optimises the outcome based upon prior knowledge of cross-resistance patterns, mutation rates, biochemical synergies, and presence or absence of drug-drug interactions. Such an approach, evolutionary dynamics-guided adaptive therapy (EDGAT) has the potential to reduce treatment failure by orders of magnitude.

Minimizing resistance with data science

If we know enough about the molecular pathology of a disease, and enough about the drugs used to treat that disease, we can attempt to predict the outcome of a clinical trial. Virtual clinical trials in bacterial infections require knowledge of the population PK/PD of the available drugs (usually in plasma, but sometimes in other compartments). We also need data on drug-drug interactions. We need a disease model that includes information on doubling times, dormancy rates, rates of mutation and of horizontal transmission and of the partitioning of the pathogen into different body compartments. For some diseases, much of this information is available [29].

There are ~ 8 x 10^9 combinations of 4 drugs selected from the 300+ known antibiotics. If we have a supercomputer that can run one virtual clinical trial per second, it will take >250 years to test every possible combination. We would like to have this information within one hour. This problem cannot be solved by brute force – it will require an AI approach. The suggested technique involves pattern recognition based upon an array of diagnostics.

The importance of early diagnostics of resistance

The traditional way of identifying an infectious pathogen, and of determining its drug sensitivity, has been to grow it in culture, and this is still considered to be the definitive way to characterise an infectious disease. Its limitations are that not all organisms can be readily cultured, and for those that do it will take at least 24 hours, and often longer, to adequately characterise the pathogen. Cultures obtained from clinical material, such as buccal swabs or sputum material, may well be heterogeneous, so that what grows may not be the organism responsible for the symptoms. While an experienced microbiologist will often be able to identify the cause of an infection, culture results may be ambiguous. A more serious concern is that, for serious infections, the disease may progress, or even be lethal, before it has been identified. If our primary concern is to determine whether antibiotic-resistance mutations are present, identifying the pathogen is a secondary concern, and heterogeneity of the sample is also irrelevant: if we want to know whether a particular three-antibiotic combination can be administered immediately, the relevant question is whether mutations (or plasmids) that cause resistance to those antibiotics are present in the clinical sample. If they are not, there is no guarantee of response, but the risk of treatment failure is reduced.

Diagnostics and information

There are tens of thousands of possible drug resistance mutations. One existing approach being explored to identify them in clinical material is rapid whole genome sequencing. This is a definitive test of whether antibiotic-resistance mutations are present that takes about 48 hours [30,31]. Using molecular hybridisation probes, it would be possible to make an Affimetrix© chip that could simultaneously and rapidly test for the presence of each of these mutations. At present, such a chip does not exist.

How useful would it be to test for a smaller number of mutations? It is possible to develop immunodiagnostics (e.g. lateral flow tests) for particular mutant sequences relatively cheaply. Of course, we need to know that that particular mutant sequence is a cause of clinical resistance. If, for a particular infectious organism, we can identify the 7 commonest mutations that cause resistance to any of the three antibiotics most active against that organism, and substitute other antibiotics for those where resistance is predicted, treatment success can still not be guaranteed, but, assuming each diagnostic contributes one bit of information, then the use of a panel of 7 diagnostics would reduce treatment failure by a factor of 2^7, or greater than 99%, using existing antibiotics, and rapid, low cost diagnostics that can be developed with existing technology.

"Bugs and drugs" databases have been compiled that have amassed current knowledge on antibiotic resistance mutations [30,32,33]. What is needed for making rapid treatment recommendations, however, does not exist. To decide rapidly upon optimal treatment after obtaining antibiotic resistance diagnostic data will require an online system that contains continually updated data on clinical outcomes. One way to approach this without waiting for several years of clinical feedback would be to construct a Bayesian system using laboratory data to make prior predictions. This would incorporate all available cross-resistance information, and should converge upon a highly predictive system as it progressively incorporates clinical outcomes data.

Conclusions

The most important lesson that evolutionary dynamics teaches us about the antibiotic resistance problem is that nature can generate new resistant mutations faster than we can develop new antibiotics. Resistance is inevitable, but we can reduce its prevalence by many orders of magnitude.

The key to overcoming resistance is use of non-cross-resistant antibiotic combinations, but they must be combined in a way that does not increase their toxicity, and that minimises unfavourable drug-drug interactions.

Optimising the treatment of infectious disease requires information about disease dynamics, about antibiotic pharmacodynamics, and about mechanisms of antibiotic resistance.

Novel diagnostics can be developed more rapidly and more cheaply than new antibiotics.

Each additional diagnostic test provides information that can reduce the failure rate of treatment.

Early use of diagnostics makes possible minimization of multiple antibiotic resistance through adaptive therapy.

Minimizing treatment failure will require a data-driven approach to rapidly predicting a treatment protocol that minimises the risk of treatment failure due to antibiotic resistance.

Combining evolutionary dynamics with adaptive therapy (evolutionary dynamics-guided adaptive therapy, EDGAT) can direct treatment by maximizing the advantage provided by diagnostics-generated information.

The clinical database that can do this does not exist, but laboratory data can provide prior parameter estimates for a Bayesian system that by feeding clinical outcomes into the database will converge upon a treatment approach that can control the antibiotic resistance problem to a much lower level.

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Supplement: Cybermycin. A computer model of bacterial growth and evolution

Cybermycin is a mathematical description of bacterial growth in humans or animals that are treated with antibiotics, including combinations of up to four antibiotics. Cybermycin can model appearance of up to 4 drug-resistance mutations, so a total of 16 bacterial populations can be tracked. Antibiotic effects can be bactericidal or bacteriostatic. The program can also model bacterial killing by the immune response. Appearance of antibiotic resistance may be by mutations in the bacterial chromosome (vertical gene transmission) or by plasmid-mediated conjugation (horizontal gene transmission). In order to model drug toxicity, four host cell populations are modelled. These are bone marrow stem cells, gastrointestinal crypt cells, keratinocytes, and B lymphocytes.

Equations of the model

Bacterial growth is described by the Gompertz equation, $dN/dt = kN/c \,.\, (A - logeN)$

where N is the population size at time t and k, the growth rate constant is $\log_e 2$/doubling time (hours). The constant A is a function of the asymptote, the final cell count at which growth levels off. If the final cell count is represented as Ninf (N at time infinity) then:

A=loge Ninf

Thus the expression (A – logeN) is a measure of how far the population is, at time t, from its final value. The constant c is a measure of how far the final state is from the initial cell number, N0: $c = A - \log_e N0$

Growth fraction, $GF = A - \log_e(N_t) / c$ (Jackson, 2016)

The number of quiescent (non-replicating) bacterial cells at time t is $N_t.(1 - GF)$

Mutations occur by inaccurate base pairing (or deletions or copy number changes) during cell replication. During time interval dt the number of instances of a particular mutation will be

$(Nt+dt - Nt).\ m$ (m = mutation rate)

Antibiotic cytotoxicity dose-response curves are described by a semi-logarithmic relationship

cell kill, ck (log10) = dose/MTD * GLK / Rfactor

where MTD = maximum tolerated single dose (mg/kg) and GLK = gross log kill of bacteria at MTD (i.e. not corrected for re-growth of surviving cells during he treatment period.

Rfactor = resistance factor, defined as GLK for resistant bacteria / GLK for wild-type. Then, for a cytotoxic drug:

$N(\text{treated}) = N(\text{control}) / 10^{ck}$ (Lloyd, 1977)

For a cytostatic drug, the doubling time (DT) for cell replication is increased. The dose-respose curve is described by a Hill equation:

$DT(\text{treated}) = DT(\text{control}) * (1 + Imax * (\text{dose}/MTD)^{nH} / (1 + (\text{dose}/MTD)^{nH}))$

where Imax is the maximal growth-inhibitory effect and nH is the Hill coefficient.

Cytotoxicity to normal mammalian tissues is calculated by the same equation, except that the therapeutic index (TI) is substituted for the resistance factor.

Selection pressure against bacterial survival is calculated by a genetic algorithm as follows:

(i) Proliferation of wild type during the treatment period is calculated.
(ii) Proliferation of mutant during the treatment period is calculated.
(iii) The proportions of wild type and mutant are expressed as fractions of the total population: $\text{fraction}_S = N_s / (N_s + N_r)$; $\text{fraction}_R = N_r / (N_s + N_r)$;
(iv) The increase in the total bacterial population, $N_{t+dt} - N_t$ is calculated from the Gompertz equation.
(v) Then, the increase in wild-type during the treatment period is $(N_{t+dt} - N_t) * \text{fraction}_S$ and increase in mutant during the treatment period is $(N_{t+dt} - N_t) * \text{fraction}_R$.

Cross-resistance refers to the situation where a mutant that was selected for resistance to a particular drug may also be resistant to another drug to which it may not previously have been exposed . Cross resistance may be total or partial. In the cybermycin model, each of the 16 bacterial cell lines is assigned four Rfactor values, where Rfactor (resistance factor), is defined as GLK for resistant bacteria / GLK for wild-type. By definition, Rfactor values for wild-type = 1.0. If the Rfactor for bact/R1 (selected for resistance to drug 1) is 10.0, and its Rfactor for drug 2 is 5.0, then it is partially cross-resistant to drug 2. It is possible for Rfactors to be <1, in which case that bacterial strain is said to be *collaterally sensitive* to that particular drug.

Kinetics of conjugation. Conjugation, which involves cell-cell contact, is a second-order process:

$$v_{hgt} = N_s \,.\, N_r \,.\, k_{hgt}$$

where N_s and N_r are numbers of sensitive and resistant cells, respectively, per unit volume, k_{hgt} is a second-order rate constant, and v_{hgt} is the rate of horizontal gene transfer, cells transfected per unit time.

Plasmid loss is modelled as a first-order process: $v_{pl} = k_{pl} * N_r$ where N_r is the number of plasmid-bearing bacteria per unit volume and k_{pl} is a first-order rate constant.

Resistance modifiers are assumed to bind saturably to their target macromolecule, which may be an enzyme or an efflux channel, and to follow a Hill equation:

$$B_{rm} = C_{rm}^{nH} / (C_{rm}^{nH} + K_i)$$

where B_{rm} is fraction of the maximal effect, C_{rm} is concentration of the resistance modifier, K_i is its binding constant, and nH is the Hill coefficient. If the resistance factor in the absence of the resistance modifier is $Rfactor_0$, and Rfactor in presence of the modifier at concentration C_{rm} is $Rfactor_{crm}$ and the maximal reversal factor is RevF, then

$$Rfactor_{crm} = Rfactor_0 / C_{rm}/K_i/(1 + C_{rm}/K_i) * RevF$$

Immune killing of bacteria:

B cells are stimulated into proliferation by bacterial antigens. This stimulation follows Michaelis- Menten kinetics: $k_{19} = k_b * \{ 1 + [V_{m,I} * bacteria / (bacteria + K_{m,I})]\}$

where k_{19} = growth rate constant of stimulated B cells, k_b is growth rate constant of unstimulated B cells, $V_{m,I}$ and $K_{m,I}$ are the V_{max} and K_m, respectively, for B cell activation, and “bacteria” is the bacterial cell count of infected tissue, in cells/g. The model assumes that activated B cells release antibody, and that antibody kills bacteria. Antibody levels decline exponentially:

$$[antibody] = [activated\ B\ cells] * k_{0,ab} – [antibody] * k_{ab,0}$$

$k_{0,ab}$ and $k_{ab,0}$ are rate constants for antibody production and turnover, respectively. Antibody-mediated cytotoxicity of bacteria is second-order:

$$dN_i/dt = N_i * [antibody] * k_{c,i}$$

where N_i is the count of each of the bacterial sublines ($i = 0 - 15$) and $k_{c,i}$ is a rate constant which is a function of the immunogenicity of bacterial subline i, and may thus differ among sublines.

Programming: Code for the cybermycin model will be made available on Github.