Assessing evolutionary risks of resistance for new antimicrobial therapies

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Abstract

New antibiotics are urgently required to combat rising rates of resistance against all existing classes of antimicrobials. We highlight key issues that complicate the prediction of resistance evolution in the real-world and outline the ways in which these can be overcome.

Introduction

Globally rising rates of antimicrobial resistance (AMR) against all existing antibiotic classes combined with a near empty pharmaceutical pipeline of new antibiotic classes has given renewed urgency to drug discovery efforts[1]. Recent high-profile discoveries, based on novel natural product screens (e.g. teixobactin[2]) or modified natural products (e.g. arylomycin[3]), are encouraging but the evolution of resistance remains a serious concern for the long-term efficacy of new antibiotics. The standard approach for assessing the risk of resistance evolution in such studies is to measure the rate of spontaneous resistance mutation using fluctuation tests: assays where bacterial cultures are spread onto agar containing the antibiotic and the number of colony forming units compared against antibiotic-free controls[4]. Whole genome or targeted sequencing of resistant mutants can then identify the genetic locus (or loci) mutated and the degree of resistance provided by different mutations. While these data provide a valuable snapshot of the potential for the tested bacterium to evolve resistance via spontaneous mutation, resistance evolution in the real-world is often more complex, as is evident from older antibiotics which induce high rates of *in vitro* resistance but remain clinically useful (e.g. rifampicin). Our recent conversations with the pharmaceutical industry suggest that it now takes resistance evolution very seriously and implements stringent cut-offs for *in vitro* resistance mutation frequencies deemed acceptable for new antibiotics. An over-simplified view of resistance evolution combined with stringent *in vitro* targets for resistance risks halting the development of potentially useful compounds if *in vitro* tests over-estimate the evolutionary success of resistant genotypes (e.g. by ignoring high *in vivo* fitness costs). Conversely, under-estimating the *in vivo* evolutionary success of resistant genotypes will lead to wasted development effort. How then can we bridge the gap between existing practice and a more realistic assessment of the risk of resistance evolution to ensure the long-term utility of new antibiotics?

Mind the reality gap

Here we identify four key issues that complicate the prediction of resistance evolution in the real-world and outline the ways in which these can be overcome (Fig. 1).

1. **Population ecology of infections:** All else being equal, resistance by spontaneous mutation is more likely in larger bacterial populations[5]. Bacterial population size varies widely between different kinds of infection, although accurate estimates of population sizes are surprisingly limited. For example, the total number of bacteria in blood stream infections caused by *E. coli* and *S. aureus* varies between approximately 10^3 and 10^8 cells (ref. 6), and pathogen densities during ventilator-associated pneumonia can exceed 10^6 CFU per mL of sputum (ref. 7). However, invasion by resistant genotypes can also be affected by differential clearance of resistant versus susceptible genotypes. For example, the number of *E. coli* cells in urinary tract infections can reach 10^{10} CFU, guaranteeing the presence of thousands of resistant mutants, but resistance evolves rarely during infection because of the high rate of turnover of bladder contents[8]. In contrast, chronic biofilm infections may sustain large population sizes, lower rates of population turnover and elevated mutation rates, giving rise to high levels of standing genetic variation available to natural selection[9-11]. Further, physiological tolerance to antibiotics can allow sufficient time for resistance mutations to arise *in vivo*[12], which may not be observed *in vitro*. Improving resistance prediction requires better data on the basic population ecology parameters of different infections.
2. **Pre-existing resistance:** Standing genetic variation (SGV) for resistance is likely to exist for new antibiotics that target cellular processes already targeted by existing drugs, or that are derived from natural antimicrobials (e.g. antimicrobial peptides[13]). For example, *S. aureus* acquired the SCC*mec* element years before the clinical introduction of methicillin, and methicillin use resulted in the very rapid spread of these MRSA lineages[14]. Selection by non-antimicrobial drugs[15] and non-clinical antimicrobial agents (e.g., triclosan[16,17]) also risks the collateral evolution of antibiotic resistance. The vast database of genome sequences of pathogenic bacteria provides a powerful way to prospectively test for SGV at genes involved in resistance to new antibiotics by genome mining. For instance, for arylomycin, 7 resistance mutations were

identified in the *lepB* gene[3]. We scanned the European Nucleotide Archive (ENA) for mutations in *lepB* using the search tool BIGSI[18] (N=447,833 whole genome sequence datasets). We found 45,009 datasets contained a *lepB* gene >97% DNA identical to the *lepB* gene in *E. coli* ATCC 25922, harbouring a total of 98/324 codons with at least one non-synonymous mutation. We did not find any of the 7 variants found to confer arylomycin resistance in the original study[3], but we did find 82 datasets containing another amino acid substitution at one of those seven positions (L142I). This demonstrates the feasibility of estimating whether resistant variants are circulating prior to an antibiotic's clinical use, if the genetic targets of resistance mutations are known. Alternatively, resistance determinants against new antibiotics can be detected using functional metagenomics[19]. Here, fragments of metagenomic DNA are expressed and screened for their effect on resistance. This approach is especially useful for detecting unknown resistance genes present in environments where natural product-derived antibiotics are naturally expressed[20,21] or in animal or human microbiomes[22,23]. Pre-existing resistance is especially problematic if these genes become mobilised on mobile genetic elements, such as transducing phages and plasmids[24]. Horizontal gene transfer via mobile genetic elements, bacteriophages or via competence for transformation can strongly contribute to the rise and spread of resistance[25,26]. Experiments estimating rates of gene mobilisation in relevant environments[27] are urgently required.

3. **Fitness costs and interplay with the host:** Population genetic models suggest that fitness costs associated with resistance limit its persistence upon removal of the antibiotic[5]. Costs arise because resistance mutations may impair the normal function of the target gene or through physiological costs of expressing resistance genes or harbouring MGEs[28]. Fitness costs can be easily quantified using growth or competition assays in lab media and animal infection models, and these should be provided in all reports of new antibiotics[28]. Although there is some degree of agreement between in *vivo* and *in vitro* measures of fitness cost[28,29], *in vitro* assays can underestimate the fitness costs of resistance that manifest in more complex infection environments[30]. For example, mutations conferring resistance to bacteriocin-derived avidocin antibiotics cause loss of the S-layer in *Clostridium difficile.* This has no effect on *in vitro* growth rate but makes resistant cells highly susceptible to innate immune effectors and avirulent in an

in vivo infection model[31]. High *in vitro* resistance mutation rate against a new antibiotic can deter further development of a new drug, even if high fitness costs *in vivo* effectively limit the success of these resistant mutants in patients because of competition or immune clearance. For example, the development of mecillinam would currently be halted by pharmaceutical companies due to high resistance mutation frequencies (ranging from 8×10^{-8} to 2×10^{-5} per cell) but resistance in the clinic is rare due to the low *in vivo* fitness of mutants[32]. Accurately modelling within-host bacterial metabolism and physiology by using more realistic experimental environments, such as host-mimicking media[33,34], *in vitro* biofilm models[35,36], animal models[37] or *ex vivo* tissue models[38,39], will offer a more accurate picture of fitness costs.

4. **Compensatory evolution:** Over time, second-site mutations that compensate for the fitness cost of the resistance mutation while leaving the resistant phenotype intact can allow persistence of resistant genotypes *in vivo* and in environmental reservoirs in the absence of antibiotic selection[28,40]. The targets of compensatory mutations can be determined using evolve-and-resequence experiments where resistant mutants are serially passaged without antibiotic until they recover ancestral-level fitness[41-43]. For example, mutations in *M. tuberculosis* that compensate for the fitness cost of rifampicin resistance *in vitro* are found in clinical isolates and contribute to the tuberculosis epidemic[44]. The extent to which lab studies provide an unbiased view of compensatory evolution in the real-world is unclear[45], but one promising solution to this problem is to use genome-wide association analyses of bacterial genomic datasets to link known resistance mutations with subsequent compensatory mutations. For example, acquisition of multidrug resistance plasmids in *E. coli* ST131 has been shown to be associated with specific regulatory mutations that may compensate the fitness cost of plasmid carriage[46].

Bridging the gap

Ideally, evolutionary analysis of resistance should accompany *in vivo* experimental and clinical trials of new antibiotics. The following changes to existing practices would allow far better understanding of the potential for resistance evolution:

- **Appropriate choice of infection models during research & development:** By considering the physicochemical environment at infection sites and its effects on bacterial growth, gene expression and physiology, will provide better estimates of likely rates of resistance emergence and associated fitness costs which may differ substantially from those observed in standard laboratory growth media[34]. For example, the leucyl-tRNA synthetase inhibitor GSK2251052 showed limited resistance development *in vitro* but failed in clinical trials because of rapid emergence of resistance in treated patients[47]. Improved estimates of the risks of *in vivo* resistance would allow drug candidates to be better prioritised for progression to clinical trials, and could therefore directly benefit the pharmaceutical industry by more effectively targeting investment.
- **Dosing to minimise resistance selection:** Massive experimental and modelling efforts are made to quantify the PK/PD of new antibiotics before clinical trial with the goal of maximizing antibiotic efficacy. Minimizing selection for resistance should also be a goal of PK/PD studies[48] which should employ appropriate infection models.
- **More intensive sampling of infections during clinical trials:** Current approaches sample single colonies, ignoring the diversity present in most infections and therefore losing the opportunity to understand evolutionary processes occurring in infections[49,50]. More extensive culture-based sampling should be combined with deep-sequencing to quantify resistance allele frequencies and capture hard to culture organisms (e.g. persister cells, polymicrobial infections). Access for scientists to the samples from completed clinical trials would allow the development of methods and analyses for efficiently characterising the evolutionary mechanisms leading to resistance in patients. Furthermore, development of culture conditions allowing maintenance of diverse bacterial populations and/or communities in the lab would facilitate understanding how resistance evolution is a function of microbial community interactions.
- **Microbiome sampling in clinical trials:** Antibiotic treatment often has collateral effects on the microbiome including driving lateral transfer of resistance elements at other body sites[51]. Combining new bioinformatics tools for resistance prediction[52] with long-read or proximity ligation sequencing technologies allows localisation of resistance genes to their genomic context and the tracking of gene mobilisation and transfer[53,54].

These new approaches will increase the scale and cost of clinical trials in the immediate term and will require that medical funders and pharmaceutical companies embrace eco-evolutionary thinking. However, there are likely to be direct benefits in terms of more effective targeting of investment and fewer potentially useful drug candidates being discarded due to misleading *in vitro* resistance estimates. It is possible therefore that taking a more realistic view of resistance evolution could help to rejuvenate the antibiotic pipeline.

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Figure legend

Figure 1 | Predicting the evolutionary risk of resistance against new antibiotics is complicated by the complexity of real-world infections