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1 **Assessing evolutionary risks of resistance for new antimicrobial therapies**

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15

16 **Abstract**

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

20

21 **Introduction**

22 Globally rising rates of antimicrobial resistance (AMR) against all existing antibiotic classes
23 combined with a near empty pharmaceutical pipeline of new antibiotic classes has given renewed
24 urgency to drug discovery efforts¹. Recent high-profile discoveries, based on novel natural product
25 screens (e.g. teixobactin²) or modified natural products (e.g. arylomycin³), are encouraging but the
26 evolution of resistance remains a serious concern for the long-term efficacy of new antibiotics. The
27 standard approach for assessing the risk of resistance evolution in such studies is to measure the
28 rate of spontaneous resistance mutation using fluctuation tests: assays where bacterial cultures are
29 spread onto agar containing the antibiotic and the number of colony forming units compared against
30 antibiotic-free controls⁴. Whole genome or targeted sequencing of resistant mutants can then identify
31 the genetic locus (or loci) mutated and the degree of resistance provided by different mutations.
32 While these data provide a valuable snapshot of the potential for the tested bacterium to evolve
33 resistance via spontaneous mutation, resistance evolution in the real-world is often more complex,
34 as is evident from older antibiotics which induce high rates of *in vitro* resistance but remain clinically
35 useful (e.g. rifampicin). Our recent conversations with the pharmaceutical industry suggest that it
36 now takes resistance evolution very seriously and implements stringent cut-offs for *in vitro* resistance
37 mutation frequencies deemed acceptable for new antibiotics. An over-simplified view of resistance
38 evolution combined with stringent *in vitro* targets for resistance risks halting the development of
39 potentially useful compounds if *in vitro* tests over-estimate the evolutionary success of resistant
40 genotypes (e.g. by ignoring high *in vivo* fitness costs). Conversely, under-estimating the *in vivo*
41 evolutionary success of resistant genotypes will lead to wasted development effort. How then can
42 we bridge the gap between existing practice and a more realistic assessment of the risk of resistance
43 evolution to ensure the long-term utility of new antibiotics?

44

45 **Mind the reality gap**

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

48

49 1. **Population ecology of infections:** All else being equal, resistance by spontaneous mutation is
50 more likely in larger bacterial populations⁵. Bacterial population size varies widely between
51 different kinds of infection, although accurate estimates of population sizes are surprisingly
52 limited. For example, the total number of bacteria in blood stream infections caused by *E. coli*
53 and *S. aureus* varies between approximately 10^3 and 10^8 cells (ref. 6), and pathogen densities
54 during ventilator-associated pneumonia can exceed 10^6 CFU per mL of sputum (ref. 7).
55 However, invasion by resistant genotypes can also be affected by differential clearance of
56 resistant versus susceptible genotypes. For example, the number of *E. coli* cells in urinary tract
57 infections can reach 10^{10} CFU, guaranteeing the presence of thousands of resistant mutants,
58 but resistance evolves rarely during infection because of the high rate of turnover of bladder
59 contents⁸. In contrast, chronic biofilm infections may sustain large population sizes, lower rates
60 of population turnover and elevated mutation rates, giving rise to high levels of standing genetic
61 variation available to natural selection⁹⁻¹¹. Further, physiological tolerance to antibiotics can allow
62 sufficient time for resistance mutations to arise *in vivo*¹², which may not be observed *in vitro*.
63 Improving resistance prediction requires better data on the basic population ecology parameters
64 of different infections.

65 2. **Pre-existing resistance:** Standing genetic variation (SGV) for resistance is likely to exist for
66 new antibiotics that target cellular processes already targeted by existing drugs, or that are
67 derived from natural antimicrobials (e.g. antimicrobial peptides¹³). For example, *S. aureus*
68 acquired the *SCCmec* element years before the clinical introduction of methicillin, and methicillin
69 use resulted in the very rapid spread of these MRSA lineages¹⁴. Selection by non-antimicrobial
70 drugs¹⁵ and non-clinical antimicrobial agents (e.g., triclosan^{16,17}) also risks the collateral evolution
71 of antibiotic resistance. The vast database of genome sequences of pathogenic bacteria
72 provides a powerful way to prospectively test for SGV at genes involved in resistance to new
73 antibiotics by genome mining. For instance, for arylomycin, 7 resistance mutations were

74 identified in the *lepB* gene³. We scanned the European Nucleotide Archive (ENA) for mutations
75 in *lepB* using the search tool BIGSI¹⁸ (N=447,833 whole genome sequence datasets). We found
76 45,009 datasets contained a *lepB* gene >97% DNA identical to the *lepB* gene in *E. coli* ATCC
77 25922, harbouring a total of 98/324 codons with at least one non-synonymous mutation. We did
78 not find any of the 7 variants found to confer arylomycin resistance in the original study³, but we
79 did find 82 datasets containing another amino acid substitution at one of those seven positions
80 (L142I). This demonstrates the feasibility of estimating whether resistant variants are circulating
81 prior to an antibiotic's clinical use, if the genetic targets of resistance mutations are known.
82 Alternatively, resistance determinants against new antibiotics can be detected using functional
83 metagenomics¹⁹. Here, fragments of metagenomic DNA are expressed and screened for their
84 effect on resistance. This approach is especially useful for detecting unknown resistance genes
85 present in environments where natural product-derived antibiotics are naturally expressed^{20,21} or
86 in animal or human microbiomes^{22,23}. Pre-existing resistance is especially problematic if these
87 genes become mobilised on mobile genetic elements, such as transducing phages and
88 plasmids²⁴. Horizontal gene transfer via mobile genetic elements, bacteriophages or via
89 competence for transformation can strongly contribute to the rise and spread of resistance^{25,26}.
90 Experiments estimating rates of gene mobilisation in relevant environments²⁷ are urgently
91 required.

92 3. **Fitness costs and interplay with the host:** Population genetic models suggest that fitness
93 costs associated with resistance limit its persistence upon removal of the antibiotic⁵. Costs arise
94 because resistance mutations may impair the normal function of the target gene or through
95 physiological costs of expressing resistance genes or harbouring MGEs²⁸. Fitness costs can be
96 easily quantified using growth or competition assays in lab media and animal infection models,
97 and these should be provided in all reports of new antibiotics²⁸. Although there is some degree
98 of agreement between *in vivo* and *in vitro* measures of fitness cost^{28,29}, *in vitro* assays can
99 underestimate the fitness costs of resistance that manifest in more complex infection
100 environments³⁰. For example, mutations conferring resistance to bacteriocin-derived avidocin
101 antibiotics cause loss of the S-layer in *Clostridium difficile*. This has no effect on *in vitro* growth
102 rate but makes resistant cells highly susceptible to innate immune effectors and avirulent in an

103 *in vivo* infection model³¹. High *in vitro* resistance mutation rate against a new antibiotic can deter
104 further development of a new drug, even if high fitness costs *in vivo* effectively limit the success
105 of these resistant mutants in patients because of competition or immune clearance. For example,
106 the development of mecillinam would currently be halted by pharmaceutical companies due to
107 high resistance mutation frequencies (ranging from 8×10^{-8} to 2×10^{-5} per cell) but resistance in
108 the clinic is rare due to the low *in vivo* fitness of mutants³². Accurately modelling within-host
109 bacterial metabolism and physiology by using more realistic experimental environments, such
110 as host-mimicking media^{33,34}, *in vitro* biofilm models^{35,36}, animal models³⁷ or *ex vivo* tissue
111 models^{38,39}, will offer a more accurate picture of fitness costs.

112 4. **Compensatory evolution:** Over time, second-site mutations that compensate for the fitness
113 cost of the resistance mutation while leaving the resistant phenotype intact can allow persistence
114 of resistant genotypes *in vivo* and in environmental reservoirs in the absence of antibiotic
115 selection^{28,40}. The targets of compensatory mutations can be determined using evolve-and-
116 resequence experiments where resistant mutants are serially passaged without antibiotic until
117 they recover ancestral-level fitness⁴¹⁻⁴³. For example, mutations in *M. tuberculosis* that
118 compensate for the fitness cost of rifampicin resistance *in vitro* are found in clinical isolates and
119 contribute to the tuberculosis epidemic⁴⁴. The extent to which lab studies provide an unbiased
120 view of compensatory evolution in the real-world is unclear⁴⁵, but one promising solution to this
121 problem is to use genome-wide association analyses of bacterial genomic datasets to link known
122 resistance mutations with subsequent compensatory mutations. For example, acquisition of
123 multidrug resistance plasmids in *E. coli* ST131 has been shown to be associated with specific
124 regulatory mutations that may compensate the fitness cost of plasmid carriage⁴⁶.

125 **Bridging the gap**

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

130

- 131 • **Appropriate choice of infection models during research & development:** By considering the
132 physicochemical environment at infection sites and its effects on bacterial growth, gene
133 expression and physiology, will provide better estimates of likely rates of resistance emergence
134 and associated fitness costs which may differ substantially from those observed in standard
135 laboratory growth media³⁴. For example, the leucyl-tRNA synthetase inhibitor GSK2251052
136 showed limited resistance development *in vitro* but failed in clinical trials because of rapid
137 emergence of resistance in treated patients⁴⁷. Improved estimates of the risks of *in vivo*
138 resistance would allow drug candidates to be better prioritised for progression to clinical trials,
139 and could therefore directly benefit the pharmaceutical industry by more effectively targeting
140 investment.
- 141 • **Dosing to minimise resistance selection:** Massive experimental and modelling efforts are
142 made to quantify the PK/PD of new antibiotics before clinical trial with the goal of maximizing
143 antibiotic efficacy. Minimizing selection for resistance should also be a goal of PK/PD studies⁴⁸
144 which should employ appropriate infection models.
- 145 • **More intensive sampling of infections during clinical trials:** Current approaches sample
146 single colonies, ignoring the diversity present in most infections and therefore losing the
147 opportunity to understand evolutionary processes occurring in infections^{49,50}. More extensive
148 culture-based sampling should be combined with deep-sequencing to quantify resistance allele
149 frequencies and capture hard to culture organisms (e.g. persister cells, polymicrobial infections).
150 Access for scientists to the samples from completed clinical trials would allow the development
151 of methods and analyses for efficiently characterising the evolutionary mechanisms leading to
152 resistance in patients. Furthermore, development of culture conditions allowing maintenance of
153 diverse bacterial populations and/or communities in the lab would facilitate understanding how
154 resistance evolution is a function of microbial community interactions.
- 155 • **Microbiome sampling in clinical trials:** Antibiotic treatment often has collateral effects on the
156 microbiome including driving lateral transfer of resistance elements at other body sites⁵¹.
157 Combining new bioinformatics tools for resistance prediction⁵² with long-read or proximity ligation
158 sequencing technologies allows localisation of resistance genes to their genomic context and
159 the tracking of gene mobilisation and transfer^{53,54}.

160

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

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- 307

308 **Figure legend**

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

311
312