Allogenous Selection of Mutational Collateral Resistance: Old Drugs Select for New Resistances Within Antibiotic Families

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Running Title: Old Antibiotics Select for Modern Resistances

Keywords: Allogenous selection, Synergistic pleiotropy, Collateral hyper-resistance, Collateral selection, Collateral expansion, Collateral evolution, Antibiotic resistance mutations.

ABSTRACT

Allogeneous selection occurs when an antibiotic selects for resistance to more advanced members of the same family. The mechanisms of allogenous selection are (a) collateral expansion, when the antibiotic expands the gene and gene-containing bacterial populations favoring the emergence of other mutations, inactivating the more advanced antibiotics; (b) collateral selection, when the old antibiotic selects its own resistance but also resistances to more modern drugs; (c) collateral hyper-resistance, when resistance to the old antibiotic selects in higher degree for populations resistant to other antibiotics of the family than to itself; and (d) collateral evolution, when the simultaneous or sequential use of antibiotics of the same family selects for new mutational combinations with novel phenotypes, generally with higher activity or broader spectrum. Note that in some cases, collateral selection derives from collateral evolution. In this study, examples of allogenous selection are provided for the major families of antibiotics. Improvements in minimal inhibitory concentrations with the newest drugs do not necessarily exclude "old" antibiotics of the same family of retaining some selective power for resistance to the newest agents. If this were true, the use of older members of the same drug family would facilitate the emergence of mutational resistance to the younger drugs of the family, which is frequently based on previously established resistance traits. The extensive use of old drugs (particularly in low-income countries and in farming) might be significant for the emergence and selection of resistances to the novel members of the family, becoming a growing source of variation and selection of resistance to the whole family. In terms of future research, it could be advisable to focus antimicrobial drug discovery more on the identification of new targets and new (unique) classes of antimicrobial agents, than on the perpetual chemical exploitation of classic existing ones.

INTRODUCTION

Within a given family of antibiotics, the acquisition of mutational resistance in the chromosomal target or in transport genes to one of the antibiotic members frequently involves resistance to many or all other members of the family (cross-resistance). The emergence of resistance is an "antibiotic-specific" phenomenon, i.e., the use of antibiotic "*a1*" of the family "*A*" preferentially favors the selection of mutations providing resistance to "*a1*" and not necessarily (or less effectively) to "*a2*". This is explained by the frequent commonality of pharmacodynamic parameters. In the case of mutations in target or antibiotic-modifying (inactivating) genes, selection with "*a1*" favors the mutational emergence of "*a1*" resistance and to a lesser degree to other members. For example, within a single family, aminopenicillins (such as ampicillin or amoxycillin) select for aminopenicillin resistance; or oxyimino-cephalosporin selects for resistance to this group of antibiotics (such as cefotaxime or ceftazidime). However, this is not always true. We will discuss this issue, analyzing the cases in which the use of an *a1* antibiotic favors the emergence and spread of resistance to an *a2* antibiotic, leading to "allogeneous selection".

The phenomenon of "collateral-susceptibility" refers to the case in which resistance to a particular antibiotic is associated with high susceptibility to another antibiotic. Collateral susceptibility not only occurs (as is commonly believed) among antibiotics of different groups *(Podnecky et al., 2018),* but also among members of the same family. Mutational resistance to an A member occasionally increases the susceptibility to other "A" family drugs, indicating an asymmetry of the phenotypes resulting from mutational events involved in the acquisition of resistance *(Sanz-García et al., 2019).* This asymmetry is the main conceptual point making allogeneous selection a particularly interesting aspect

of the classic “selection by cross-resistance”. In fact, pleiotropy occurs when a single mutation affects multiple phenotypic traits. In other words, allogenous selection occurs when a given antibiotic selects mutations that confer resistance to another, often more potent or advanced, member of the same antibiotic family, either directly or by enriching the frequency of mutations at the population level that facilitate the emergence of resistance to the second antibiotic.

FOUR CASES DETERMINING ALLOGENOUS SELECTION

In this review, we consider several cases determining allogenous selection, as illustrated in Figure 1. First, the case of “collateral expansion” (Figure 1a), in which the use of a particular antibiotic increases the absolute frequency of a resistance determinant, which results in a greater chance of evolution to increased resistance to other novel member(s). For example, the use of ampicillin leads to enrichment of *bla* genes such as $bla_{\text{TEM-1}}$ or $bla_{\text{SHV-1}}$. The expansion of these *bla* genes could facilitate the mutational emergence of evolved variants called extended-spectrum beta-lactamases (ESBLs). This emergence facilitates the mutational emergence of TEM-1- or SHV-1- derived ESBLs, inactivating oxyimino-cephalosporins and resulting in an allogenous selection of these novel drugs. Second, the case of “collateral selection” (Figure 1b), in which allogenous selection occurs between subgroups of antibiotics of the same family. For example, the use of ampicillin or cefalexin (first-generation cephalosporins) selects for resistance to third generation cephalosporins and *vice versa*. Third, the case of “collateral hyper-resistance” (Figure 1c) within different families of antibiotics, in which exposure to an antibiotic “*a1*” favors the emergence of mutational resistance, resulting in a higher mean inhibitory concentration (MIC) of another antibiotic of the same family “a_n” absent in the selective process. For example, ceftazidime selects for strains with high efficiency carbapenemases more efficiently than carbapenems themselves. Fourth, the case of “collateral evolution”

98 (Figure 1d), in which the combined effect of 2 or more antibiotics from the same family
99 favors the emergence and further evolution and selection of novel resistance phenotypes,
100 eventually more efficient to inactivate on the ensemble of causative drugs.

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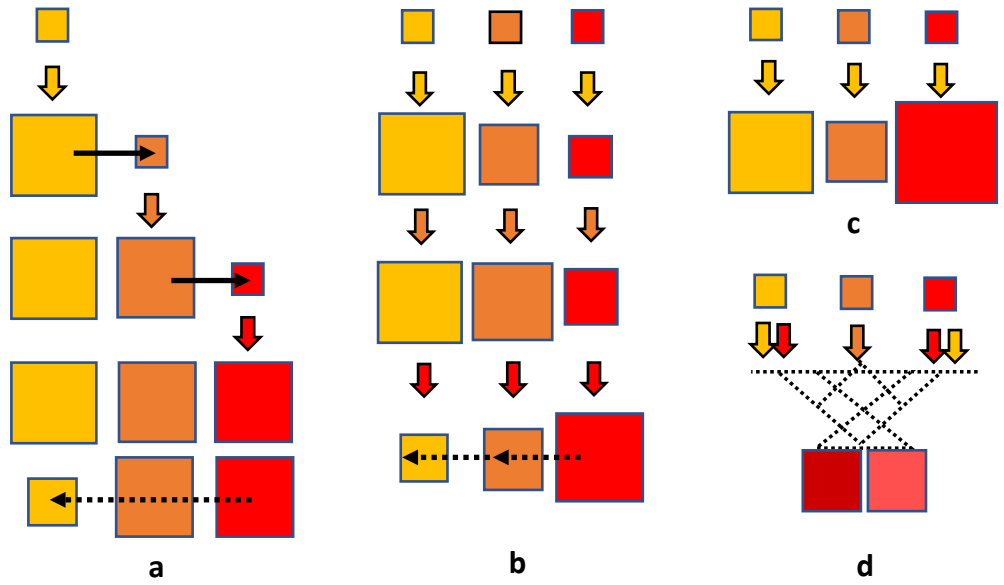

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103 **Figure 1. Four cases of allogeneous selection**, in which the use of an antibiotic (solid-
104 colored arrows, the potency of the antibiotic increasing in order from yellow to brown to
105 red) results in the emergence of mutants with resistance to other antibiotics of the same
106 family, even the more advanced ones. **(a) collateral expansion:** the yellow antibiotic
107 expands the gene and species populations with mutational resistance to the yellow drug
108 (yellow square), favoring the emergence of other mutations (black arrow) and inactivating
109 the brown antibiotic; the brown antibiotic expands these populations, favoring new
110 mutations producing resistance to the red antibiotic, which are selected by this drug. Note
111 that this final effect starts with use of the yellow (less potent) antibiotic, and results in
112 resistance to the red one (more potent). Eventually, resistance to the red antibiotic might
113 reduce the efficiency of resistance to the yellow one, by antagonistic pleiotropy or

collateral susceptibility (dotted black arrows). **(b) collateral selection:** the yellow antibiotic selects the populations with yellow resistance but also (to a minor extent) the brown and red resistances. However, further exposure to brown and red drugs selects for these resistant populations, but the trigger of the process is the weaker antibiotic; again, collateral susceptibility might occur (dotted black arrows). **(c) collateral hyper-resistance:** the yellow antibiotic selects for populations resistant to other antibiotics to a higher degree than to itself. **(d) collateral evolution:** when the simultaneous or sequential use of antibiotics of the same family selects for new mutational combinations with novel phenotypes, generally with higher activity or broader spectrum. In some cases, collateral selection derives from collateral evolution.

ALLOGENEOUS SELECTION WITHIN BETA-LACTAMS

Collateral expansion has strongly influenced the spread of antibiotic resistance within beta-lactams. The emergence of TEM-1- or SHV-1-derived ESBLs, hydrolyzing oxyimino cephalosporins, has occurred because of the wealth (absolute abundance) of $bla_{\text{TEM-1}}$, $bla_{\text{TEM-2}}$ or $bla_{\text{SHV-1}}$ genes resulting from the overprescription of aminopenicillins. Beta-lactamases are enzymes with a high tolerance to amino acid changes (*Zaccolo and Gherardi, 1999*), providing a highly plastic genetic background for mutational variation. The antibiotic-driven abundance of TEM-1 and SHV-1 enzymes favored the selection of mutational events when cefotaxime (CTX) became available and was widely used. Selection for CTX resistance in TEM-1-harboring strains is accompanied by smaller increases in ceftazidime (CAZ) resistance than *vice versa* (*Schenk et al., 2015*). Thus, selection by aminopenicillins has contributed to ESBL evolution. It will be of interest to search for TEM-derived ESBLs before 1983, the year when cefotaxime was launched. The number of natural TEM enzymes is much larger and could have led to cefotaxime-resistant variants that originated in TEM-1 or TEM-2, which

are just one part of a complex network of TEM enzymes with related sequences (*Zeil et al., 2016*). In addition, there is collateral susceptibility to aminopenicillins in strains harboring ESBLs such that alleles inactivating CTX become more sensitive to these drugs, a phenomenon of antagonistic pleiotropy. However, it can be suspected that the continued use of aminopenicillins could contribute to the selection of ESBLs. In fact, the ampicillin specificity constant, *kcat/Km*, which allows a comparison of the activity of various beta-lactamases on this substrate, is only 40 times lower for the ESBLs TEM-5 and TEM-10 than for TEM-2 (*Quinn et al., 1989*). In the case of ESBL derivatives of SHV-1 harboring the changes Gly238Ser or Gly238Ala, the difference is even lower, only between 6 and 3 times (*Hujer et al, 2002*). That difference suggests to us and others (Karen Bush, personal communication) that aminopenicillins might be sufficiently hydrolyzed by ESBLs to select for ESBL-containing organisms. However, most ESBL-containing organisms retain early aminopenicillin-hydrolyzing TEM or SHV enzymes even in the presence of ESBLs, which probably minimizes specific selection of ESBL-only strains, as has been indicated by membrane-computational models (*Campos et al, 2019*). In any case, the frequent coexistence of genes encoding early TEM-1 or SHV-1 beta-lactamases with ESBLs ensures that strains with ESBLs are also selected with aminopenicillins or first-generation cephalosporins. Certainly, first-generation cephalosporins contribute to the emergence and selection of *in vivo* resistance to CTX (*Kimura et al., 2017*). Even ampicillin can select for metallo-beta-lactamases such as NDM-1, which inactivate aminopenicillins (*Zhang and Hao, 2011*).

Another example of collateral expansion leading to allogenous selection within beta-lactams is the evolution of mutations in *S. pneumoniae* penicillin-binding proteins (PBPs) leading to resistance to third-generation cephalosporins. The use of penicillins from the 1940s, and particularly the overuse of aminopenicillins from the 1960s, has selected *S.*

pneumoniae low-level resistant strains. This also occurs with *in vitro* selection, yielding mutants in PBP2x and PBP2b: strains with higher levels of penicillin resistance occur because of added cumulative changes in PBP1a. PBP2x mutants are also involved in CTX resistance (*Maurer et al., 2008*), and penicillin-triggered multiple mutants, including those in PBP1a, significantly increase resistance to CTX (*Smith et al., 2001; Schrag et al, 2004*). In summary, the use of penicillins expanded the PBPs' genetic substrates, leading to the selection of CTX resistance. Finally, the emergence of the SCCmec element, giving rise to methicillin-resistant *Staphylococcus aureus* was also probably associated with the use of penicillins in the 1940s and 1950s and not necessarily with exposure to methicillin-oxacillin, launched 15 years later (*Harkins et al., 2017*).

Collateral hyper-resistance (Figure 1c) occurs in beta-lactams. Although it seems obvious that the ability to hydrolyze carbapenems would have resulted from an increasing exposure to carbapenems, several studies have demonstrated that oxyimino-cephalosporins are more efficient in this evolutionary process. Using VIM enzymes as a model, we demonstrated that CAZ, better than CTX or any carbapenem (imipenem, meropenem or ertapenem) was most probably responsible for the evolution of variants toward more efficient carbapenem hydrolysis (*Martínez-García et al, 2018; Galán et al., 2013*). The same can be observed in experimental evolution studies on KPC-2 carbapenemase, in which evolved single and double mutational variants showed a higher catalytic efficiency toward CAZ than toward carbapenems (*Mehta et al., 2015*).

It has also been observed that the exposure to ceftazidime favors certain mutational events in CTX-M enzymes that increase MIC to CAZ but with very different effects depending on the aminoacid position(s) involved. Positive selection on specific amino acid mutation sites is required to obtain high fitness peaks toward efficient CAZ hydrolysis, but also secondary mutations are needed to guarantee a more balanced CTX-CAZ resistance

189 phenotype, whereas other beta-lactams, such as cefuroxime, are much less affected
190 (*Novais et al., 2008*). This observation uncovers the role of the combinatorial exposure to
191 different beta-lactams (cefuroxime, cefotaxime, ceftazidime and/or cefepime) on CTX-
192 M collateral evolution, as described below.

193 Collateral evolution is also observed within beta-lactam resistance determinants. The
194 simultaneous or sequential exposure to beta-lactams contributes to the emergence and
195 selection of new beta lactamases through various combinatorial mutational trajectories,
196 giving rise to a plethora of intermediates from which evolution can occur From multiple
197 possible evolutionary trajectories, the emergence (*Novais et al., 2010)* of the most
198 evolved and efficient variants (e.g., CTX-M-32, CTX-M-58) as well as several CTX-M-
199 3 derivatives already described, can be easily explained by a fluctuating exposure to both
200 cefotaxime and ceftazidime that would favor some of these trajectories. This would favor
201 an increase in frequency of a series of mutational variants and their subsequent evolution
202 toward a balanced hydrolysis of both CTX and CAZ, which constitutes an example of
203 collateral evolution.

204 From the diverse possible evolutionary paths, some were predicted to occur more
205 frequently, such as those including the mutation D240G, precisely because they more
206 frequently generate variants that consistently confer an increased catalytic efficiency to
207 both oxyimino-cephalosporins, CTX and CAZ. In fact, data from the beta-lactamase
208 database as of July 2021 (http://bldb.eu/BLDB.php?prot=A#CTX-M) confirm these
209 predictions. given almost half (n=102/213; 48%) of the CTX-M-described variants
210 contain the D240G mutation (*Novais et al, 2010*). This study showed that P167S variants
211 are those that confer higher catalytic efficiency to these drugs. Experimental evolution
212 assays based on serial passages under increased concentrations of an oxyimino-
213 cephalosporins frequently yielded mutants that did not correspond to those more

commonly detected in nature (*Novais et al., 2008*). This result might indicate that the most successful long-term strategy follows slower trajectories, such as the D240G mutation, a phenomenon known as the "survival of the flattest" (*Day et al., 2015*). The fittest organisms in terms of MIC (selected under high antibiotic concentrations) could be less robust than those with lower increases in enzymatic activity.

It is also reasonable to hypothesize that the exposure of a wealth of CTX-M variants (with favorable phenotypes to CTX, CAZ or both) to beta-lactam/beta-lactamase inhibitor combinations would also extend the spectrum of these beta-lactamases. As another example of collateral evolution giving rise to combined phenotypes, specific mutations (S237G, K234R) on specific genetic backgrounds are able to produce slight increases in MIC to either amoxicillin-clavulanate or piperacillin-tazobactam combinations while maintaining the efficiency toward cephalosporins (*Ripoll et al., 2011*). However, these MIC levels are still in the susceptibility range and might go unnoticed by routine procedures or detected only when combined with additional mechanisms (e.g., production of OXA-1, permeability defects, or insertion sequences upstream of an antibiotic-resistance gene) (*Ripoll et al., 2011*). However, this situation is not very common, given the same or other mutations in variable CTX-M backgrounds drastically reduce the MIC to second- and third-generation cephalosporins (*Ripoll et al, 2011, Rosenkilde et al. 2019*). This collateral sensitivity effect could constrain the evolution of variants toward these combined phenotypes, which is why certain combinations (cephalosporins combined with beta-lactam inhibitors) could be explored to counteract/block evolution of antibiotic resistance. In fact, *in vitro,* the combination of CTX and mecillinam prevented CTX-M-15 to evolve toward combined resistance to other beta-lactams compared with each of these antibiotics alone (*Rosenkilde et al., 2019*).

We also need to consider that some of these conclusions arise from a small number of experimental replicates and that collateral responses might be more diverse. Experimental evolution assays with mathematical modelling assessing SHV-1 evolutionary trends under CTX exposure have shown not only a divergent collateral response but also that sensitivity to a second drug depends on the type of first mutation that arises (*Nichol et al., 2019*). By simulating diverse sequential combinations of beta-lactams, the authors also showed that cross-resistance, and thus collateral selection, frequently occur to cefazolin and ceftolozane-tazobactam. The cross-resistance reported between ampicillin (first drug) and CTX (second drug) suggests also that ampicillin exposure could have favored diversification of SHV-1 and selection of mutants with increased advantage over CTX, contributing partly to the selection of *Klebsiella pneumoniae* strains.

Multidrug efflux pumps contribute to intrinsic resistance to several antibiotics. These pumps also contribute to an MIC increase to several beta-lactams when they are overexpressed. In recent years, it has been shown that mutants in the structural elements of efflux pumps might modify their substrate profile. A good example of this is mutations in SmeH, a resistance-nodulation-division efflux pump transporter from *Stenotrophomonas maltophilia*, resulting in small increases in MICs to cephazolin (probably also to other second-generation cephalosporins), CTX and cefoxitin. These mutations can certainly be selected by these antibiotics, but the result is a significant increase in resistance to more “advanced” antibiotics such as CAZ and occasionally aztreonam (*Blanco et al., 2019*).

ALLOGENEOUS SELECTION WITHIN MACROLIDES

Most cases of allogenous selection of macrolide-resistant mutants involving other macrolide-lincosamide-streptogramin B (MLS) antibiotics correspond to complete (all MLS) or incomplete symmetrical cross-resistance, allogenous collateral selection. In any

case, the selection of strains with mutational resistance to old drugs can lead to increases in resistance to some of the novel ones. In fact, mutational resistance to old drugs (such as erythromycin) might produce increases in MICs to other MLS drugs, but this is not always the case (particularly with the newest macrolides), and infrequently the MIC increase for other drugs surpasses the MIC increase for erythromycin. However, allogenous hyper-resistance was found in azithromycin-resistant *Streptococcus pyogenes* mutants, increasing spiramycin resistance to a higher degree than that of azithromycin itself (*Malbruny et al., 2002*). Several mutations in the ribosomal macrolide binding site L4/L22 proteins and the 23S rRNA domains II/V selected under long-term serial erythromycin exposure in *Staphylococcus aureus* increased the MICs to ketolides and lincosamides (telithromycin or solithromycin), but not greater than the level of erythromycin resistance (*Yao et al., 2018*). Mutations in the 23S rRNA gene (such as A2142G) of *Helicobacter pylori* are generally associated with cross-resistance to all MLS antibiotics, but not in all cases: erythromycin-resistant strains might maintain susceptibility to clarithromycin (*Wang and Taylor, 1998; García-Arata et al., 1999).* In *Streptococcus pneumoniae*, erythromycin-resistant mutations obtained by serial passages are expected to contribute to selection of the resistant strain by exposure to 15- and 16-membered macrolides, streptogramin, and less frequently to lincosamides, but not to telithromycin (*Tait-Kamradt et al., 2000*), and this trend is expected to occur with other organisms.

Macrolides are common substrates of multidrug efflux pumps. The study of clinical *Haemophilus influenzae* isolates has shown that various mutations in the AcrR regulator, which triggers AcrAB pump expression, slightly increase azithromycin susceptibility without reaching breakpoints defining resistance and renders clarithromycin resistance (*Seyama et al, 2017*). These cases suggest that the use of azithromycin might have fewer

consequences on azithromycin resistance than on resistance to the allogenous agent, clarithromycin, indicating a possible case of allogenous hyper-resistance.

ALLOGENEOUS SELECTION WITHIN TETRACYCLINES

E. coli resistance determinants encoding tet(M), tet(K), tet(A) and E. coli tet(X) detoxify first-generation tetracycline (high MICs) but increase MICs to second-generation drugs such as doxycycline and minocycline, and also to those of the third generation, tigecycline and eravacycline (*Grossman, 2016*). Tigecycline and eravacycline MICs remain unchanged in strains with tet(B). The tet(X) resistance protein, a flavin-dependent monooxygenase, inactivates first- and second-generation tetracyclines (*Moore et al., 2005*), but some of its mutational variants increase minocycline or tigecycline MICs more than tetracycline itself (*Cui et al., 2021*). As in the case of classic antagonistic pleiotropy beta-lactamases evolving to ESBLs or inhibitor-resistant TEMs, Tet(M) mutants selected for increased tigecycline MICs might lose resistance against earlier tetracycline antibiotics (*Linkevicius et al., 2015*). Accordingly, with the Imamovic and Sommer map of "collateral sensitivity", in which a mutant to one antibiotic results in increased susceptibility to another (*Imamovic and Sommer, 2013),* minocycline-resistant mutants might increase resistance to tigecycline to a greater extent than to minocycline (its predecessor molecule) itself.

Undoubtedly, the main allogeneic selection by tetracyclines is the expansion of tetracycline resistance determinants, fostering the emergence of genetic variants that increase tetracycline resistance, which also influences (inactivates) the newest members of the family. These genetic variants probably include *tet* mosaic genes yielding higher MICs to tetracycline, such as tet(O)-tet(W) mosaic genes (*Stanton et al., 2004*).

ALLOGENEOUS SELECTION IN QUINOLONES-FLUOROQUINOLONES

Collateral expansion of quinolone-fluroquinolone resistance is evident. The emergence of fluoroquinolone resistance followed the previous selection (enrichment) of quinolone resistance genes by drugs such as nalidixic acid, oxolinic acid, cinoxacin, and pipemidic acid, widely used in the community in the 1960s and 1970s *(Emmerson and Jones, 2003, Ruiz, 2019).* These quinolones provided selective power for resistant variants harboring a significant first mutational step, paving the way toward fluoroquinolone resistance by acquisition of a second mutation. Therefore, at the time of launching norfloxacin in 1986, this enriched genetic background was ready for the unexpected rapid emergence and spread of fluoroquinolone resistance *(Aguiar et al., 1992).* One striking example can be that of *qnrB* alleles described in isolates from as early as in the 1930's (*Saga et al., 2013*) well before quinolones' market introduction. Their diversification within *Citrobacter* spp. and other *Enterobacteriaceae* after horizontal gene transfer mobilization explains their high allele diversity and current abundance (*Ribeiro et al, 2015*). Many of these variants have stable nalidixic acid phenotypes (*Rodríguez-Martínez et al., 2009*) and could eventually be selected by first generation quinolones, increasing the opportunity for resistance to newer generation quinolones to arise.

Collateral hyper-resistance might occur within quinolones-fluoroquinolones. Selection of double mutations in topoisomerases by ciprofloxacin (typically involving positions 83 and 87 of *gyrA*) probably increases the MIC of nalidixic acid five times more than the original ciprofloxacin MIC (*Vila et al., 1994*). Thus, the continued use of quinolones (such as nalidixic acid, oxolinic acid, cinoxacin and pipemidic acid) in the 1960s and 1970s should have facilitated an efficient selection of fluoroquinolone resistance, introduced in the late 1980s or later, such as norfloxacin, ciprofloxacin, ofloxacin, moxifloxacin, tosufloxacin, or sitafloxacin (*Honda et al., 2020*). Nalidixic acid is used in

South-East Asian countries mainly to treat *Shigella* infections (*Hoge et al., 1995*). Today, nalidixic acid is still consistently found in European rivers (*Castrignanò et al., 2020*) and will continue to serve as a selector for resistance to the newest fluoroquinolones. Similarly, exposure to second-generation fluoroquinolones such as ciprofloxacin and ofloxacin typically selects mutants that show increased resistance to these drugs, but even higher levels of resistance to more recent fluoroquinolones, such as sparfloxacin (a third-generation fluoroquinolone). Again, exposure to sparfloxacin selects mutants that lead to greater resistance to gatifloxacin (a fourth-generation fluoroquinolone) (*Fukuda et al., 1998; Sanders, 2001*).

In addition to mutations in genes encoding their targets, quinolone resistance can be achieved by the activity of resistance determinants that can be intrinsic (multidrug efflux pumps) or acquired (Qnr, QepA, OqxAB or AAC(6')-Ib-cr). These resistance determinants are more proficient against the first generation of quinolones, and their activity against fluoroquinolones and later generations is less, but clinical resistance can be reached through overexpression (*Garoff et al., 2018*). In the case of Qnr, which shields the target of quinolones, there are mutational alleles presenting various degrees of protection against ciprofloxacin (*Tavio et al., 2014, Rodríguez-Martínez, 2009*); whether they act in parallel in all quinolones remains unclear. In addition, it has also been shown that the presence of transferable quinolone resistance determinants, often conferring non-clinically relevant phenotypes, favors the selection of mutations in other chromosomal targets that act cooperatively to increase MIC to quinolones (*Cesaro et al., 2008; Li et al., 2019*). However, it is clear that older quinolones (such as nalidixic acid) should efficiently select for QnrA1, QnrB1, QnrS1, AAC(6')-Ib-cr and QepA (*Hooper and Jacoby, 2016*). Each of these genes might present different alleles, and the effect of each of these alleles in the resistance profile can vary. The study of environmental microbiomes has detected

362 an AAC(6')-Ib-cr allele (the WY variant) that presented increased activity against
363 gemifloxacin and was less active against ciprofloxacin compared with the “wild-type” -
364 cr allele. Note that these alleles are unlikely to be the result of selection in clinics, given
365 they have not yet been encountered in isolates from patients (*Kim et al., 2018*). Also, it
366 has been shown for the first time that *in vitro* substitutions causing mutations in the
367 structural elements of intrinsic multidrug efflux pumps can alter their substrate specificity
368 profile within members of the same family of antibiotics (*Blair et al., 2015*). Amino acid
369 substitution within the drug binding pocket of an efflux pump protein (AcrB) caused
370 selection of relevant ciprofloxacin resistance in *Salmonella Typhimurium*, and in some
371 cases, nalidixic acid resistance was increased more than for ciprofloxacin (allogeneous
372 hyper-resistance). However, mutations in the pump SmeH of *S. maltophilia* (previously
373 discussed) increased norfloxacin resistance without altering the susceptibility to nalidixic
374 acid or ofloxacin (*Blanco et al, 2019).*

375

376 **ALLOGENEOUS SELECTION WITHIN AMINOGLYCOSIDES**

377 Allogeneous selection appears to be rare or nonexistent in aminoglycosides. Despite
378 prolonged use of members of this family of antibiotics, nothing comparable to the
379 emergence of hundreds of mutant extended-spectrum beta-lactamases under beta-lactam
380 exposure has occurred, and no mutant derivatives of aminoglycoside-modifying enzymes
381 have been reported in clinical isolates (*Toth et al., 2010*). Among the more frequent
382 inactivating enzymes are the AAC (6') enzymes, with three families that have been
383 recognized by phylogenetic analysis. The possibility of the *aac(6')-Iaa* gene evolving to
384 increased levels of resistance to gentamicin, tobramycin, kanamycin or amikacin and to
385 acquire resistance to gentamicin was assessed by *in vitro* evolution experiments, which
386 did not succeed in obtaining alleles with increased MICs (*Salipante and Hall, 2003*).

In addition to classical inactivating enzymes, several traits unrelated to classical antibiotic resistance determinants (most of them participating in pathways of bacterial metabolism) contribute to intrinsic resistance to aminoglycosides. Given their role in basic bacterial physiology, their activity should be similar to all aminoglycosides. Unexpectedly, in a study on *P. aeruginosa* seeking changes in MICs of four aminoglycosides by transposon-tagged insertion mutagenesis, the majority of mutants did not show changes in MICs for any of four studied aminoglycosides (streptomycin, kanamycin, tobramycin, amikacin), suggesting a certain degree of specificity of these "metabolism-derived resistance-traits" (*Sanz-García et al., 2019*). Apramycin-resistant mutants might have reduced MICs to streptomycin, kanamycin or neomycin; again, this is a possible case of antagonistic pleiotropy *(Walton, 1978*).

ALLOGENEOUS SELECTION IN OTHER ANTIBIOTIC FAMILIES: GLYCO-LIPOPEPTIDES, INHIBITORS OF FOLATE METABOLISM

Vancomycin and avoparcin selects for VanA and VanB glycopeptide resistance determinants in enterococci. In the resistance process, there are two-component regulatory systems (VanR-VanS and VanRB-VanSB) that are inducers of the expression of resistance genes. Teicoplanin is a poor inducer (badly recognized); thus, strains with VanA or VanB might remain teicoplanin-susceptible. However, mutations in the genes involved in these regulatory systems might evolve teicoplanin resistance (*Baptista et al., 1996*). Vancomycin exposure might yield *Staphylococcus capitis* mutants with increased resistance to daptomycin (*Butin et al., 2015).* In *Staphylococcus aureus*, vancomycin exposure selects several mutations giving rise to a vancomycin-intermediate phenotype (VISA), some of which also reduce the effect of daptomycin. However, vancomycin exposure rarely selects for mprF mutations in reduced daptomycin-vancomycin susceptibility (*Thitiananpakorn et al., 2020*).

Daptomycin-resistant mutants have been detected in clinical coagulate-negative *Staphylococcus*. Here again, mutations influence the effect of a two-component regulator, WalKR (*Jiang et al., 2019*). As previously mentioned, daptomycin-resistant mutants based on the phospholipid flippase MprF emerges under daptomycin exposure (*Ernst et al., 2018*). In vancomycin-resistant *Enterococcus faecium*, mutations in both *liaFSR* and cardiolipin synthase (*cls*) genes presented a high level of resistance to daptomycin (*Wang et al., 2018*).

Allogenous selection has not been detected among mutants to antifolate inhibitors. Sulfonamide mutational resistance (mostly by alterations in dihydropteroate synthase) appeared to have no impact on the level of trimethoprim resistance, given the trimethoprim MICs for four different strains resistant to sulfonamides but susceptible to trimethoprim and which were transformed to trimethoprim resistance remained unchanged from their original MICs (*Adrian and Klugman, 1997).*

SYNERGISTIC MUTATIONAL PLEIOTROPY IN RESISTANCE WITHIN MEMBERS OF ANTIBIOTIC FAMILIES: AN EVOLUTIONARY ACCELERATOR?

Allogenous selection results from a pleiotropic effect that can be considered the opposite (synergistic pleiotropy) of antagonistic pleiotropy within a family ("*A*") of antibiotics, in which a mutational event increasing resistance to the *a1* antibiotic reduces resistance to another *a2* antibiotic. The typical case of antagonistic pleiotropy can be illustrated by mutations of classic TEM-1, TEM-2, SHV-1 or ROB-1 beta-lactamases, which are extremely active on aminopenicillins. Mutations influencing the beta-lactamase omega loop, which are found in oxyimino-cephalosporin-resistant variants, reduce enzymatic stability in these beta-lactamases (*Poirel et al., 2001*). Classic beta-lactamases are thus converted into ESBLs, but at the expense of drastically reducing hydrolytic efficiency

toward aminopenicillins and first-generation cephalosporins. (*Matagne and Frere, 1995; Raquet et al., 2004; Galán et al., 2003*). Similarly, acquisition of mutational resistance to inhibitors of beta-lactamases reduces the hydrolyzing activity against aminopenicillins and ESBLs, including CTX-M enzymes *(Ripoll et al., 2011).* A similar case occurs in the development of new mutational variants of the beta-lactamase (carbapenemase) KPC-1; KPC-2 or KPC-3 reduce carbapenem MICs but also affect the inhibitor capacity of avibactam. Avibactam, however, is not a member of the beta-lactam family (*Gaibani et al., 2018; Giddings et al, 2018)*). Of course, antibiotic antagonistic pleiotropy (also called collateral susceptibility, but which includes antibiotics from other families) tends to slow the evolution of resistance with use of multiple antibiotics of the same family.

Antibiotic synergistic pleiotropy within families could eventually accelerate the development of resistance, because of the simultaneous evolution of resistance traits. It has been shown that those additive interactions and epistatic interactions resulting from exposure to different cephalosporins increase the ability of a TEM enzyme to provide higher fitness to the host cell than any single cephalosporin (*Mira et al., 2021*). The predictability of resistance phenotypes resulting from mutations to different antibiotics (*Knopp and Andersson, 2018*) suggests that a similar approach could be applied to mutations within a single antibiotic family.

Synergistic pleiotropy has been proposed to explain the evolution of adaptive traits in plants and animals (*Frachon et al., 2017*) and should be more effective in simpler organisms, such as bacteria. There is a “cost of complexity” that results in complex organisms adapting more slowly than simple ones when using mutations of the same phenotypic size (*Orr et al, 2000; Rocabert et al., 2020)*.

PRACTICAL CONCLUSIONS: BEING AWARE OF COMMON MISTAKES

Within a single-family *A* of antibiotics, when resistance to an *a1* antibiotic emerges, a new *a2* antibiotic active in *a1* resistant organisms is often developed and introduced in clinical practice. However, it is frequently not known (untested) whether the introduction of *a2* will select *a2* mutants with increased resistance (MIC) to *a1* in *a1*-susceptible or low-level resistance strains. Thus, the increase in the use of *a2* (because of the hypothetical prevention of *a1* resistance) will eventually help to reduce the overall effect of the older drug. On the other hand, improvements in MICs with the newest drugs of the same family do not necessarily preclude the "old" antibiotics from retaining some selective power for resistance to the newest agents. If this is true, the use of older members of the same family would facilitate the emergence of mutational resistance to the younger drugs of the family, which is frequently based on previously established resistance traits.

It could be mistaken to consider the "older drugs" that have been replaced in high-income countries by more advanced ones (generally much more expensive) of the same family as "accessible" (cheaper) antibiotics to be used in low-income countries. These drugs will probably select for "modern resistance traits," inactivating the novel members of the family. This phenomenon could explain (together with lack of proper sanitation) the increase in "modern resistances" such as NDM-1 beta-lactamase in low-income countries (see above) among low-income countries to beta-lactamases such as NDM-1 (see above). The same is true for the "old antibiotics" used in livestock or agriculture.

The rapid worldwide propagation of certain multiresistant bacterial clones, such as *E. coli* ST131 harboring CTX-M-15, or in *Klebsiella* and *E. coli* clones harboring NDM-1, even in continents with scarce use of expensive extended-spectrum cephalosporins or carbapenems, could be due to local selection of these clones by old, inexpensive, widely used antibiotics such as aminopenicillins or first-generation cephalosporins (*Ghiglione et*

al., 2018; Walsh et al, 2005) and their subsequent spread in poor sanitary conditions (*Iskandar et al., 2020*).

Policies based on restricting the use of specific antibiotics as a response to increases in resistance might be misleading, because the continued use of "old" antibiotics of the same family could provide a powerful selection field for antibiotic-resistant mutants to the newest ones. The mixture of "old" and "new" antimicrobials of the same family, or even different types of "new" ones in a local setting is frequently synergistic for the evolution of antibiotic resistance. In addition, in recent times there has been a "vintage trend" to revive old antibiotics (*Theuretzbacher et al., 2015, Falagas et al., 2008)*; however, we should be aware of the risks of such an approach. In Figure 2, we show the key role of first-generation antibiotics ("old ones") within a family of drugs in maintaining and extending antibiotic resistance.

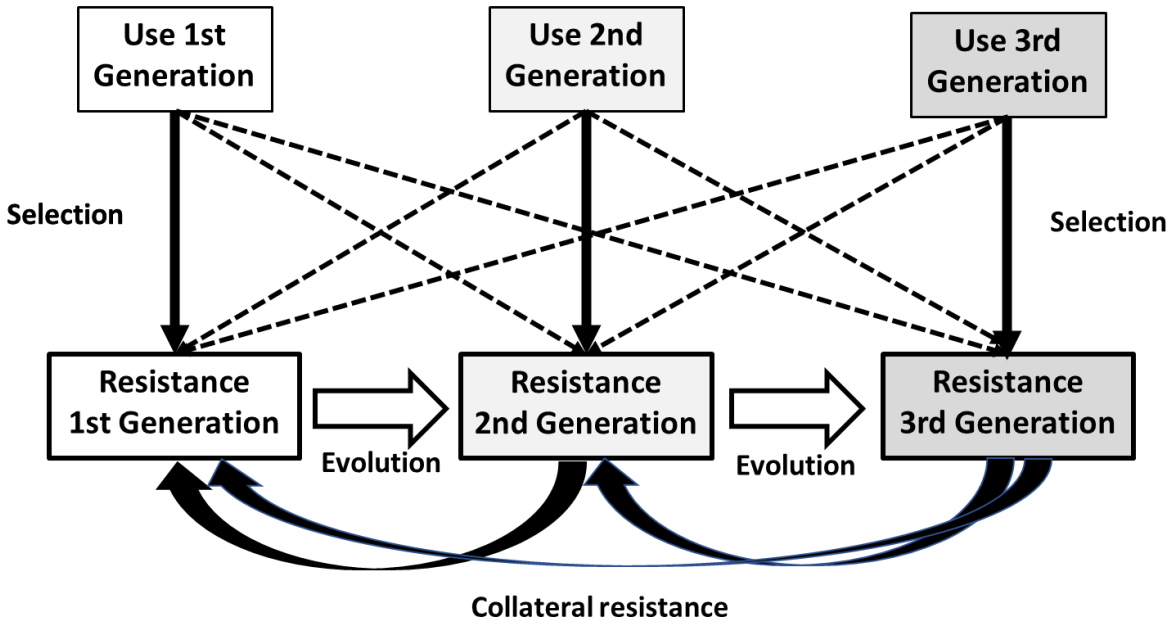

Figure 2. Key role of first-generation antibiotics within a family of drugs in maintaining and extending antibiotic resistance. Each generation of drugs preferentially selects

resistance to the corresponding generation (vertical thick arrows) but might select for resistance to other generations (broken arrows). Quantitative expansion of resistance to first-generation drugs provides a wealth of genetic sequences from which evolution to resistance to second- and third generation drugs takes place (white horizontal arrows). Resistance to second- and third-generation antibiotics frequently inactivates (and then selects by collateral resistance, curved arrows) resistance to first-generation drugs, which is amplified by the constant use of all members of the family and is thus maintained as a growing source of variation and selection of resistance to the whole family.

In terms of future research, it might be advisable to focus antimicrobial drug discovery more on the identification of new targets and new (unique) classes of antimicrobial agents than on the perpetual chemical exploitation of existing classic ones. Of course, that will not prevent the evolution of resistance to the members of these novel families, except if they are used very prudently or in combination therapy. In any case, the consideration of complex networks of collateral susceptibility *(Imamovic and Sommer, 2013),* and collateral resistance (allogenous selection) merits further exploration to reduce the dynamics of evolutionary paths and trajectories in antibiotic resistance (*Baquero et al., 2021*).

FUNDING

This work was supported (FB, TMC) by Fundación Ramón Areces and the Instituto de Salud Carlos III (PI18/1942) and co-funded by the European Regional Development Fund (ERDF, 'A way to achieve Europe'). Also supported by InGEMICS-CM (B2017/BMD-3691), funded by Comunidad de Madrid (Spain) and CIBER (CIBER in Epidemiology and Public Health, CIBERESP; CB06/02/0053), integrated in the Spanish 2013-2016 I+D+i State Plan and funded by

Instituto de Salud Carlos III. ÂN is supported by national funds through FCT in the context of the transitional norm [DL57/2016/CP1346/CT0032].

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

Adrian, P. V., Klugman, K. P. (1997). Mutations in the dihydrofolate reductase gene of trimethoprim-resistant isolates of *Streptococcus pneumoniae*. *Antimicrob Agents and Chemother*. 41, 2406-2413.

Aguiar, J.M., Chacon, J., Canton, R., Baquero, F. (1992). The emergence of highly fluoroquinolone-resistant *Escherichia coli* in community-acquired urinary tract infections. *J Antimicrob Chemother*. 29:349-50.

Baquero, F., Martinez, J. L., F. Lanza, V., Rodríguez-Beltrán, J., Galán, J. C., San Millán, A., et al. (2021). Evolutionary pathways and trajectories in antibiotic resistance. *Clin Microbiol Rev*, 34(4), e00050-19

Baptista, M., Depardieu, F., Courvalin, P., Arthur, M. (1996). Specificity of induction of glycopeptide resistance genes in *Enterococcus faecalis*. *Antimicrob Agents Chemother*. 40:2291-2295

Blair, J. M., Bavro, V. N., Ricci, V., Modi, N., Cacciotto, P., Kleinekathöfer, U., et al (2015). AcrB drug-binding pocket substitution confers clinically relevant resistance and altered substrate specificity. *Proc Natl Acad Sci U.S.A.*, 112: 3511-3516.

548 Blanco, P., Corona, F., Martínez, J.L. (2019). Involvement of the RND efflux pump
549 transporter SmeH in the acquisition of resistance to ceftazidime in *Stenotrophomonas*
550 *maltophilia*. *Sci Rep*, 20; 9(1):4917.

551 Butin, M., Martins-Simoes, P., Picaud, J.C., Kearns, A., Claris, O., Vandenesch, F., et al.
552 (2015). Adaptation to vancomycin pressure of multiresistant *Staphylococcus*
553 *capitis* NRCS-A involved in neonatal sepsis. *J Antimicrob Chemother* 70:3027–3039

554 Campos, M., Capilla, R., Naya, F., Futami, R., Coque, T., Moya, A. et al. (2019).
555 Simulating multilevel dynamics of antimicrobial resistance in a membrane computing
556 model. *MBio*, 10(1), e02460-18.

557 Castrignanò, E., Kannan, A.M., Proctor, K., Petrie, B., Hodgen S, Feil, E.J., et al. (2020).
558 (Fluoro)quinolones and quinolone resistance genes in the aquatic environment: A river
559 catchment perspective. *Water Res*. 182:116015)

560 Cesaro, A., Bettoni, R.R., Lascols, C., Mérens, A., Soussy, C.J., Cambau, E. (2008). Low
561 selection of topoisomerase mutants from strains of *Escherichia coli* harbouring plasmid-
562 borne *qnr* genes. *J Antimicrob Chemother* 61:1007–1015

563

564 Cui, C.Y., He, Q., Jia, Q.L., Li, C., Chen, C., Wu XT, et al. (2021). Evolutionary
565 Trajectory of the Tet(X) Family: Critical Residue Changes towards High-Level
566 Tigecycline Resistance. *mSystems*. 6(3),e00050-21.

567 Day, T., Huijben, S., Read, A.F. (2015). Is selection relevant in the evolutionary
568 emergence of drug resistance?. *Trends Microbiol*. 23,126-133.

569 Emmerson, A.M., Jones, A.M. (2003). The quinolones: decades of development and use.
570 *J Antimicrob Chemother*.51 Suppl 1:13-20.

571 Ernst, C. M., Slavetinsky, C. J., Kuhn, S., Hauser, J. N., Nega, M., Mishra, N. N., et al
572 (2018). Gain-of-function mutations in the phospholipid flippase MprF confer specific
573 daptomycin resistance. *MBio*, 9(6), e01659-18).

574 Falagas, M. E., Grammatikos, A. P., Michalopoulos, A. (2008). Potential of old-
575 generation antibiotics to address current need for new antibiotics. *Expert Rev Antiinfect*
576 *Ther*, 6, 593-600)

577 Frachon, L., Libourel, C., Villoutreix, R., Carrère, S., Glorieux, C., Huard-Chauveau, C.,
578 et al. (2017). Intermediate degrees of synergistic pleiotropy drive adaptive evolution in
579 ecological time. *Nat Ecol Evol*. 1,1551-1561

580 Fukuda, H., Hori, S., Hiramatsu, K.. (1998). Antibacterial activity of gatifloxacin (AM-
581 1155, CG5501, BMS-206584), a newly developed fluoroquinolone, against sequentially
582 acquired quinolone-resistant mutants and the *norA* transformant of *Staphylococcus*
583 *aureus*. *Antimicrob Agents Chemother*. 42,1917-1922.

584 Galán, J.C., Morosini, M.I., Baquero, M.R., Reig, M., Baquero, F. (2003). *Haemophilus*
585 *influenzae bla*ROB-1 mutations in hypermutagenic delta-*ampC Escherichia coli*
586 conferring resistance to cefotaxime and beta-lactamase inhibitors and increased
587 susceptibility to cefaclor. *Antimicrob Agents Chemother*. 47, 2551-2557.

588 Galán, J. C., González-Candelas, F., Rolain, J. M., Cantón, R. (2013). Antibiotics as
589 selectors and accelerators of diversity in the mechanisms of resistance: from the resistome
590 to genetic plasticity in the β-lactamases world. *Front Microbiol*, 4, 9.

591 García-Arata, M. I., Baquero, F., de Rafael, L., de Argila, C. M., Gisbert, J. P., Bermejo,
592 F., et al. (1999). Mutations in 23S rRNA in *Helicobacter pylori* conferring resistance to
593 erythromycin do not always confer resistance to clarithromycin. *Antimicrob Agents*
594 *Chemother*, 43, 374-376.

595 Gaibani, P., Campoli, C., Lewis, R.E., Volpe, S.L., Scaltriti, E,, Giannella, M, et al..
596 (2018). In vivo evolution of resistant subpopulations of KPC-producing *Klebsiella*
597 *pneumoniae* during ceftazidime/avibactam treatment. *J Antimicrob Chemother* 73,1525–
598 1529.

599 Garoff, L., Yadav, K., Hughes, D. (2018). Increased expression of Qnr is sufficient to
600 confer clinical resistance to ciprofloxacin in *Escherichia coli*. *J Antimicrob Chemother*
601 73:348–352.

602 Ghiglione, B., Rodríguez, M. M., Curto, L., Brunetti, F., Dropa, M., Bonomo, R. A., et
603 al. (2018). Defining substrate specificity in the CTX-M family: the role of Asp240 in
604 ceftazidime hydrolysis. *Antimicrob Agents Chemother*, 62, e00116-18

605 Giddins M.J., Macesic, N., Annavajhala, M.K., Stump, S., Khan, S., McConville, T.H.,
606 et al. (2018). Successive emergence of ceftazidime-avibactam resistance through distinct
607 genomic adaptations in *bla*KPC-2-harboring *Klebsiella pneumoniae* sequence type 307
608 isolates. *Antimicrob Agents Chemother* 62(3):e02101-17

609 Grossman, T. H. (2016). Tetracycline antibiotics and resistance. *Cold Spring Harb*
610 *Perspect Med*, 6(4), a025387.

611 Harkins, C.P., Pichon, B., Doumith, M., Parkhill, J., Westh, H., Tomasz A, et al, (2017)
612 Methicillin-resistant *Staphylococcus aureus* emerged long before the introduction of
613 methicillin into clinical practice. *Genome Biol* 18(1):130.

614 Hoge, C. W., Bodhidatta, L., Tungtaem, C., Echeverria, P. (1995). Emergence of nalidixic
615 acid resistant *Shigella dysenteriae* type 1 in Thailand: an outbreak associated with
616 consumption of a coconut milk dessert. *Int J Epidemiol*, 24, 1228-1232.

Honda, H., Sato, T,, Shinagawa, M., Fukushima, Y., Nakajima, C., Suzuki, Y. et al. (2020). In Vitro Derivation of Fluoroquinolone-Resistant Mutants from Multiple Lineages of *Haemophilus influenzae* and Identification of Mutations Associated with Fluoroquinolone Resistance. *Antimicrob Agents Chemother* 64(2), e01500-19

Hooper, D.C., Jacoby, G.A. (2016). Topoisomerase Inhibitors: Fluoroquinolone Mechanisms of Action and Resistance. *Cold Spring Harb Perspect Med*. 6(9):a025320.

Hujer, A. M., Hujer, K. M., Helfand, M. S., Anderson, V. E., Bonomo, R. A. (2002). Amino acid substitutions at Ambler position Gly238 in the SHV-1 β-lactamase: exploring sequence requirements for resistance to penicillins and cephalosporins. *Antimicrob Agents Chemother,* 46: 3971-3977.

Imamovic, L., Sommer, M. O. (2013). Use of collateral sensitivity networks to design drug cycling protocols that avoid resistance development. *Sci Transl Med*, 5, 204ra132

Iskandar, K., Molinier, L., Hallit, S., Sartelli, M., Catena, F., Coccolini, F., et al (2020). Drivers of antibiotic resistance transmission in low-and middle-income countries from a "one health" perspective—a review. *Antibiotics*, 9(7), 372.

Jiang, J.H., Dexter, C., Cameron, D.R., Monk, I.R., Baines, S.L., Abbott, I.J., et al . (2019) Evolution of daptomycin resistance in coagulase-negative Staphylococci involves mutations of the essential two-component regulator WalKR. *Antimicrob Agents Chemother*. 63(3):e01926-18).

Kim, D. W., Thawng, C. N., Choi, J. H., Lee, K., Cha, C. J. (2018). Polymorphism of antibiotic-inactivating enzyme driven by ecology expands the environmental resistome. *ISME J*, 12, 267-276.

639 Kimura, A., Yossapol, M., Shibata, S., Asai, T,. (2017). Selection of broad-spectrum
640 cephalosporin-resistant Escherichia coli in the feces of healthy dogs after administration
641 of first-generation cephalosporins. *Microbiol Immunol*. 61(1):34-41

642 Knopp, M., Andersson, D.I. (2018). Predictable phenotypes of antibiotic resistance
643 mutations. *mBio* 9:e00770-18

644 Li, X., Zhang, Y., Zhou, X., Hu, X., Zhou, Y., Liu, D. et al., (2019). The plasmid-borne
645 quinolone resistance protein QnrB, a novel DnaA-binding protein, increases the bacterial
646 mutation rate by triggering DNA replication stress. *Mol Microbiol* 111:1529–1543.

647 Mira, P.M, Østman, B., Guzman-Cole, C., Sindi, S., Barlow, M. (2021) Adaptive
648 Processes Change as Multiple Functions Evolve. *Antimicrob Agents Chemother*.
649 65(4):e01990-20.

650 Podnecky, N. L., Fredheim, E. G., Kloos, J., Sørum, V., Primicerio, R., Roberts, A. P., et
651 al. (2018). Conserved collateral antibiotic susceptibility networks in diverse clinical
652 strains of Escherichia coli. *Nat Comm*, 9(1), 1-11

653 Poirel, L., Naas, T., Le Thomas, I., Karim, A., Bingen, E., Nordmann, P. (2001). CTX-
654 M-type extended-spectrum β-lactamase that hydrolyzes ceftazidime through a single
655 amino acid substitution in the omega loop. *Antimicrob Agents Chemother* 45,3355–3361.

656 Quinn, J. P., Miyashiro, D., Sahm, D., Flamm, R., Bush, K. (1989). Novel plasmid-
657 mediated beta-lactamase (TEM-10) conferring selective resistance to ceftazidime and
658 aztreonam in clinical isolates of *Klebsiella pneumoniae*. *Antimicrob Agents*
659 *Chemother*, 33, 1451-1456.

660 Linkevicius, M., Sandegren, L., Andersson, D.I. (2015) Potential of tetracycline
661 resistance proteins to evolve tigecycline resistance. *Antimicrob Agents Chemother*
662 60,789-96.

663 Malbruny, B., Nagai, K., Coquemont, M., Bozdogan, B., Andrasevic, A.T., Hupkova, H.,
664 et al. (2002). Resistance to macrolides in clinical isolates of *Streptococcus pyogenes* due
665 to ribosomal mutations, *J Antimicrob Chemother*, 49:935–939.

666 Martínez-García, L., González-Alba, J. M., Baquero, F., Cantón, R., Galán, J. C. (2018).
667 Ceftazidime is the key diversification and selection driver of VIM-type
668 carbapenemases. *MBio*, 9(3), e02109-17

669 Matagne, A., Frère, J.M.. (1995). Contribution of mutant analysis to the understanding of
670 enzyme catalysis: the case of class A beta-lactamases. *Biochim. Biophys. Acta* 1246:109–
671 12.

672 Maurer, P., Koch, B., Zerfass, I., Krauss, J., van der Linden, M., Frère, J.M. et al. (2008).
673 Penicillin-binding protein 2x of *Streptococcus pneumoniae*: three new mutational
674 pathways for remodelling an essential enzyme into a resistance determinant. *J Mol*
675 *Biol.* ;376:1403-16.

676 Mehta, S. C., Rice, K., Palzkill, T. (2015). Natural variants of the KPC-2 carbapenemase
677 have evolved increased catalytic efficiency for ceftazidime hydrolysis at the cost of
678 enzyme stability. *PLoS Path*, 11(6), e1004949.

679 Moore, I.F., Hughes, D.W., Wright, G.D. (2005). Tigecycline is modified by the flavin-
680 dependent monooxygenase TetX. *Biochemistry*. 44(35):11829-35

Nichol, D., Rutter, J., Bryant, C., Hujer, A. M., Lek, S., Adams, M. D., et al. (2019). Antibiotic collateral sensitivity is contingent on the repeatability of evolution. *Nat Commun*, 10:1-10.

Novais, Â., Cantón, R., Coque, T. M., Moya, A., Baquero, F., Galán, J. C. (2008). Mutational events in cefotaximase extended-spectrum β-lactamases of the CTX-M-1 cluster involved in ceftazidime resistance. *Antimicrob Agents Chemother*, 52, 2377-2382.

Novais, Â., Comas, I., Baquero, F., Canton, R., Coque, T. M., Moya, A., et al. (2010) Evolutionary trajectories of beta-lactamase CTX-M-1 cluster enzymes: predicting antibiotic resistance. *PLoS Path*, 6(1), e1000735.

Orr, H.A. Adaptation and the cost of complexity. (2000). *Evolution* 54,13-20

Raquet, X., J. Lamotte-Brasseur, E. Fonze, S. Goussard, P. Courvalin, Frère, J.M (1994). TEM b-lactamase mutants hydrolysing third-generation cephalosporins. *J. Mol. Biol*. 244, 625–639

Ribeiro, T.G., Novais, Â, Branquinho, R., Machado, E., Peixe, L. (2015). Phylogeny and comparative genomics unveil independent diversification trajectories of *qnrB* and genetic platforms within particular *Citrobacter* species. *Antimicrob Agents Chemother*. 59,5951-5958.

Ripoll, A., Baquero, F., Novais, Â., Rodríguez-Domínguez, M. J., Turrientes, M. C., Cantón, R., et al. (2011). In vitro selection of β-lactam plus β-lactamase inhibitor resistant variants in CTX-M β-lactamases: predicting the in-vivo scenario? *Antimicrob Agents Chemother*. 55, 4530-4536

Rocabert, C., Beslon, G., Knibbe, C., Bernard, S. (2020). Phenotypic noise and the cost of complexity. *Evolution*. 74,2221-2237.

Rodríguez-Martínez, J.M., Briales, A., Velasco, C., Conejo, M.C., Martínez-Martínez, L., Pascual, A. (2009). Mutational analysis of quinolone resistance in the plasmid-encoded pentapeptide repeat proteins QnrA, QnrB and QnrS. *J Antimicrob Chemother*, 63,1128-34

Rosenkilde, C.E.H., Munck, C., Porse, A., Linkevicius, M., Andersson, D.I., Sommer M.O.A. (2019). Collateral sensitivity constrains resistance evolution of the CTX-M-15 β-lactamase. *Nat Commun*. 10(1) ,618.

Ruiz, J. (2019). Transferable mechanisms of quinolone resistance from 1998 onward. *Clin Microbiol Rev*. 32(4), e00007-19.

Saga, T., Sabtcheva, S., Mitsutake, K., Ishii, Y., Tateda, K, Yamaguchi, K. et al., (2013). Characterization of *qnrB*-like genes in *Citrobacter* species of the American Type Culture Collection. *Antimicrob Agents Chemother* 57,2863–2866,

Salipante, S.J., Hall, B.G. (2003). Determining the limits of the evolutionary potential of an antibiotic resistance gene. *Mol Biol Evol* 20,653–659.

Sanders, C.S. Mechanisms responsible for cross-resistance and dichotomous resistance among the quinolones (2001) *Clin Infect Dis* 32, Suppl.1, S1–S8.

Sanz-García, F., Alvarez-Ortega, C., Olivares-Pacheco, J., Blanco, P., Martínez, J.L., Hernando-Amado S. (2019). Analysis of the *Pseudomonas aeruginosa* aminoglycoside differential resistomes allows defining genes simultaneously involved in intrinsic antibiotic resistance and virulence. *Antimicrob Agents Chemother*. 63e00185-19.

Schenk, M.F., Witte, S., Salverda, M.L., Koopmanschap, B., Krug, J., de Visser, J.A. (2015) Role of pleiotropy during adaptation of TEM-1 β-lactamase to two novel antibiotics. *Evol Appl.* 8, 248-60.

727 Schrag, S. J., McGee, L., Whitney, C. G., Beall, B., Craig, A. S., Choate, M. E., et al.
728 (2004). Emergence of *Streptococcus pneumoniae* with very-high-level resistance to
729 penicillin. *Antimicrob Agents Chemother*, 48, 3016-3023.

730 Seyama, S., Wajima, T., Nakaminami, H., Noguchi, N. (2017). Amino acid substitution
731 in the major multidrug efflux transporter protein AcrB contributes to low susceptibility
732 to azithromycin in *Haemophilus influenzae*. *Antimicrob Agents Chemother*, 61, e01337-
733 17.

734 Smith, A. M., Botha, R. F., Koornhof, H. J., Klugman, K. P. (2001). Emergence of a
735 pneumococcal clone with cephalosporin resistance and penicillin
736 susceptibility. *Antimicrob Agents Chemother*, 45, 2648–2650

737 Stanton, T.B., McDowall, J.S., Rasmussen, M.A. (2004). Diverse tetracycline resistance
738 genotypes of *Megasphaera elsdenii* strains selectively cultured from swine feces. *Appl*
739 *Environ Microbiol*., 703754-7.

740 Tait-Kamradt, A., Davies, T., Cronan, M., Jacobs, M. R., Appelbaum, P. C., Sutcliffe, J.
741 (2000). Mutations in 23S rRNA and ribosomal protein L4 account for resistance in
742 pneumococcal strains selected in vitro by macrolide passage. *Antimicrob Agents*
743 *Chemoth*, 442118-2125

744 Tavío M.M., Jacoby, G.A., Hooper, D.C. (2014). QnrS1 structure-activity relationships.
745 *J Antimicrob Chemother*. 69,2102-2109

746 Theuretzbacher, U., Van Bambeke, F., Cantón, R., Giske, C. G., Mouton, J. W., Nation,
747 R. L., et al. (2015). Reviving old antibiotics. *J Antimicrob Chemother*, 702177-2181

748 Thitiananpakorn, K., Aiba, Y., Tan, X. E., Watanabe, S., Kiga, K., Sato'o, Y., et al. (2020).
749 Association of *mprF* mutations with cross-resistance to daptomycin and vancomycin in
750 methicillin-resistant *Staphylococcus aureus* (MRSA). *Sci Rep*, 101-15.

751 Toth, M., Frase, H., Chow, J. W., Smith, C., Vakulenko, S. B. (2010). Mutant APH (2 ″)-
752 IIa enzymes with increased activity against amikacin and isepamicin. *Antimicrob Agents*
753 *and Chemother*, 54, 1590-1595.

754 Vila, J., Ruiz, J., Marco, F., Barcelo, A., Goni, P., Giralt, E., et al (1994). Association
755 between double mutation in *gyrA* gene of ciprofloxacin-resistant clinical isolates of
756 *Escherichia coli* and MICs. *Antimicrob Agents Chemother*, 38, 2477-2479.

757 Wagner, G., Kenney-Hunt, J., Pavlicev, M., Peck J.R., Waxman, D., Cheverud,
758 J.M. (2008). Pleiotropic scaling of gene effects and the 'cost of
759 complexity'. *Nature*, 452**,** 470–472.

760 Walton, J. R. (1978). Apramycin, a new aminocyclitol antibiotic: I. In vitro
761 microbiological studies. *J Antimicrob Chemother*, 4, 309-313

762 Walsh, T.R., Toleman, M.A., Poirel, L., Nordmann, P. (2005). Metallo-beta-lactamases:
763 the quiet before the storm? *Clin Microbiol Rev*18,306-325

764 Wang, G., Yu, F., Lin, H., Murugesan, K., Huang, W., Hoss, A.G., et al (2018). Evolution
765 and mutations predisposing to daptomycin resistance in vancomycin-resistant
766 *Enterococcus faecium* ST736 strains. *PLoS One*,13(12):e0209785.

767 Wang, G. E., Taylor, D. E. (1998). Site-specific mutations in the 23S rRNA gene of
768 Helicobacter pylori confer two types of resistance to macrolide-lincosamide-
769 streptogramin B antibiotics. *Antimicrob Agents Chemother*, 42, 1952-1958

Yao, W., Xu, G., Bai, B., Wang, H., Deng, M., Zheng, J., et al. (2018). In vitro-induced erythromycin resistance facilitates cross-resistance to the novel fluoroketolide, solithromycin, in *Staphylococcus aureus*. *FEMS Microbiol Lett*, 365 (12).

Zaccolo, M., Gherardi, E. (1999). The effect of high-frequency random mutagenesis on in vitro protein evolution: a study on TEM-1 beta-lactamase. *J Mol Biol*. 285,775-83

Zhang, G., Hao, Q. (2011). Crystal structure of NDM-1 reveals a common β-lactam hydrolysis mechanism. *FASEB J*, 25, 2574-2582.

Zeil, C., Widmann, M., Fademrecht, S., Vogel, C., Pleiss, J. (2016). Network Analysis of Sequence-Function Relationships and Exploration of Sequence Space of TEM β-Lactamases. *Antimicrob Agents Chemother*. 60:2709-2717.