Antiviral drug resistance as an adaptive process

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Abstract
Antiviral drug resistance is a matter of great clinical importance that, historically, has been investigated mostly from a virological perspective. Although the proximate mechanisms of resistance can be readily uncovered using these methods, larger evolutionary trends often remain elusive. Recent interest by population geneticists in studies of antiviral resistance has spurred new metrics for evaluating mutation and recombination rates, demographic histories of transmission and compartmentalization, and selective forces incurred during viral adaptation to antiviral drug treatment. We present up-to-date summaries on antiviral resistance for a range of drugs and viral types, and review recent advances for studying their evolutionary histories. We conclude that information imparted by demographic and selective histories, as revealed through population genomic inference, is integral to assessing the evolution of antiviral resistance as it pertains to human health.

Key words: antiviral resistance, genetic barrier, mutagenesis, fluctuating selection, compensatory mutation, cost of adaptation

1. Resistance as an evolutionary process
Viral evolution, with its many public health consequences, is increasingly investigated within a population genetic framework. The growing availability of high quality molecular data has changed the scale at which these evolutionary processes can be monitored. Parameters such as nucleotide diversity, the strength of selection, and effective population size (Ne) can be monitored almost in real time using experimental evolution, and can be measured at increasingly frequent intervals in patients, informing treatment decisions (Chevillotte et al. 2010; Newman et al. 2013; Renzette et al. 2014). New mutations can also be observed at a fine scale, and of particular interest are those conferring drug resistance (Moya et al. 2004; zur Wiesch et al. 2011).

An increasing number of viral infections that impair host health are treated using antiviral drugs, typically targeting mechanisms of viral replication (Fig. 1). If the treatment is robust and viral fitness is impaired sufficiently, no viral genomes will be successfully replicated, but if treatment is not as effective and some genomes replicate, selective pressure may result in rapid adaptation toward resistance. This is exacerbated by the large population sizes and high rates of mutation characterizing many viruses: if resistance-conferring polymorphisms do not already exist in the population at the onset of treatment, they will likely arise soon thereafter. This problem has forced antiviral drug development to remain innovative, including combining existing drugs, and establishing new drug classes.

We here present a brief review of resistance mechanisms and their evolution, with a focus on viruses relevant to human health; we continue by describing evolutionary forces that are uniquely exemplified by viral resistance, and thus merit special attention from both the population genetics and virology fields.
2. Variation in viral biology and resistance mechanisms

Differences in viral replication biology drive the generation of new drug classes targeting different parts of the viral life cycle. Understanding these differences is critical not only for synthesizing new drugs, but also for predicting and countering evolution towards resistance. Below, we briefly review common modes of resistance (and relevant underlying biology) in six exemplary viruses: RNA viruses hepatitis C and influenza A virus (IAV), DNA viruses herpes simplex virus (HSV) and human cytomegalovirus (HCMV), retrovirus HIV, and unconventionally replicating hepatitis B virus (HBV).

Hepatitis C virus (HCV) is characterized by a high mutation rate and subsequently high genomic diversity (Simmonds 2004) (Table 1). This mutation rate is facilitated by frequent replication and poor proofreading function of the virally encoded RNA polymerase. In fact, the virus likely exists as a quasispecies, or a group of genomes forming a structured ‘cloud’ in sequence space, with replication near the maximum error rate allowed before genomic integrity is lost (Sanjuán et al. 2004; Eigen 2002). Within a host, HCV replicates in different compartments, such as different organs or bodily fluids (Halfon and Locarnini 2011). Bottlenecks are common during both inter-host transmission and intra-host compartmentalization (Bull et al. 2011); these bottlenecks reduce population size, thus amplifying the role of genetic drift in HCV evolution (Table 1). The most common antiviral drugs used against HCV are direct-acting antiviral agents (DAAs), which usually inhibit either protease or polymerase activity. Those inhibiting protease have a low genetic barrier to resistance, meaning that resistance is easily achieved through only one or a few mutations (see Section 4). Indeed, given the high rate of mutation, it is likely that these polymorphisms may already exist in a given population (Bull et al. 2011). However, new combinations of the DAAs, such as ledipasvir and sofosbuvir (marketed as Harvoni), appear to have a high genetic barrier to resistance, with very little (if any) cross-resistance between the two drugs, and are thus promising for future treatment (Gritsenko and Hughes 2015).

IAV is an RNA virus whose small genome codes for at least eleven proteins including two proteins on the virion surface: hemagglutinin (HA) and neuraminidase (NA). HA and NA allow viral envelopes to fuse with and separate from the cell membrane, respectively. As surface antigens, HA and NA tend to evolve relatively rapidly, with dN/dS ratios much higher than in the other viral proteins (Chen and Holmes 2010; Bhatt et al. 2011). The most common antivirals used to treat IAV are...
HIV (9.8 kb) $1.2 \times 10^{-4}$ 0.0143 2.33 Recombination rare Bull et al. (2011), Jabara et al. (2014)

IAV (13.5 kb) $2 \times 10^{-6} – 2 \times 10^{-4}$ 0.0008-0.0016 2.806 homologous recombination Copeland et al. (2011)

HSV (152 kb) $1.8 \times 10^{-8}$ 0.0022-0.0056 -1.05 2.5 $10^{-3b}$ Sakaoka et al. (1994), Dowden et al. (2004), Duffy et al. (2008)

HCMV (236 kb) $2 \times 10^{-7}$ 0.0022 0.0185 0.999 9.8 $10^{-7f}$ Gong and Padhi (2011), Renzette et al. (2011), Renzette et al. (2015)

HIV (9.8 kb) $1.1 \times 10^{-5} – 3.4 \times 10^{-5}$ 0.005 0.018 -1.9 1.9 -0.4 1 $10^{-32}$ Mansky and Temin (1995), Nora et al. (2007), Abram et al. (2010), Neher and Leitner (2010), Batorsky et al. (2011)

HBV (3.3 kb) $1.4 \times 10^{-5} – 3.2 \times 10^{-5}$ -0.008 $i$ – 2.19 $10^{-2f}$ Locarnini et al. (2004), Zhou and Holmes (2007), Zoulim and Locarnini (2009), Bang and Kim (2014), Ramachandran et al. (2014)

Estimates for several population genetic parameters for the six viruses highlighted here. Shading indicates viral type: purple represents RNA viruses, green represents DNA viruses, and beige represents those that utilize both DNA and RNA. Genome sizes are given under virus names. Most studies were based on clinical samples, but some relied on databases such as GenBank or the Los Alamos HIV database. Estimates of Tajima’s D in Hepatitis B have not been found to be reported. References are listed with respect to the order of entries per row; those within a single pair of brackets represent a single table entry. We emphasize that this table serves as a guide only, and that the original studies should always be consulted for technical details.

- $i$: mutation rate: given as nucleotide substitutions/base/replication (genome-wide), from experimental measures.
- $j$: nucleotide diversity: between-host estimates either from regions of interest with regards to resistance or from the full-genome.
- $k$: Tajima’s D, a test-statistic to distinguish neutrally evolving populations from those evolving under non-random process(es); between-host estimates from either specific regions or the full-genome.
- $l$: recombination rate: given as recombination events/site/generation, based on population-level sequence diversity.
- $m$: Protease (0.13 kb, n = 20).
- $n$: Genom-wide.
- $o$: See Han and Worobey (2011) for discussion.
- $p$: Us (2 kb, n = 42).
- $q$: US28 (2.5 kb, n = 103).
- $r$: (Reverse) Transcriptase (0.6 kb, n = 28).
- $s$: envelope (1.3 kb, n = 28).
- $t$: nucleotide substitutions/base/replication (genome-wide), from experimental measures.

NA-inhibitors, which prevent detachment of viral envelopes from the cell membrane. The popular NA inhibitor oseltamivir has been used since the early 2000s to treat seasonal IAV cases, but around 2007 became associated with the mutation H274Y, which confers very high levels of resistance at very little fitness cost (see Section 5) (Moscona 2009). It is thought that other compensatory mutations (see Section 6) alleviated these costs, allowing it to circulate uncurtailed in the population (Bloom et al. 2010). Another NA inhibitor, zanamivir, has been successful in IAV treatment and has not been observed to readily develop resistance, but is not prescribed as frequently due to difficulty of administration; this may contribute to the relative rarity of resistance mutations in vivo (Thorlund et al. 2011). Finally, a newer antiviral called favipiravir is thought to act by inducing mutagenesis in the IAV genome (see Section 7). Thus far, resistance to favipiravir has yet to be observed in vivo, and has only been generated in the laboratory under high selection pressure (Baranovich et al. 2013; Furuta et al. 2013; Cheung et al. 2014).

HSV is a herpesvirus containing a large genome with low diversity as compared with RNA viruses, promulgated in part by high replication fidelity and low recombination rates (Andrei et al. 2013) (Table 1). Infections are lifelong, cycling between periods of latency and viral shedding, though the virus is thought to be transmissible even in asymptomatic hosts (James and Prichard 2014). Systemic antiviral therapy is usually only necessary in immune-compromised patients, where it is relatively successful. The most commonly used drugs are Acyclovir and its related analogs, all nucleoside inhibitors, for which resistance mutations are known, affecting either a thymidine kinase necessary for prodrug activation by phosphorylation or the DNA polymerase (Griffiths 2011).

HCMV is also a herpesvirus for which infections are lifelong and generally asymptomatic. However, as with HSV, health is impaired when hosts are immuno-compromised including congenitally infected infants. Infection begins in a single compartment, such as plasma or saliva, before spreading to others; these subsequent divisions act as strong bottleneck events, curtailting population size. After compartmentalization, differentiation continues to the extent that populations from differing compartments within a single host are as diverse as those from between hosts (Renzette et al. 2013). The HCMV genome is generally diverse within-host, with levels of polymorphism comparable to RNA viruses, even though the DNA polymerase exhibits higher fidelity than RNA virus polymerases (Lurain and Chou 2010; Renzette et al. 2011) (Table 1). Approximately 5% of open reading frames show signatures of positive selection (Renzette et al. 2011). In particular, loci associated with envelope proteins tend to have elevated dN/dS, as diverse envelopes help to evade host immune defense, while loci for replicative proteins tend to be conserved. If treatment is necessary for HCMV, it usually involves antivirals acting as nucleoside analogs, including ganciclovir and cidofovir. Resistance mainly occurs either in a viral
kinase necessary for phosphorylation of the prodrug or in the DNA polymerase (Gilbert and Boivin 2005). As mentioned earlier, resistance mutations are usually only observed in immune-compromised hosts, such as tissue transplant recipients or those co-infected with HIV. This observation could however be biased considering the population treated with antivirals in the first place: (usually) those with impaired immunity who show symptoms of viral infection. The causality in this relationship deserves further study.

HIV is a retrovirus encoding an RNA genome within the virion, but after infecting a host cell, it replicates its genome using reverse transcription, generating a copy of DNA and eventually double-stranded DNA that is inserted in the host genome and can then be transcribed back to RNA. Reverse transcriptase is highly error prone, leading to a high rate of nucleotide substitutions, increased population diversity, and frequent resistance mutations when drug treatment is ongoing (Götze 2012) (Table 1). In fact, at the onset of drug treatment, it is estimated that every single-point mutation in the viral genome is likely to exist in some infected cell (Goldberg et al. 2012). This affords the possibility for selection on standing variation: when selection is introduced (i.e. drug treatment), a standing polymorphism may become immediately adaptive and rise to fixation (Pennings et al. 2014). The most common antiviral therapy for HIV today is a multi-drug regimen; these often combine drugs from different classes, preferably without known cross-resistance mutations (Menéndez-Arias 2013). Such a regimen increases the genetic barrier by requiring several mutations in order for resistance to be conferred. The most common combinations include nucleoside reverse-transcriptase inhibitors, non-nucleoside reverse-transcriptase inhibitors, protease inhibitors, and/or integrase inhibitors. Several resistance mutations are known within each class, but combination treatment requires these to occur simultaneously for full resistance to be conferred (though fitness can be partially restored by only one mutation; see Pennings 2012).

HBV is an enveloped DNA virus whose replication involves transcription to RNA intermediates that are then reverse transcribed back to DNA. Despite its DNA genome, HBV is thought to exist as a quasispecies; high levels of diversity are maintained through lack of proofreading during reverse transcription (Fig. 1, Table 1). This means that, like HIV and many RNA viruses, HBV infections maintain polymorphism at almost all nucleotide positions within a host at a given time (Khudyakov 2010; Bang and Kim 2014), paving the way for resistance mutations to increase in frequency upon drug treatment. HBV is most commonly treated with reverse transcriptase inhibitors, particularly lamivudine. However, several resistance mutations against lamivudine have been observed, many of which have low genetic barriers. Given this, a second reverse transcriptase inhibitor is often supplemented (Bang and Kim 2014). This method of drug-switching, if done within a single class, is potentially problematic: it encourages more robust resistance mutations that are either effective across many drugs and/or suffer lower fitness costs when drug treatment (selection) ceases (Locarnini et al. 2004; Shaw et al. 2006; Bang and Kim 2014). Initial combination therapy is becoming more frequent in clinical trials, but is not usually the standard treatment.

3. Inferring population genetic parameters in viruses

Before discussing the evolutionary mechanisms and consequences of antiviral resistance, it is important to consider the interpretation of standard parameters in light of viral biology. For instance, the standard definition of N_e is the size of an imaginary population that would experience the same rate of genetic drift as the population in question. However, this can be inferred using several methods (see Charlesworth 2009), and can represent intra-host or global populations (Moya et al. 2004), be given as genome-wide or region-specific estimates (Rambaut et al. 2008), or given as short-time scale estimates versus those over deeper time (Pennings et al. 2014). Importantly, there is a growing literature suggesting the importance of incorporating multiple merger coalescent models in analysis of viral populations, given their biology; these models will also alter estimates of N_e (Neher and Hallatschek 2013; Tellier and Lemaire 2014). Similarly, recombination can be measured and/or reported in several ways (Schlub et al. 2010), which may require different interpretation based on the type of recombination event (i.e. homologous vs. non-homologous vs. reassortment). Patterns of nucleotide diversity and linkage disequilibrium will differ according to the coding density of viral genomes. Finally, it is unclear how latent virus copies affect estimates of these parameters, and debated whether they should be included at all. Therefore, while it is evident that these parameters are best evaluated on a population-by-population basis (especially in cases of direct clinical relevance), we attempt here to arrive at some generalizations for their application in various viruses (as seen in Table 1). In the future, more universal procedures for measuring these parameters across viruses would be of great value to the field.

4. Genetic barriers to resistance

The number and type of substitution(s) necessary to confer resistance often differ according to antiviral target and class; this is often quantified as the genetic barrier to resistance (Götze 2012). Many older classes of antivirals are now known to have low genetic barriers: viruses usually only require one or two substitutions to gain resistance (e.g. first generation reverse-transcriptase inhibitors used for HIV infections, Perno et al. 2008; Menéndez-Arias 2013). A common goal of new drug development is to increase this genetic barrier by developing new drugs or combinations of drugs such that resistance does not arise so readily.

Genetic barriers affect the rates at which resistance mutations are observed. If a certain pathway to resistance requires only one substitution, but another requires more, we would expect the former to occur more often. These pathways can even differ by viral genotype; for example, in HCV, the common resistance-conferring mutation R155K requires only a single nucleotide change in genotype 1a, but two in genotype 1b, where it is rarely observed (Götze 2012). Genetic barriers involve not only the substitution quantity, but also type; for instance, resistance mutations requiring transitions should be more common than those requiring transversions (Powdrill et al. 2011). These step-wise mutations can be conceptualized as an adaptive walk within the context of Fisher’s geometric model (Bank et al. 2014, 2015; Foll et al. 2014; Tenaillon 2014). Upon environment change (i.e. when selective pressure changes), the distance to the optimum phenotype increases. When new mutations arise, they can move the phenotype closer to the optimum (i.e. a beneficial mutation), maintain the same distance relative to the optimum (i.e. a neutral mutation), or move the phenotype farther from the optimum (i.e. a deleterious mutation). Which of these occurs depends on the effect size of the mutation and the population’s current position in phenotypic
space, but we know from the distribution of fitness effects of new mutations that beneficial mutations are less probable than neutral or deleterious ones (though this is a function of distance from the optimum, see Hietpas et al. 2013). We thus know that a greater proportion of multiple step combinations will be deleterious compared with single steps. This suggests that when selection is introduced, resistance is more likely to come about through a small number of mutations (low barrier) than through multiple mutations (high barrier). Therefore, rather than tracking already-known mutations, the model’s utility would lie in exploring new, potential paths on fitness landscapes that could be taken in response to the environmental changes induced by drug treatment. Questions when deriving new antiviral drugs could include: for a given target, or viral trait, what is the shape of the distribution of fitness effects? Can we predict the likelihood of single step versus multi-step adaptive walks? Considering genetic barriers through the lens of Fisher’s geometric model can help inform which viral traits should be targeted through selection in order to avoid rapidly developing resistance, though some natural extensions, such as accounting for the genetic code (Lourenço et al. 2013) may be required.

5. Fitness costs

Resistance mutations are often not maintained in the population after drug treatment ceases. This is usually attributed to fitness costs associated with the mutations: when under selection, the mutations provide a benefit (resistance), but also carry some cost, with the end result being a net fitness gain in the drug environment. However, when the environment changes and a benefit is no longer provided, the fitness costs are fully realized (Tanaka and Valckenborgh 2013) (Figure 2). These cost/benefit tradeoffs can take the form of, for example, binding site alteration resulting in decreased enzymatic production (e.g. TK mutations in HSV, Piret and Boivin 2014), binding site alteration resulting in failed binding of intended partners (e.g. mutations in HCV affecting protease inhibitor activity, Halfon and Locarnini 2011), and removal of chain-terminating analogs rendering lower replication rates (e.g. thymine analog mutations in HIV, [Hu et al. 2006]).

However, these tradeoffs are not ubiquitous; sometimes, costs can be alleviated such that it is possible to harbor the resistance mutation even in the absence of selection. For example, the H274Y mutation in influenza (H1N1) confers resistance to oseltamivir, the most popular NA inhibitor. Until fairly recently, H274Y was associated with the usual fitness costs in the absence of the drug; however, since the 2008–09 flu season, these costs have diminished greatly in seasonal influenza, such that the majority of H1N1 strains can now carry H274Y at little or no cost (Renzette et al. 2014; Hauge et al. 2009; Poland et al. 2009; Baz et al. 2010). This fitness restoration is thought to be due to the development of compensatory mutations (see Section 6) (Bloom et al. 2010; Duan et al. 2014).

Fitness costs also co- vary with the degree of resistance conferred. Usually, mutations providing greater resistance carry higher fitness costs in the absence of drug, and vice-versa (Croteau et al. 1997; De Luca 2006). To illustrate this frequently cited correlation, previously published data on resistance (measured as IC50 ratio) and fitness (replication rate relative to the wild-type in a drug-free environment) were aggregated (Fig. 2). A linear model showed a non-significant relationship across all viruses. Within a given virus, though non-significant correlations between resistance and fitness were found for many, a significant negative correlation was seen for HCV ($P = 0.0499$).

Surprisingly, for seasonal H1N1 (a strain of IAV), a positive relationship between fitness and resistance was seen. This uniquely positive correlation may be influenced by the inclusion of only H274Y mutations, as these were the only data available fitting inclusion criteria (see Fig. 2 legend; no mutants were from pandemic strains and none were on compensatory backgrounds). As mentioned above, H274Y has been found to have little or no fitness costs in the absence of drug owing to compensatory mutations. Since H274Y is indeed a unique resistance mutation in this respect, we re-analyzed the correlation between fitness cost and resistance when those data are omitted; indeed, an analysis of the remaining data indicates a significant negative correlation among all viruses ($P = 0.0088$). However, these results should be interpreted with caution, as they are aggregated from studies with various experimental procedures, using various viral strains with different genetic backgrounds. A single experiment assessing this relationship would be much more informative, and would indeed be an asset to the field in the future.

6. Compensatory mutations

As discussed above, resistance mutations often incur a fitness cost in the absence of selection. This deficit can be alleviated...
through the development of compensatory mutations, often re- 
estoring function or structure of the altered protein, or through 
reversion to the original (potentially lost) state. Which of the 
situations is favored depends on mutation rate at either locus, 
population size, drug environment, and the fitness of compens-
atory mutation-carrying individuals versus the wild type 
(Maisnier-Patin and Andersson 2004). Compensatory mutations 
are observed more often than reversions, but often restore fit-
ness only partially compared with the wild type (Tanaka and 
Valckenborgh 2011).

In a fluctuating environment (e.g. incomplete adherence, see 
Section 8), the fate of compensatory mutations is uncertain. 
These mutations are generally either neutral or deleterious in 
the absence of the target mutation, and are thus likely to be lost 
if the primary resistance mutation is removed. However, if the 
compensatory mutations are antagonistically epistatic with the 
primary ones, then they may be maintained: by conferring a re-
latively higher fitness than if both the primary and compensa-
tory mutations were absent, they may be conserved in the popula-
tion (Khudyakov 2010; zur Wiesch et al. 2011). These 
compensatory mutations could then act as permissive (sec-
ondary) mutations, allowing the frequency of the primary resis-
tance mutation to fluctuate (Bloom et al. 2010).

7. Mutagenesis

Efforts to prevent drug resistance, however promising, some-
times prove futile. An alternative approach to antiviral action is 
to target mutational processes, rather than replicative ones, in 
order to lower population fitness. More specifically, as popu-
lation mutation rates increase—and particularly as the major-
ity of new mutations are deleterious—the influx may have a nu-
mer of effects. First, particularly in non-recombining viruses, 
Muller’s Ratchet (the process by which the most fit class is lost 
via genetic drift) is expected to operate, reducing fitness 
through time (Muller 1964). Secondly, Hill-Robertson inter-
ference (the process by which, owing to linkage, the probability of 
fixation of beneficial mutations and the probability of loss of 
deleterious mutations are reduced) will become stronger (Hill 
and Robeterson 1966). Notably, both effects may compound 
through time—with reduced fitness leading to reduced effective 
population size, which further speeds the Ratchet; this leads to 
compounding reductions in fitness, which may ultimately lead 
to mutational meltdown (i.e. population extinction).

Many RNA viruses replicate under conditions very close to 
the error threshold, or the error rate beyond which meltdown is 
likely (e.g. IAV and HCV, see Holmes 2003). New drug designs 
take advantage of this by acting to increase the base mutation 
rate such that populations exceed this threshold (Crotty et al. 
2001; Mullins et al. 2011; Pauly and Lauring 2015). The most 
promising of these drugs is favipiravir, and while its exact mode 
of action is debated, it is thought to act on the RNA polymerase 
to decrease fidelity in nucleoside incorporation (Baranovich 
et al. 2013; Furuta et al. 2013). This would lead to a higher rate of 
mutilator virion production and a decrease in specific infectivity 
(Arias et al. 2014). Though resistance to other mutagenic drugs 
has been observed before (usually through mutation to restore 
fidelity of the polymerase, e.g. Pfeiffer and Kirkegaard 2003), it 
is thus far uncommon in favipiravir. In fact, for IAV (a virus 
against which favipiravir shows great potential), no significant 
in vivo resistance has yet been detected in clinical trials, making 
favipiravir a promising new antiviral candidate. Finally, though 
the in vivo favipiravir resistance studies are encouraging, it 
should be noted that NA inhibitors like oseltamivir were once 
thought to be less likely to select for resistance mutations than 
previous generations of antivirals (Moscona 2005). It is therefore 
not implausible that resistance against mutagenic therapies 
could likewise arise in the future.

8. Adherence and fluctuating selection

As with antibiotics, interruptions to antiviral drug regimens 
pose a risk to both personal and public health as they allow viral 
populations that are not completely eliminated to repopulate 
within a host (Rong et al. 2007; Gardner et al. 2010; Chotiyaputta 
et al. 2012). The initial treatment creates a bottleneck in the viral 
population, through which the drug-resistant individuals are 
most likely to survive. When drug use is interrupted, repopula-
tion will stem from these individuals, population size will in-
crease dramatically, and the new population will likely have 
higher resistance and be more difficult to control (e.g. Wang et al. 
2011). In a clinical sense, the occurrence and frequency of such 
interruptions is summarized as adherence (Fox et al. 2008; Sethi 
et al. 2003). Non-adherence includes not only complete inter-
ruption of treatment, but also dosage and timing non-
compliance. These can lead to treatment failure, often accom-
panied by novel resistance mutations (Fox et al. 2008).

The dynamic intra-host drug concentrations resulting from 
non-adherence can create a case of fluctuating selection in which 
the magnitude or even sign of selection can change as 
the environment fluctuates (though changes in population size 
may be equally important; see Pennings 2012). Population 
genetic models of fluctuating selection are not new, and several 
empirical examples are known (see Bell 2010), for review). 
Previously, estimation of fluctuating selection coefficients was 
based on phenotypic, labor-intensive, time-series data sets, but 
methds now exist based on polymorphism data from a single 
collection (e.g. Miura et al. 2013), and traditional tests for selec-
tion have been updated to accommodate such estimates (e.g. 
MK test, Gossmann et al. 2014). Therefore, the fate of an allele 
that fluctuates from being beneficial to neutral to deleterious, 
such as a resistance mutation with changing drug adherence, 
can be tracked using current methods.

In the context of antimicrobial drug resistance, fluctuating 
selection has been examined previously (zur Wiesch et al. 2010; 
Hall et al. 2011; Tanaka and Valckenborgh 2011), but application 
of these methods to antiviral resistance is lacking. Also, selec-
tion on resistance alleles or the allele frequencies themselves 
are rarely treated as parameters of interest; rather, whole-
organism fitness is tracked. Therefore, the population genetic 
methods alluded to above could be applied to antiviral resis-
tance, though not without some modification. One potential av-
venue would be to tailor future studies to known resistance 
mutations, in order to characterize their frequencies through 
periods of adherence and non-adherence.

9. Genetic screening

Before the onset of treatment with a new-to-host drug, screens 
for pre-existing resistance mutations are frequently adminis-
tered, with respect to the drug in question (particularly in HIV 
and HCV). The presence of such mutations pre-treatment may 
be due to previous exposure to similar drugs, transmission of a 
strain already containing resistance mutations, or simply due to 
a high rate of mutation. Given the genomic data collected in 
these screens, a further goal may be to detect new, not yet re-
ported resistance mutations in addition to scanning for already 
known mutants. This is analogous to searches for signatures of
positive selection commonly employed in studies of adaptation. Many methods exist currently that could be adopted by clinical researchers for purposes of improving mutation lists (Beerenwinkel et al. 2005; Nielsen 2005; Jensen et al. 2007); methods could also be extended to unveil novel adaptive events as viruses adapt to new environments (e.g. Pennings 2012; Foll et al. 2014).

10. Conclusion
As reviewed here, the application of evolutionary theory or metrics to the study of antiviral drug resistance is often illuminating. Tests for positive selection can potentially identify resistance mutations, fluctuating selection models may predict non-adherence outcomes, and phenomena such as mutational meltdown can guide the development of promising mutagenic drugs. Though evolutionary studies are increasingly addressing viral populations, the fields of virology and evolutionary biology could mutually benefit from further collaboration: the former through more elaborate methods for predicting and preventing resistance (and associated clinical outcomes), and the latter through better model systems for testing new theory or methods, including parameter ranges rarely observed in other taxa, such as large effective population sizes and high mutation rates (Table 1).

The clinical implications of antiviral resistance must not be underestimated. Both human and monetary costs of resistance remain high, particularly for IAV (Howard and Scott 2005; Moscona 2009). From a public health perspective, unpredictability of viral evolution and drug resistance means that antiviral treatments also remain costly (Lipsitch et al. 2013). Addressing clinical and public health questions with the tests and metrics mentioned here may indeed alleviate some of these costs by better illuminating resistance patterns and facilitating prediction of epidemics or pandemics.

Though we have reviewed here only a handful of viruses, and focused on commonly applied antivirals and associated mutations, the consequences of resistance are of course much further reaching. Viruses detailed here are those with well-studied resistance patterns, usually due to their serious human health effects; however, antiviral failure certainly occurs elsewhere, including in viruses with more minor effects (for which failure is not perilous to health and therefore not thoroughly studied) and in animal and plant viruses with agricultural impact. We have also only briefly summarized the drugs employed for treatment and their associated mutations, but reports of all known resistance mutants for a given virus are readily available, as are growth or fitness data (Chevillotte et al. 2010; Nguyen et al. 2012; Wyles 2013; Wensing et al. 2014) (Fig. 2).

Finally, while we here make some generalizations about antiviral resistance, it is imperative to note that the process differs, sometimes dramatically, according to viral biology, and should be accounted for in any analysis of resistance conferral. It is our aim that the review presented here will motivate further research on resistance evolution, and highlight the fact that clinically motivated and evolutionary-motivated studies often have overlapping and complementary goals which ought to be embraced.

Supplementary data
Supplementary data are available at Virus Evolution online.

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