Biomedical implications of viral mutation and evolution

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Abstract

Mutation rates vary hugely across viruses and strongly determine their evolution. In addition, viral mutation and evolution are relevant to several fields of biomedicine, including the study of pathogenesis, vaccine design or antiviral research. In this review, we outline how viral mutation rates are estimated and how they vary across viral groups, paying special attention to general trends. We review recent advances positing a direct association between viral mutation rates and virulence, or the use of high fidelity variants as attenuated vaccines. The implications of mutation rates for the design of rational antiviral therapies and for efficient epidemiological surveillance are also discussed.

Keywords: mutation rate, replication fidelity, evolutionary biology, viral escape, viral fitness, lethal mutagenesis, vaccine.

Main text

Viruses are amongst the fastest evolving entities, with several nucleotide substitutions occurring in the global population each year in most RNA and some DNA viruses [1,2]. This implies that the genetic diversity produced in a few years of viral evolution is often equivalent to millions years of evolution in their eukaryotic hosts. The mutation rate of an organism is defined as the per-generation per-nucleotide probability of erroneous replication. Since viral generations are poorly defined, the mutation rate is expressed per replication cycle or per cell infection cycle [3]. Viruses show highly variable mutation rates and, therefore, widely different evolutionary properties [1]. Although there are other important factors determining evolution such as generation times, population sizes, selection strength, or recombination rates, mutation is of particular interest because it is the ultimate source of genetic variation.

Viral mutation has important biomedical implications. For instance, the high mutation rate of HIV-1 implies that any single substitution conferring drug resistance takes place within a single day and makes multidrug treatments necessary [4], an argument that applies similarly to other RNA viruses producing chronic infections such as hepatitis C virus (HCV). The viral mutation rate also plays a role in the assessment of vaccination strategies [5] and influences the stability of live attenuated vaccines [6]. Additionally, mutation rates can determine viral fitness and virulence [7,8] and thus the ability of the host immune system to clear the infection. At the epidemiological level, the viral mutation rate can determine the risk of emergence of new diseases [2,9]

This review focuses on the evolutionary and biomedical implications of viral mutation. Firstly, we briefly outline some mechanistic aspects of viral mutation and review a few methods for measuring mutation rates. Secondly, we summarize the general trends emerging from current mutation rate data. Finally, we discuss the

relevance of viral mutation to pathogenesis, vaccination strategies, and antiviral research.

Measuring viral mutation rates

There are three basic approaches to determining viral mutation rates: cell-free replication fidelity assays (*in vitro*), cell culture systems (*ex vivo*), and analysis of samples taken directly from hosts (*in vivo*). Abundant *in vitro* information is available for HIV-1 and other RNA viruses from studies of the incorporation kinetics of correct and incorrect bases and from replication assays using reporter sequences [10–12]. These have provided invaluable insights into the mechanistic aspects of replication fidelity. A salient result is that all viral RNA-dependent RNA polymerases (RdRp) except those of coronaviruses and all reverse transcriptases (RT) studied to date lack 3' exonuclease proofreading activity and incorporate mismatches into the nascent chain relatively efficiently [10,13–15]. However, this does not imply that viral RdRps and RTs lack any mechanism of fidelity. Their base-selection specificity is similar to that of cellular DNA polymerases and key amino acids involved in fidelity have been identified [10,16]. The sequence context of the template is also important. For instance, template-primer misalignment at homopolymeric runs can cause frameshift mutations or nucleotide substitutions [17].

However, the mutation rates inferred *in vitro* tend to differ substantially from those obtained in cell cultures and qualitative differences exist as well. One possible explanation is that *in vitro* assays use saturating amounts of essential compounds such as nucleotides or divalent cations, which can affect replication fidelity and alter the mutational spectrum. For instance, in HIV-1, G-to-A is the most frequent type of mutation *in vivo*, but not *in vitro* [10]. Another reason for this inconsistency is that mutations depend on factors other than the viral polymerase. In retroviruses, dUTPases

reduce mutation levels by preventing the incorporation of uracil into DNA [18], and the Vpr protein is known to recruit the host-encoded uracil DNA glycosylase UNG2, a protein involved in DNA repair, into the virion [19]. Furthermore, mutations are also a consequence of spontaneous nucleic acid damage and host-mediated editing.

Apolipoprotein B mRNA-editing catalytic polypeptide-like enzymes (APOBEC3) are cytidine deaminases that produce G-to-A hypermutation in retroviruses [20–22], and the double-stranded RNA-dependent adenosine deaminase (dsRAD) may produce A-to-G hypermutation in several RNA viruses, including rhabdoviruses [23], paramyxoviruses [24], and retroviruses [25–27].

Mutation rates have been determined in cell cultures for over twenty viruses using a variety of methods [3]. For instance, a genetically homogeneous inoculum (e.g. a plaque or an infectious clone) can be propagated for one or a few passages and the genetic variation appeared during this time interval quantified by PCR, molecular or biological cloning and sequencing of a large number of clones encompassing one or several genome regions. By dividing by the number of infection cycles elapsed, this variation can be converted to a mutation rate. However, deleterious mutations will be underrepresented relative to neutral mutations. This selection bias will increase with time and has to be corrected appropriately [3]. For some viruses, particularly retroviruses, culturing systems have been developed to allow the virus to replicate in the absence of selection, thus avoiding this problem. Mutations introduced by the polymerases used for PCR amplification are yet another source of inaccuracy in molecular clone sequencing [28]. To minimize this problem, the cloning step can be carried out before the PCR by picking isolated plaques (i.e. isolated plaques) or limiting-dilution PCR.

An alternative approach to mutation rate estimation is the classical Luria-Delbrück fluctuation test [3,29,30]. To carry out this test, one has to seed a large number of parallel cultures with a small initial number of individuals each, let them replicate in a permissive medium until a given titer is reached, and plate in the presence of a selective agent such as a monoclonal antibody, an antiviral, or a non-permissive cell type, in which only certain mutants can form plaques. The distribution of the number of resistant plaques per assayed culture can then be used for estimating the mutation rate (Box 1, Fig .1). This test has the advantage of avoiding PCR errors and selection bias, but necessarily focuses on a few genome sites (i.e. those encoding the resistant phenotype).

There are very few *in vivo* mutation rate estimates for viruses. In two plant virus studies, a transgenic host was engineered to redundantly express one viral gene, such that the viral copy was released from selection by trans-complementation and could accumulate mutations neutrally [31,32]. However, this method needs an estimation of the number of viral generations elapsed in the host, which makes it essentially not applicable to human and most animal viruses. A more widely applicable strategy is to focus on mutations that suppress viral infectivity (lethal mutations). Since such mutants leave no progeny, their population frequency remains equal to the mutation rate at any time. By determining the within-host frequency of putative lethal mutations in large collections of molecular clones, it was possible to determine the *in vivo* mutation rate of a viroid [33] and to demonstrate that the standard ribavirin/interferon treatment against HCV is mutagenic for the virus [34]. However, this method requires considerable sequencing effort and careful controls to distinguish naturally occurring mutations from sequencing errors.

Variability in mutation rates across viruses

In RNA viruses, mutation rates estimated in cell cultures or *in vivo* range from 10^{-6} to 10^{-4} substitutions per nucleotide per cell infection (s/n/c), whereas the range for DNA viruses is from 10^{-8} to 10^{-6} s/n/c [3]. Therefore, there is little or no overlap between these two large groups (Fig. 2). The biochemical basis for this dichotomy is well-established because the vast majority of RNA virus polymerases lack 3' exonuclease proofreading activity. The immediate consequence is that, generally speaking, RNA viruses evolve faster than DNA viruses [1,2]. However, there are at least two other important factors to be considered when making generalizations about viral mutation rates: genome size and whether the genome is single- or double-stranded.

Drake's rule establishes that the genomic mutation rate of DNA-based microorganisms, including viruses, is roughly constant and approximately equal to 0.003 [35]. This implies that there is an inverse relationship between the per-nucleotide mutation rate and genome size spanning several orders of magnitude. Single-stranded (ss) DNA viruses and some double-stranded (ds) DNA viruses have genome sizes below 10 kb, implying that their per-nucleotide mutation rates are close to 10^{-6} s/n/c and thus not extremely different from those of some RNA viruses. Consistently, ssDNA viruses have been shown to evolve relatively fast [1,2]. For instance, the evolutionary rates of canine parvovirus, panleukopenia virus [36] and human B19 erythrovirus [37] are on the order of 10^{-4} substitutions per nucleotide site per year (s/s/y), which is within the typical range of 10^{-2} to 10^{-5} s/s/y accepted for RNA viruses [38]. In RNA viruses, in contrast, genomic mutation rates are highly variable and evidence supporting a negative correlation between per-nucleotide mutation rates and genome sizes is weak [3]. Interestingly, however, the chrysanthemum chlorotic mottle viroid has a minimal genome of 0.4 kb and the highest per-nucleotide mutation rate described [33]. Furthermore, the genome of bacteriophage Q β is one of the smallest

among RNA viruses and its estimated mutation rate is one the highest [3]. Finally, coronaviruses, which are the RNA viruses with the largest genomes, encode for a 3' exonuclease which is probably used for proofreading [14, 15].

Positive-stranded, negative-stranded, and double-stranded viruses differ profoundly in their replication and gene expression strategies. For instance, in ss(+)RNA viruses, the genomic RNA is used directly as mRNA, whereas in ss(-)RNA viruses the genome needs to be transcribed first. The genomes of ss(+)RNA viruses interact less strongly with nucleoproteins, tend to form more RNA secondary structures, and recombine more frequently by replication template switching [39]. Given these differences, it seems reasonable that we might also see differences in mutation rates among major viral groups. Indeed, available mutation rate estimates indicate that ssDNA viruses mutate faster than dsDNA viruses (Fig. 2). Although ssDNA viruses use host-encoded DNA polymerases for replication, their relatively high mutation rates can still be explained by the lower stability of single-stranded nucleic acids or the absence of post-replicative repair. For instance, GATC motifs are required for methyl-directed mismatch repair in *Escherichia coli* [40], but the genome of its phage ϕ X174 has few or no such motifs. In fact, the mutation rate of ϕ X174 can be significantly reduced by adding GATCs to the viral genome [41].

In contrast, differences between ssRNA and dsRNA viruses have not been demonstrated. Mutation rates have been determined for seven ss(+)RNA viruses and four ss(-)RNA viruses [3], but the only estimate for a dsRNA virus corresponds to bacteriophage ϕ 6 [42]. Interestingly, while the median mutation rate is 7×10^{-5} s/n/c for ss(+)RNA viruses and 2×10^{-5} s/n/c for ss(-)RNA viruses, the estimate for bacteriophage ϕ 6 is only 2×10^{-6} s/n/c, at the lower limit of the accepted 10^{-6} - 10^{-4}

range. This suggests that there may be differences between ssRNA and dsRNA similar to those established for DNA viruses, but more data are required to test this hypothesis.

Viral mutation and virulence

The link between mutation rates and virulence in the microbial world has been best characterized with bacteria, for which mutator and antimutator strains have been described over three decades. Naturally occurring and genetically engineered mutator strains of numerous bacterial species have permitted *in vitro* and *in vivo* studies that reveal a positive link between mutation rate, adaptation and virulence [43–46]. In bacteria, the temporary, positive selection of hypermutators, with mutation rates up to 10000-times higher than normal [47], correlates with the development of antibiotic resistance and adaptive radiation leading to colonization and persistence in hosts. Given the large variability in the mutation rates of viruses the question of a link between mutability and virulence seems all the more pertinent. The recent isolation of variants of a number of RNA viruses presenting higher and lower fidelity RdRps has opened the way to experimentally examine these concepts both *in vitro* and *in vivo* [16,48].

Currently, the clearest connection between mutation rate and virulence has been obtained in a body of work involving the G64S high fidelity variant of poliovirus [6–8,16]. With only a single amino acid difference from wild type virus, this variant replicated with increased fidelity and generated approximately 3-6-fold fewer mutations; while retaining wild type-like replication kinetics and all of the known determinants for pathogenesis. The resulting decrease in genetic diversity generated by this variant was shown to correlate with a loss in adaptability, in the ability to generate resistance mutations to antiviral compounds and escape mutants to neutralizing antibody. Most surprisingly, despite making more perfect copies of the pathogenic sequence of wildtype poliovirus, this virus population was found to be moderately [7] to

highly [8] attenuated in the murine infection model. The researchers proposed that, in order to successfully infect a host, an RNA virus must maintain a high mutation rate to adapt to changing conditions within the host environment (e.g. cell type, tissue tropism) and that even a small decrease in this mutability could have dramatic fitness costs *in vivo*. A recent confirmation of this observation was obtained with a higher fidelity variant of chikungunya virus that also presented wildtype-like replication kinetics but reduced mutation frequencies. In this case, infection and dissemination in the natural mosquito host was compromised and viremia in the mammalian model was negatively impacted [49]. These works reveal that moderate changes in mutation rates of RNA viruses can result in significant fitness costs. Interestingly, a low fidelity polymerase of poliovirus has been generated *in vitro* that is non-viable *in vivo*, suggesting that increasing mutation rates beyond the already high levels of natural RNA virus populations may have equally negative effects [50] (see below).

Viral mutation and vaccines

Although innate, non-specific immunity plays an essential role in blocking viral infection and limiting replication and dissemination, it is the adaptive, specific immune responses that are required for virus clearance and long-term protection from future infections. Since adaptive immune responses recognize specific peptide sequences or protein structures, RNA viruses have limited options available to them to keep ahead of developing immunity. Some have acquired the ability to down-regulate some branches or effectors of immunity, but their small genomes and repertoire of proteins cannot host the immune evasion genes that are found in large DNA viruses or larger non-viral microbes. Thus, they must largely rely on their mutation rates to introduce amino acid changes at key antigenic sites that abrogate recognition by pre-existing immunity. This mutational drift is well documented, for example, in seasonal influenza viruses where

the surface glycoproteins targeted by the antibody response undergo continual antigenic change [51]. The influenza vaccines therefore, must be updated annually to take into account this change, since differences approaching 5% at antigenic sites result in little or no cross-protection. Further complicating this is the global variability of the virus, where geographic regions may not harbor the same antigenic variants and vaccine composition must be locally adapted. Other key examples where the interplay between mutation rates and immune selective pressures driving virus evolution have strong implications for vaccination include the viruses HIV and HCV, chronically infecting viruses that present both a huge global variability in the strains that circulate, as well as a considerable within-host variability of antigenic variants. In these chronic infections, the development of a universal vaccine against all strains is a very challenging task [52].

Given this key obstacle in vaccine design, a better understanding of a specific virus' mutation rate, how to best measure it and how to manipulate it would widen the options on the rational design of vaccines or other antiviral strategies. The recent work on higher fidelity variants of RNA viruses that reveal an attenuating effect suggests that increasing replication fidelity could constitute a novel component in the development of live virus vaccines. The high fidelity variants of poliovirus, for example, were completely attenuated in mice and protected against lethal challenge by homologous virus up to six months post immunization [6]. The potential safety benefits of adding such a component to live virus vaccines are two-fold: the virus, being less adaptable, is seemingly compromised in its ability to disseminate to secondary tissues and be shed into the environment by the vaccinated individual, and the likelihood of accumulating new virulent mutations or reverting other attenuating mutations to wildtype sequences is significantly reduced. Such features are particularly desired for live vaccines such as

the poliovirus Sabin strains, for which vaccine-derived virus shed by vaccinees frequently carry reverted sequences that are pathogenic to naïve hosts [53].

Viral mutation and antiviral therapies

Antiviral drugs inhibit some essential step in the viral infection cycle such as attachment and entry, uncoating, replication, integration into the host genome in retroviruses, or virus release [54]. Many of these drugs are highly effective in the short-term but drug-resistant viruses evolve in most cases making the treatment unsuccessful [55,56]. Indeed, due to their effectiveness, antiviral drugs impose a strong selective pressure to the virus. Therefore, once a mutation conferring drug resistance has appeared its population frequency increases fast. Since many resistance mutations only involve a single amino acid change, the waiting time until the mutation appears is often short, particularly in RNA viruses. Furthermore, the high mutation rates of RNA viruses make that the viral population infecting a single individual usually harbors all possible single-nucleotide mutants and many double such mutants [57]. Therefore, resistance mutants often exist before the antiviral treatment starts, albeit as minority variants. Although most resistance mutations have a fitness cost for the virus, evolution in the continuous presence of the inhibitor permits the selection of secondary mutations that compensate this cost, which facilitates the transmission of resistant virus among individuals [58,59].

In light of the above facts, a successful strategy to diminish the spread of resistant mutants is the use of combination therapies where several drugs that inhibit different steps of the virus infection cycle are simultaneously administered, thus making resistance less likely. The clearest example of the advantages provided by combination therapies is represented by the highly active antiretroviral therapy (HAART) for HIV [60]. HAART regimes include one or two nucleoside reverse transcriptase inhibitors,

one non-nucleoside reverse transcriptase inhibitor, and an additional drug that is usually a protease inhibitor. Combination therapy is also used to treat HCV.

The high mutation rates of RNA viruses make them highly adaptable to new environments, including those produced by antiviral therapies. However, the relationship between mutation and adaptation is not straightforward. Increasing the mutation rate accelerates adaptation by reducing the waiting time before the appearance of advantageous mutations. However, a very high mutation rate can hamper adaptation because beneficial mutations will tend to occur in genetic backgrounds loaded with deleterious mutations and thus fail to spread in the viral population [61]. The reason for this interference is that deleterious mutations are universally more likely than advantageous ones. This sets the stage for an antiviral strategy called lethal mutagenesis, which consists of increasing the mutation burden of a population using specific mutagens [57,62,63]. Since spontaneous mutation rates are highest among RNA viruses, the latter are the best candidates for this kind of therapy.

The idea of lethal mutagenesis as a feasible goal in RNA viruses was first inspired by the quasispecies theory, which explores the population dynamics of highly error prone self-replicating molecules and predicts an error threshold beyond which genetic information cannot be sustained [64]. Early experiments demonstrated that slight increases in the replication error rate of poliovirus and vesicular stomatitis virus were associated to strong decreases in viral infectivity [65]. Lethal mutagenesis has proved effective in cell cultures and animal models against several RNA viruses [62] including enteroviruses [66,67], aphtoviruses [68], hantaviruses [69], arenaviruses [70], and lentiviruses [71,72], and is currently being tested in patients [73]. Interestingly, ribavirin, a nucleoside analogue used to treat several viral diseases, is a mutagen for

RNA viruses in cell cultures [66] and this mutagenic effect has also been shown *in vivo* in patients treated with interferon and ribavirin [34].

Sequence analysis of populations at stages previous to extinction through lethal mutagenesis shows a progressive increase in both the mutation frequency and the complexity of the mutant spectrum as a function of the number of generations experienced in the presence of the mutagen [68,74], even if the average or consensus sequence of the virus may remain unchanged [75] (Fig. 3). Studies carried out with lymphochoriomeningitis virus [70] and foot-and-mouth disease virus (FMDV) [76] have also shown that, at increased mutation rates, defective genomes become frequent and can parasitize functional viruses, favouring the extinction of the whole population [70]. Therefore viral extinction through mutagenesis can be a complex process [77].

Mutagenesis can also lead to the emergence of resistances. This was first demonstrated when poliovirus passaged in the presence of ribavirin evolved a single amino acid substitution that increased the overall replication fidelity of the virus [16]. Fidelity variants have also been isolated in coxsackie virus replicating in the presence of different mutagens [48]. In FMDV, in contrast, resistance to ribavirin was attained through a mutation that increased the RdRp ability to discriminate between the correct nucleotide and the base analog [78]. This mutant, when exposed to higher ribavirin concentrations, evolved another resistance mechanism consisting in the modulation of the types of mutations induced by the mutagen [79]. Recently, resistance to azacytidine in bacteriophage Q β has been associated with the fixation of a substitution in the readthrough protein of the virus, which is not involved in replication [80]. Finally, another mechanism that could be used by RNA viruses to avoid the negative effects of mutagenesis is increased mutational robustness [81]. To minimize the probability of resistance, mutagens could be combined with other antiviral drugs, since theory predicts

that this increases the probability of viral clearance [63]. Recent work suggests that a sequential treatment first with the inhibitor and then with the mutagen may be more effective than the administration of both compounds in combination [82].

Viral mutation, evolution, and epidemiology

In fast evolving RNA viruses, epidemiological and evolutionary processes take place in the same timescales, and isolates taken only months apart or sometimes even days apart undergo enough genetic divergence to infer their phylogenetic relationships with confidence [83,84]. One practical implication is that molecular epidemiology and phylogenetic analysis can be used to address forensic issues. For instance, this made it possible to assess the role of medical staff in transmitting HIV-1 to patients in a trial case in Lybia [85] or to demonstrate patient-to-patient transmission of HCV during colonoscopy diagnosis in a Spanish hospital [86].

Secondly, viral phylogenetics and epidemiology allow us to address issues such as where and when did new viruses crossed the species boundary, identify reservoir species or even make predictions about viral emergence. Several general results have been reached in this field. For instance, viruses that cause acute infections frequently jump between host species and evolve rapidly, whereas viruses that cause persistent infections tend to infect a single host species and slowly coevolve with their host [84]. The former are usually RNA viruses whereas the latter tend to be DNA viruses, although there are exceptions to this rule. RNA viruses have caused several zoonoses in the past century, including among others, transmission of HIV-1 from chimpanzees to humans, swine/avian influenza pandemics, or the 2003 SARS outbreak (which involved bats as the putative reservoir hosts and palm civets as the source of transmission to humans) [2,9]. Evolutionary analysis combined with ecological, genetic and virological

studies may allow us to build efficient surveillance programs in order to prevent pandemics in the future.

Future perspectives

Viral mutation rates have been measured using widely different strategies. They sometimes contain several sources of bias and error and are expressed using different, non-convertible, units. Moreover, *in vitro* and *ex vivo* viral mutation rate studies have frequently yielded disparate results. Our knowledge of *in vivo* rates is still very limited, but it would be not surprising that they differed from those obtained *ex vivo* and *in vitro*. Therefore, a more comprehensive empirical knowledge of viral mutation is necessary.

Evolutionary theory establishes a direct link between mutation and molecular evolutionary rates [87]. Broadly speaking, data obtained with viruses confirm this link, because RNA viruses mutate and evolve faster than DNA viruses. However, a finer-grain correlation between these two parameters has not been demonstrated yet, possibly due to large estimation errors. More surprisingly, no systematic comparison has been undertaken yet between viral mutation and evolution rates.

Another important challenge is to clarify the forces driving the evolution of mutation rates. Particularly, the evolutionary basis for Drake's rule remains uncertain, and the mechanisms that allow ssDNA viruses to mutate significantly faster than their hosts or than dsDNA viruses remain to be elucidated in most cases. It is also unknown whether the inverse relationship between mutation rates and genome sizes can be extended to RNA viruses, and whether other factors such as genome polarity play a role in determining viral mutation and evolution.

The demonstration that the mutation rate of a virus determines its fitness and virulence is an important advance in the field. However, until very recently, most of this body of work built on a single RdRp variant and was restricted to members of the

Picornaviridae family. Generalizing these results will strongly improve our understanding of viral pathogenesis and provide new basis for vaccine design.

Manipulating a virus' intrinsic mutation rate could be of potential benefit not only to live virus vaccine development, but to the production of inactivated virus vaccines as well. A high fidelity variant that replicates at wildtype levels will expectedly express larger quantities of correctly folded viral proteins, which could potentially increase production yields as well as elicit more specific immune responses. Alternatively, a lower fidelity variant with increased mutation rates may generate a more diverse population of antigens that might increase the likelihood of cross-protection against related strains presenting moderately different antigenic sites.

Lethal mutagenesis of viruses has a solid theoretical basis and has been extensively demonstrated in the laboratory. Although it has proved effective against many RNA viruses in cell cultures and occasionally in animal models, its usefulness in the clinical setting remains to be shown. In addition, resistance to this type of treatment and its potential perils should be carefully considered, given that alterations of the mutation rate may have important effects in viral adaptability, fitness, or virulence. More broadly speaking, new drugs should be evaluated not only for efficacy but also for the probability of resistance and its consequences.

Finally, a better understanding of how mutation rates are set and how they could be manipulated may open the way to using fidelity variants of RNA viruses to experimentally accelerate or decelerate evolution as a means of forecasting future strains emerging in response to developing immune responses within an individual and at the human population level.

Executive summary

Mechanisms of viral mutation

- The absence of 3' exonuclease activity in most RdRps provides the clearest single mechanistic basis for the high mutation rates of RNA viruses, compared with DNA viruses.
- However, the mutation rate of a virus depends on processes other than the fidelity of its polymerase. Additional sources of mutations include spontaneous damage of the genetic material, proteins and co-factors that modulate polymerase fidelity, repair, or host-mediated editing.

Measuring viral mutation rates

- Viral mutation rates determined in vitro and ex vivo have frequently produced discrepant results. In vivo studies are far less abundant, yet necessary.
- Common problems in mutation rate estimation are the bias introduced by selection, sequencing errors, analysis of a few potentially non-representative genome sites, and the use of different definitions and measurement units.

Variation in mutation rates across viruses

- RNA viruses mutate faster than DNA viruses, ssDNA viruses mutate faster than dsDNA viruses, and mutation rates are inversely correlated with genome sizes across DNA viruses and microorganisms.
- Genome size and polarity play a less obvious role in determining mutation rates among RNA viruses.

Viral mutation and virulence

- A single amino acid replacement can simultaneously reduce the mutation rate and the virulence of a poliovirus in mice. A likely explanation for this result is that

high fidelity variants have reduced adaptability. Recently, a similar finding has been obtained with a togavirus.

Viral mutation and vaccines

- RNA virus rapid mutation and evolution poses an important challenge to vaccine development, as illustrated by the cases of influenza virus, HIV-1 or HCV, although there also RNA viruses for which effective vaccines exist.
- Another downside of RNA virus high mutation rates is that live attenuated vaccines have the potential to revert to virulence. Attenuated high fidelity variants would have the advantage of reverting less often than currently used vaccines.

Viral mutation and antiviral treatments

- Although highly efficient in the short-term, most antiviral drugs fail in the long-term due the emergence of drug-resistant viruses.
- A potentially useful antiviral strategy consists of artificially increasing the genetic load of a virus using specific mutagens. This strategy, called lethal mutagenesis, has proved effective against many RNA viruses in controlled laboratory conditions, but its therapeutic value remains to be shown.

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Boxes and Figure legends

Box 1. Measuring viral mutation rates using the Luria-Delbrück fluctuation test

Carrying out a Luria Delbrück fluctuation test requires a virus that can be plated and a selective agent in which only certain mutants can form visible plaques (a monoclonal antibody, an antiviral, a non-permissive cell type, etc.). A large number of replicate cultures are seeded with a small initial number of individuals N_i each and after viral replication has occurred, N_f viruses are plated in the presence of the selective agent. The statistical distribution of the number of resistant mutants per culture depends on the mutation rate, but also on the fitness effect of the mutations in the permissive medium and on the replication mechanism of the virus. A simple and convenient method for inferring the mutation rate that is largely independent of selection and replication mechanism is the so-called null-class method, which consists of counting the fraction of cultures with zero resistant mutants (P_0). Since mutation is a rare event, the number of mutations per culture is expected to follow a Poisson distribution with parameter $\lambda = m(N_f - N_i)$, where m is the mutation rate to from sensitivity to resistance. A property of this distribution is that $P_0 = \exp(-m(N_f - N_i))$. Once m has been determined, mutants have to be sequenced to obtain the number of possible different mutations leading to the selected phenotype (mutation target size, T). The mutation rate can be expressed as substitutions per nucleotide per round of copying (μ) using the following equation: $\mu = 3m/T$ [3].

Figure 1. Schematic representation of a Luria-Delbrück fluctuation test (to be merged with Box 1).

Figure 2. Viral mutation rate estimates obtained *ex vivo* and *in vivo*. Data are represented versus genome size and major virus groups are indicated. Values for viroids and bacteria, the two adjacent levels of biological complexity, are also plotted. Mutation rate is expressed as the number of substitutions per nucleotide per cell infection. Adapted from [3].

Figure 3. Effect of mutagenesis on a viral population. In RNA viruses, replication of a single genome rapidly gives rise to a genetically heterogeneous population often termed quasispecies. Cloning is often required to observe this variability, because at each given genome position the most frequent nucleotide or consensus is often the same as in the founder sequence. Chemically-induced mutagenesis increases the genetic variability of the population but may not alter the consensus sequence. Both the mutation frequency and the number of variants per site increase with time. Since most mutations are deleterious, this reduces the average fitness of the viral population and an extinction threshold may eventually be reached.

Fig.1

Fig. 2

Fig. 3