

Spotlight

Influenza Evolution:
New Insights into an
Old Foe

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Influenza viruses steadily evolve to escape detection by antibodies, necessitating vaccine updates. A new study uses a massively parallel approach, deep mutational scanning, to catalogue antibody escape mutations. This approach defines potential pathways of viral evolution, beyond those already observed in natural infections, and may help predict its future directions.

Humans are first infected with influenza viruses in early childhood, and while this infection typically induces lasting immunity, most of us are periodically re-infected throughout our lifetimes. Each year, influenza causes significant morbidity and mortality worldwide, even in vaccinated populations [1]. Influenza viruses thus continue to elude the concerted efforts of scientists, epidemiologists, clinicians, and even our own immune systems, to contain its global circulation.

Influenza viruses undergo constant antigenic evolution, accumulating changes in their surface proteins, hemagglutinin (HA) and neuraminidase (NA), that mediates 'escape' from antibodies raised against previous strains. Considerable effort is made each year to predict which influenza virus strains will predominate in circulation, so that these strains can be incorporated into the seasonal influenza vaccine. Integration of large-scale phylogenetic and antigenic analyses has made possible an increasingly sophisticated understanding of the mechanisms by which new influenza

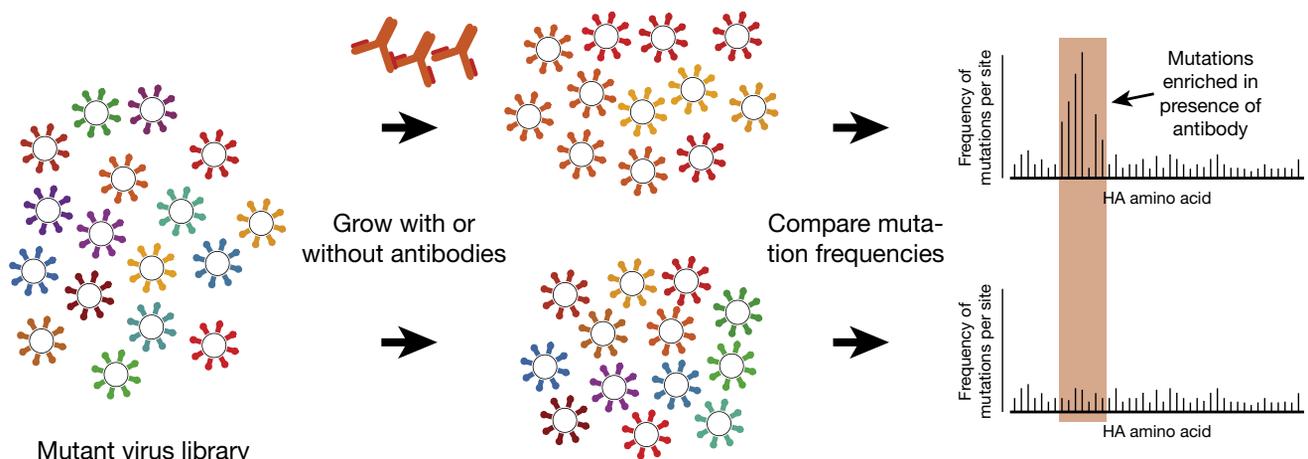
variants escape immune detection and circulate globally [2,3]. However, this understanding has been based on studying the prior evolution of influenza virus, which likely represents only one of many potential evolutionary pathways the virus could have taken. Predicting influenza's future therefore remains difficult. A new study by Doud, Hensley, and Bloom aims to help by developing a method to comprehensively catalogue influenza escape mutations [4].

HA mediates attachment and entry of influenza virus to host cells. It is also the principal target of neutralizing antibodies, that is, antibodies that prevent viral infection of target cells. Beginning in the early 1980s, researchers 'mapped' sites on the HA molecule bound by neutralizing antibodies by growing influenza viruses in the presence of suboptimal doses of monoclonal neutralizing antibodies. Viruses with mutations that disrupted antibody binding grew out; because the mutations presumably disrupted antibody binding, they revealed an antigenic 'footprint' of the antibody on the HA molecule [5]. These classical experiments were powerful, but limited in that their approach typically amplified pre-existing mutations within the viral sample. Therefore these experiments revealed some, but not all, mutations that could lead to antibody escape.

Doud *et al.* cast a wider net, using deep mutational scanning [4]. This approach [6] creates massive libraries of mutant influenza viruses bearing virtually all amino acid changes in the gene of interest (in this case, HA) that are not lethal to the virus as the starting point of their studies. Deep mutational scanning greatly expands the number of variants that can be tested in a single experiment [7]. It differs from traditional approaches like alanine scanning, which uses site-directed mutagenesis to create point mutations that systematically substitute alanine for other amino acids in a protein and does not fully explore the effects of different amino-acid substitutions. Doud *et al.* grow their virus libraries in cell

culture, either without antibodies or together with some of the same antibodies from the classical mapping experiments. The authors are careful to show that the antibody concentrations they use substantially reduce, but do not eliminate, virus growth; these conditions should favor the outgrowth of any mutant viruses in the library that can escape antibody detection. Using high-fidelity next-generation sequencing, they detect and quantify mutations that are enriched in the presence of antibody, providing an estimate for how well each mutation escaped antibody detection (Figure 1).

The authors find that each antibody selects for mutations in a relatively small number of amino acid sites in HA, with patterns of mutation that are remarkably similar across replicate experiments. This is consistent with the findings of the classical experiments, and with the interpretation that antibodies contact HA at a discrete set of amino acid sites. Analysis of individual mutant viruses selected from the library confirms that the mutations identified do indeed reduce the ability of antibodies to neutralize viral infectivity. Notably, some of the HA amino acid sites under the strongest natural selection pressure in these experiments were not identified in earlier studies, underscoring the ability of this approach to reveal pathways of viral escape from antibodies not identified by traditional means. Mutations detected by the investigators occurred both within and outside of the classically defined antigenic regions of HA. In the most striking example, two antibodies that bind the same classical antigenic region select for different sets of mutations – both antibodies select for variants at amino acid 89, which was not identified as a site of variability in classical experiments. Each antibody also selects for a different set of additional mutations, suggesting that the virus can use different 'escape routes' to avoid detection by antibodies with very similar binding sites.



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Figure 1. Identifying Influenza Antibody Escape Mutations Using Deep Mutational Scanning. Doud *et al.* [4] first generated a library of influenza viruses bearing every possible amino acid substitution in the HA attachment protein, which is the principal target of neutralizing antibodies. They then grew these virus libraries in tissue culture in the presence or absence of monoclonal neutralizing antibodies. Viruses with mutations that permitted escape from antibody binding were enriched in the presence of antibody, while susceptible viruses failed to grow. The investigators used high-fidelity next-generation sequencing to detect and quantify mutations in HA in each culture. This allowed them to measure the extent to which escape mutations were enriched in the presence of antibody, which they used to estimate each mutation's selective advantage. The experiments allowed the investigators to comprehensively map the escape routes by which influenza virus can evolve to avoid antibody detection.

Because evolution involves random processes, the future evolution of influenza is inherently difficult to predict. Doud *et al.* provide critical new information for antigenic prediction models because they show that mutations outside of classically defined antigenic sites can contribute to immune escape. Their study also has some important limitations. As the authors note, they evaluate only one viral strain – it is likely that escape routes depend on the starting viral genetic background and will therefore differ among strains. Their approach may also miss pathways of escape that require multiple mutations, including those that compensate for fitness costs associated with escape mutations.

Recent advances in technology are enabling many complementary approaches to understand how influenza evolves and improve prediction. Population-level virus sequencing and antigenic profiling has suggested that only a few specific amino acid substitutions may be responsible for most of the antigenic changes observed in circulating viruses [3], a conclusion that has found

some support when tested in animal models [8]. Next-generation sequencing showed little evidence for selection of antibody escape mutants in individual infected humans [9,10]. Together these studies suggest that many escape variant viruses identified by deep mutational scanning may have other fitness defects, such as reduced transmissibility, that would limit their ability to emerge and spread in humans. Nonetheless, Doud *et al.* provide a comprehensive view into the possible pathways of viral evolution that is not limited by the vicissitudes that result in the particular ones we happen to have observed in nature. It is easy to envision how this deep mutational scanning approach could be deployed in animal models to even better understand the complexities of immune escape by influenza and other antigenically variable viruses.

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