CAN VIRUS EVOLUTION BE PREDICTED? - APPLIED EVOLUTIONARY BIOLOGY IN VACCINE DEVELOPMENT OF THE SEASONAL FLU

Predicting influenza virus models review and their implementation in the yearly vaccine design.

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Abstract

Influenza viruses causes high mortality and morbidity across the globe every year. A/H3N2, A/H1N1, B/Yamagata and B/Victoria are the subtypes that have affected human health so far, causing severe illness and leading to epidemics, and in the case of A/H3N2, even pandemics. The natural reservoir of these viruses are swine and avian species. These viruses present two main surface proteins, hemagglutinin (H or HA) and neuraminidase (N or NA). The first one is the responsible for the entrance of the virus to the host cell, and therefore, the adaptability of human immune system, making it subjective to high selective pressure. The antigenic properties of a virus may change from season to season, and the antibodies developed by an already infected host may not recognize or be efficient enough to neutralize the virus that is predominantly circulating year(s) later. The best tool developed so far in order to combat this pathogen or at least ease the spread/severity of the illness are vaccines. Nowadays, a quadrivalent vaccine is the gold standard, it contains one representative strain for each subtype. The vaccine design is a complicate process, the World Health Organization (WHO) gathers experts on the subject twice a year (one for each hemisphere) to discuss which strain would be the most representative one circulating, so that the vaccine would contain that one, and consequently the efficiency will be enhanced. However, due to technological constrains, the decision needs to be made one year in advance, making it harder to predict which strain would be the fittest one year in the future. To help in this task, modeling influenza evolution have proved to be a powerful tool with lot of potential. In fact, using retrospective data, most models developed to this day have been able to predict which strain would be most prevalent with more efficiency than the WHO predictions.

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1. Influenza virus

1.1 Characteristics and history of Influenza viruses

Influenza viruses are responsible for the infectious disease known as the seasonal flu. They belong to the family *Orthomyxoviridae,* and encompass 4 of the 7 genera within that family. They have a negative-sense, **single-stranded RNA** genome divided in 8 segments. Influenza A and B viruses are the most important ones for human health and therefore understanding their dynamics is of utmost importance to develop strategies to prevent epidemics/pandemics with fatal consequences. The first human influenza virus was isolated in 1933 (Smith et al. 1933) and since then the study of these viruses remains a great challenge in the scientific community.

The genomes of influenza A and B viruses encode for 10 major proteins (Figure 1). Three segments encode for three subunits of the RNA-dependent RNA polymerase (RdRp) complex, an **error-prone polymerase** which is essential for viral replication in the host. Other segments encode for the matrix protein (M1) and the membrane protein (M2), the non-structural protein (NS1), the nuclear export protein (NEP), and the viral nucleoprotein (Krammer et al. 2018). Finally, two proteins with important roles in the capacity of the virus to infect and replicate in the host are encoded in two separate segments. **Hemagglutinin** (H or HA) is a surface protein that allows the fusion of the virion with the cell membrane by binding to the sialic acid receptors. And **neuraminidase** (N or NA), which cleaves the sialic acid molecule, therefore allowing the recently replicated virions to break away the host cell and infect more cells. In addition, there are alternative open reading frames that encodes for various accessory proteins (Hay et al. 2001).

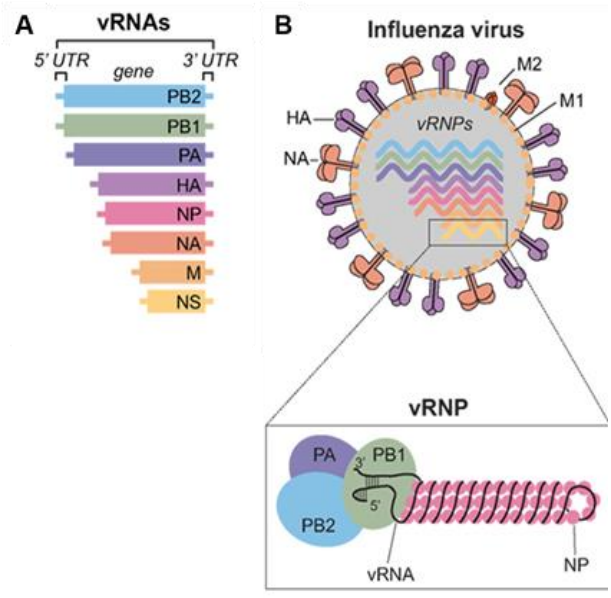

Figure 1. Structure of influenza A/B virus. The virus consists of a capsid with the surface proteins HA and NA, the matrix protein (M1) and membrane protein (M2) A) The eight RNA coding segments inside the virus capsid B) Structure of A/B influenza virus with the amplification to the polymerase coded by the segments PA, PB1 and PB2. Adapted from Dou et al. (2018)

Influenza A viruses are responsible for severe illness, seasonal epidemics and, occasionally, pandemics. These viruses circulate mainly in avian species, although they can sometimes be transmitted to other species, including mammals. They count a large variety of genetically distinct subtypes defined by their antigenic properties. So far, the combinations of 18 HA and 11 NA subtypes have been described in avian species, which establishes a large reservoir of potential pandemic viruses (Suttie et al. 2019). To this date, only subtypes H1-3 and N1-2 are known to have circulated in humans (Krammer et al. 2018).

Influenza B viruses have been described to mainly infect humans. Instead of subtypes, two main antigenically distinct lineages of influenza B viruses have been characterized; **B/Victoria** and **B/Yamagata**. They are associated with seasonal epidemics and disproportionately affect children. Currently, two subtypes of influenza A virus (**H1N1** and **H3N2**) are in circulation, as well as the two lineages of influenza B viruses (Krammer et al. 2018).

These viruses cause epidemics every year due to the serial antigenic change driven by the mutability of the RNA genome and more sporadic pandemics caused by the rise of antigenically new influenza A subtypes. In other words, new antigenic variants from previously circulating viruses and new jumps from animal reservoirs to humans followed by a human-to-human transmission facilitate the outbreak of epidemics and pandemics. Therefore, good surveillance methods together with improvements of strategies to prevent (e.g., vaccines) and to treat infections are important to fight against this pathogen.

1.2 Evolution of influenza viruses

The different variants of influenza virus differ in their evolutionary dynamics and antigenic changes. Every 3-5 years a new A/H3N2 variant appears, while new A/H1N1 and Influenza B viruses appear less frequently, every 3-8 years (Bedford et al. 2015).

Lots of effort has been put into understanding how these viruses evolve. These studies have highlighted that there are some important sources of evolutionary selective pressures acting on influenza viruses in **different scales** (Figure 2).

Molecular dynamics - Antigenic drift and shift

The rise of new antigenic variants to which the population may be immunologically naive every year is due to two main processes known as **antigenic drift** and **antigenic shift**. The former refers to the gradual accumulation of mutations given by the error-prone polymerase. Because of this punctuated nature of antigenic evolution there are graphical depictions of antigenic variation and the appearance of clustered glycoprotein structures occurs, which are known as antigenic clusters (Smith et al. 2004). The new individual antigenic variants are referred to as **strain**, especially in the field of vaccine development, and multiple strains of the different subtypes and lineages circulate every year.

The latter, antigenic shift, also known as **genome reassortment**, happens when two influenza variants exchange genomic segments when infecting the same cell, resulting in genetically novel viruses (Lowen, 2017). This is of vital significance in the emergence of completely novel antigenic variants and in the transition from one host species to another.

Both processes are determinant in the influenza virus immune escape as the host may not present an adaptive immune response to the new antigenic variants, increasing the likelihood of the virus to be able to replicate in the host cells before being neutralized, and thus causing a severe illness.

Mutations and rearrangements can happen along the whole genome; change in aminoacids in most flu genes will reduce the fitness of the strain by weakening its functionality. However, the part which is more vulnerable and significant for human health to these changes is the HA protein, as it is the target for the human adaptive immunity and therefore it is found under high selective pressures. The HA protein is a homotrimeric protein, and each of the monomers contains a **globular head** (HA1) and a **stalk** (HA2) domain (Bizebard et al. 1995). The main response is developed against the globular head although antibodies targeting the stalk domain are contrived occasionally in older individuals, probably as a consequence of long exposure to different influenza viruses (Raffael et al. 2016). As a matter of fact, seven amino acids around the receptor binding site of HA1 were primarily responsible for antigenic change in A/H3N2 viruses from 1968 to 2003, as measured by Haemagglutination Inhibition (**HAI**) assays, plaque assays and microneutralization assays (Koel et al. 2013). These assays are all based on the same principle: red blood cells, a sample of isolated virus and serum (with antibodies) are mixed. The virus can interact with the blood cells membrane proteins, causing their agglutination, which is a visible outcome. When a serum containing antibodies that can recognize the virus are added, the agglutination can be inhibited. Different dilutions of serum are added in serial wells, the outcome of the test is HAI titers, which signify the highest diluted serum (antibodies) that can prevent the haemagglutination (Microbe online, 2021).

Nevertheless, other mutations which may be distant from the receptor binding domain of HA, may also be important players in immune escape (Linderman et al. 2014).

Selective pressure on antigenicity and effects of virus population in the transmission

The selective pressure on antigenicity of the virus lies primarily on the adaptation of the immune system of the host after vaccination or infection.

As reviewed in Petrova and Russell (2018), it is of high interest to study the immune response of the different hosts (naives and hosts that have been already in contact with the pathogen) in order to understand constraints to the rise of new antigenic variants. Additionally, as more knowledge is being gathered about the dynamics of influenza transmission, its evolution is now understood as a multi-scale process, as selection can also occur at a molecular, population, regional and global scale (Figure 2).

Regarding **within-cell evolution**, the virus undergoes several bottlenecks. Once the virus is inside the cell and has replicated, different variants can be found, some are genetically identical to the one that infected the cell, others are unfit and more infrequently, fit variants. Most of the new ensembled viruses will be captured by the mucus when leaving the cell.

In **within-host selection**, both innate and adaptive immunity need to be taken into account to understand the population dynamics of the virus. The strength of the **innate immune selection** is dependent on the infectious dose, the virus immunogenicity that leads to a pro-inflammatory state and the genetics and condition of immune health of the host. On the other hand, the

adaptive immune selection mainly depends on the history of the pathogen-host relation, which accounts for the history of virus or vaccine antigen exposure, the antigenic resemblance between strains (as there can be cross-immunity, meaning that the antibodies targeting a variant may protect to a certain extent from another variant), and the extent of immune waning. In summary, the innate immunity acts as a limiting factor for the rise of new variants, whereas adaptive immunity comes into play when the new variants have already raised.

Another level of dynamics can be found in **between-host** transmission. Most of the diversity created in one host will be lost in the spreading of the virus by the donor as not all of the particles may get to the recipient. Moreover, only a minority of the viruses that get to the new host will pass the mucus and the first immune barrier. At this level, the relative number of people that are naive against the virus, immune or infected, plays an important role. Additionally, in the long-term virus maintenance requires that the viruses arising in a population and causing an epidemic are capable of starting another one in a new location, **between-populations** bottleneck (Petrova and Russell, 2018).

Finally, as influenza is a global pathogen, it is important to reflect on **global dynamics**, like the seasonality of the disease. This seasonality may be due to the fluctuations in human immunity, the pro-inflammatory responses have been described to be upregulated notably during the winter months (Dopico et al. 2015), and other social-related risk factors, such as inside gatherings in crowded and poorly ventilated places. All these bottlenecks are illustrated in Figure 2.

This results in multiple bottlenecks and may be the main explanation of why there are not as many new variants as it would be expected (according to Petrova and Russell (2018)) considering the relatively easy appearance of new antigenic variants through antigenic drift and shift and the important number of infections. Indeed, it is estimated that 5-15% of the global population is infected with the virus every year (Krammer et al. 2018). The evolutionary advantages of fit variants can be trampled by transmission bottlenecks that interrupt selective forces, causing stochastic changes. Most of them will result in a loss of fitness and, hardly ever, in a gain (i.e., more virulent virus).

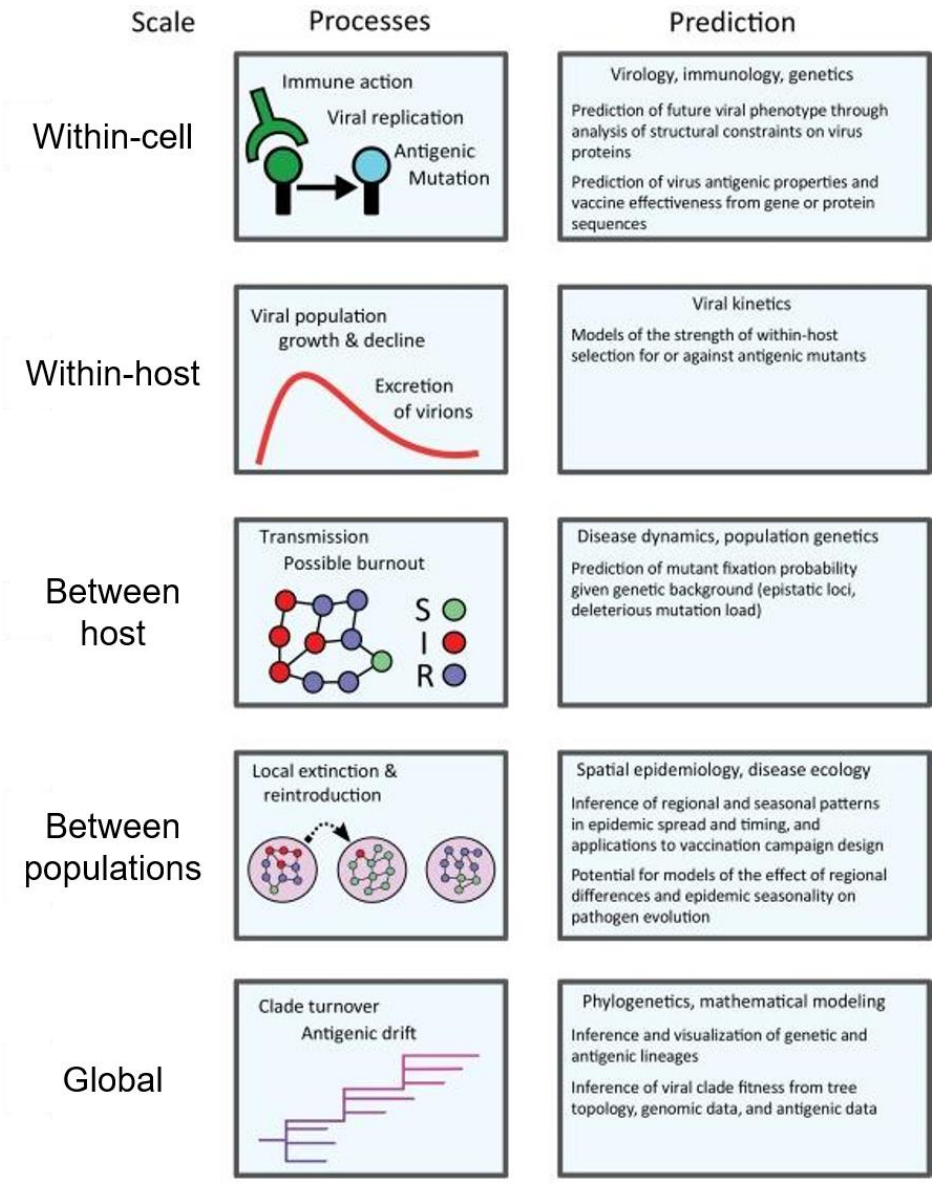

Figure 2. Multi-scale process of influenza virus evolution. Adapted from Morris et al. (2018)

Clonal interference

One dynamic that affects thoroughly asexual populations is **clonal interferenc**e and it may be taken into account for modeling influenza virus evolution (Lässig et al. 2017). This phenomenon occurs when different strains display beneficial mutations on the same segment, but as recombination will very rarely combine these mutations, the strains **compete** with each other. This can explain why beneficial mutations may take longer to fixate or even disappear. Clonal interference probably governs influenza A/H3N2 evolution, as a **sweep pattern** was observed, where at least one strongly beneficial amino acid substitution happened per year, accompanied by three to four driving mutations. The authors of that study concluded that besides positive selection on the antigenic variant, background selection outside antigenic epitopes, immune

adaptation and conservation of other viral functions have been described to interfere with each other when clones are competing (Strelkowa and Lässig, 2012). Clonal interference appears to be a wide-spread evolutionary dynamics in all **RNA viruses**, that would also include A/H1N1 and influenza B (Miralles et al. 1999).

1.3 Current influenza vaccine

The World Health Organization (**WHO**) is well aware of the importance of influenza virus for public health and established a Global Influenza Surveillance and Response System (**GISRS**) with two main aims; (i) the early detection and characterization of novel subtypes of human influenza A to prevent pandemics and (ii) the identification of new antigenic variants among currently circulating influenza A and B viruses to better design the **yearly vaccine** (Hay et al. 2001). The WHO is also in charge of assembling scientists to design the flu vaccine. More recently, with the increased accessibility to genome sequencing, large data bases of circulating influenza have been gathered (Hadfield et al. 2018).

The flu vaccine is manufactured every twice a year, it consists of a **trivalent** or **quadrivalent** intramuscular injection which contains the inactivated form of the virus and needs to be updated every year to keep up with antigenic shift. It contains one strain representing each subtype of influenza, one A/H3N2, one A/H1N1 and one or two influenza B. Due to the manufacturing constraints, the vaccine design needs to be completed one year before the flu season. Therefore, twice a year (February for the North hemisphere and September for the South) scientists assemble at the World Health Organization (WHO) to decide which strain will contain the vaccine. They discuss which strain will have the highest fitness and dominate next year's flu season. Although knowledge acquired by **applied evolutionary biology** models have been used in the decision making for the last seasons (Morris et al. 2018), to this day, the strain decision is mainly based on data from serological assays. These techniques, however, have their limitations, as they are inconsistent across labs, labor intensive, not publicly available, and difficult to interpret or scale up (Perofsky and Nelson 2020).

Since developing, manufacturing, and distributing the vaccine takes many months, it is of public health interest to **forecast** the evolution of influenza (Klingen et al. 2018; Morris et al. 2018). There is indeed a dire need to avoid antigenic mismatch between the vaccine and the circulating virus strains, as the effectiveness decreases when the circulating strains differ from the ones included in the vaccine. In fact, the flu vaccine has the most variable performance of all the vaccines licensed in the US (CDC, 2016). The mean vaccine **effectiveness** from 2004 to 2015 was only 61% for H1N1pdm and 54% for influenza B viruses; the case is not as good for H3N2, with an effectiveness of 33% (Belongia et al. 2016).

Due to the importance of the disease, improving the efficacy of the vaccine through multiple strategies have been intensely addressed by the scientific community. One approach includes the development of a **universal vaccine**, targeting the stalk domain (Sautto et al. 2018, Yang et al. 2017) while another proposes a **mosaic vaccination** strategy, so that groups of individuals in

a given population are immunized to different strains (McLeod et al. 2021). Nevertheless, more accurate measures and predictions about which will be the most prevalent strain for the next season remains a crucial tool to help in the vaccine design and improvement of the efficiency.

2. Prediction of influenza evolution

2.1 Predicting evolution - What is predictable in evolution?

What is predictable in evolution?

A prediction is a bet about the future that should strike a balance between surprise and truth (Lässig et al. 2017). In order to address the question of **what is predictable** in evolution, we need to ask ourselves what is the core principle behind any organisms evolving. A species (or strain) evolves to change a functional trait which increases their fitness in a specific environment. Normally, this change consists of a certain mutation on the genome. The sequence space is huge and even different mutations can result in the same functional output, consequently, it is unquestionably hard to predict the evolutionary path in the sequence space. However, that is not an indispensable requirement to forecast functional changes in a population. In principle, evolution is predictable as long as selection leads phenotypic evolution towards a single dominant path.

Nevertheless, to build a predictive analysis from data, we need to have access to a representative database and to relate genetic or phenotypic data to fitness differences in a population (Lässig et al. 2017).

What is predictable in influenza virus evolution?

There are some specific features in the case of influenza virus evolution which make modeling very challenging but also **predictable**. The evolutionary dynamics that probably governs influenza evolution is clonal interference, although not the only one, as it has been seen in viral laboratory populations and explained above. In RNA viral populations new mutants are produced at high rate (reducing stochastic waiting times) and it has been observed that the rise and decline of clade frequencies is driven by selection, rather than genetic drift or environmental noise; which makes it more predictable (Miralles et al. 1999). Nevertheless, there are some intrinsic stochastic processes to evolution that are hard to predict/expect, like the rise of new variants.

Fortunately, the case gets simplified when applying it to vaccine production. Instead of predicting functional changes, the main aim of the models is to accurately guess which will be the **most prevalent** circulating strain next year. In order to do that genetic and phenotypic information about the circulating viruses is required.

In this essay, we **focus** on predicting influenza virus evolution, specifically aiming to question if the models are capable of foreseeing which strains will be dominant in the next year(s), therefore increasing the efficiency of the vaccine.

2.2 Predicting influenza evolution models

There have been multiple attempts to overcome the challenge from different angles. Researchers have constructed **explicit fitness models** (Łuksza and Lässig, 2014; Huddleston et al. 2020; Barrat-Charlaix et al. 2021), use historical patterns of evolution (Łuksza and Lässig, 2014), phylogenetic patterns (Neher et al. 2014), genomic sequencing data (Steinburg et al. 2014) or phenotypic assays (Neher et al. 2016) to predict the strain that would be the most representative of the future population (Morris et al. 2018).

Understanding which variable(s) explain better the fitness remains one of the main challenges when modeling a population. Neher et al. (2014) use the **"local branching index"** (LBI) to estimate viral fitness. According to the authors, genealogical trees have information about the relative fitness of the sequences and can be used to predict successful strains. The child nodes (branching point) fitness distribution is characterized by branch propagators of the fitness of the ancestral node (Figure 3), clades with high LBI are expected to get expanded.

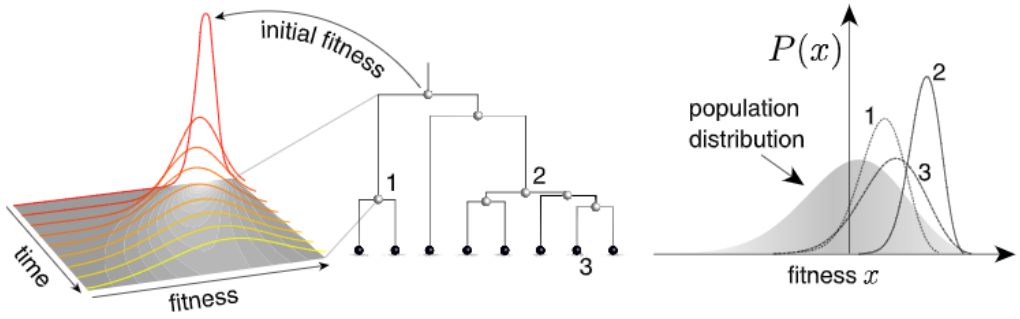

Figure 3. Inferring fitness from genealogical trees. The algorithm is based on branch propagators associated with each branch of the reconstructed tree (in the middle). The child nodes fitness is calculated given the fitness of the ancestral node (left). For instance, on the right it is shown that internal node 2 would have higher marginal fitness than node 1, as it has more children. Neher et al. 2014.

This model works under two main assumptions: (i) the population is under persistent directional selection and (ii) the accumulation of small effect mutations, in a step-manner evolution, is the driver of evolutionary change. In the specific case of human seasonal A/H3N2 influenza, there are two main observations regarding the evolution of the virus sequence that are in line with these assumptions: amino-acid substitutions in the antigen can have large phenotypic effects (Koel et al. 2013) and the accumulation of mutations with small or no antigenic effects but affecting overall fitness (Strelkowa and Lässig 2012). The authors validated the model with historical haemagglutinin A/H3N2 data, from which they constructed a genealogical tree. They predict the progenitor lineage of the upcoming influenza season with near optimal performance in 30% of

cases, and make informative predictions in 16 out of 19 years. However, the predictions were suboptimal in years when the assumption of step-manner evolution did not hold (e.g., 1995, 2002, and 2004) and the antigenic properties of a cluster changed drastically.

Overall, this shows that the shape of reconstructed genealogies can be used to infer relative fitness of the sampled individuals and exploited to predict genetic composition of future populations. These predictions might be improved by adding antigenic information (Bedford et al. 2014), biophysical and structural knowledge (Koel et al., 2013), patterns of past evolution (Łuksza and Lässig, 2014), and plausible geographic sources (Lemey et al. 2014). Interestingly, for the application of the model, only a static set of sequences from a single time point is required, instead of extensive historical data. LBI has been used by other authors, where other estimates have been evaluated as well (Huddleston et al. 2020). The predictions were also reviewed by Barrat-Charlaix et al. (2021) where they showed that the consensus sequence (regardless the fitness) performed as well as the LBI, suggesting that the reason under the predictive power of LBI may not be related to its approximation of the fitness.

Steinbruck et al. (2014) took a different approach which does not precisely rely on evolutionary models, but rather on a framework that uses antigenic and genomic sequence data to select a suitable strain for production of the vaccine. The authors refer to this model as **AACP (antigenic allele-based computational predictions).** From phylogenetic trees they identified the HA alleles (from A/H3N2) that have increased the most in comparison to others in the previous epidemic season and that were not predominant before, therefore assuming that these ones have a selective advantage and will become predominant in the future. The model is therefore based on measures of antigenic novelty and rate of propagation of the viral strains throughout the population. To make it more realistic, they infer a tree, allele dynamics and antigenic weight for each influenza season only with the data collected one month before the WHO decision. They provided a yearly recommendation based on which allele was most likely to be on the rise and had an estimated antigenic impact sufficient to justify a vaccine strain update. If there was no such a strain, the vaccine composition should be left unchanged.

The performance of the model between the years 2003 and 2007 is displayed in Figure 4, where the recommendations by WHO and AACP analysis are compared. Out of 9 seasons, 6 were correctly predicted by the AACP and the recommendations from WHO matched 4 out of nine seasons (Figure 4). Steinbruck et al. (2014) concluded that the computational decision procedure generated good matches of the vaccine strain to the circulating predominant strain for most seasons and it could be a powerful tool for experts from the WHO that decide the composition of the vaccine.

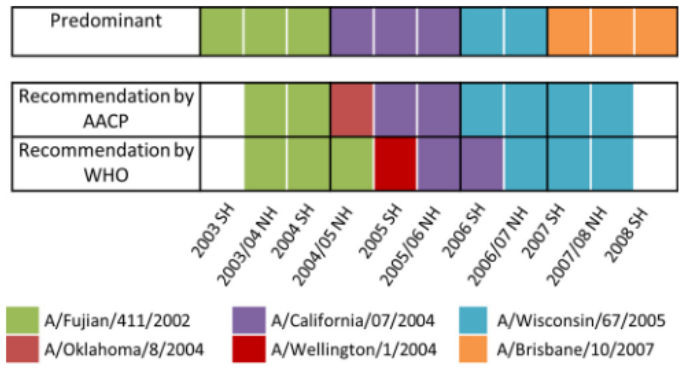

Figure 4. Evaluation of the performance of the antigenic allele-based computational prediction (AACP) of vaccine strains in the case of human influenza A/H3N2 compared with the WHO recommendations. In the upper line the actual predominant strain is represented. In the middle the recommendations by AACP are marked, in comparison with the WHO recommendation of that year (bottom line). Out of 9 seasons, 6 were correctly predicted by the AACP and the recommendations from WHO matched 4 out of nine seasons.

Instead of predicting the most representative A/H3N2 virus of the future population Łuksza and Lässig (2014) explicitly predicted the **future frequencies of entire clades**. They also use only sequencing data to infer the evolution of influenza. The fitness equation is described here as a balance between **positive selection** for substitutions that enable escape from adaptive immunity and **purifying selection** on domains that are required to maintain the protein's primary functions of binding and membrane fusion. It is therefore a predictive fitness model mainly defined by mutations in the epitope and non-epitope regions of the HA. In the case of positive selection for immunity escape, possible cross-immunity between species is taken into consideration. Unlike Neher et al. (2014), the authors of this paper assumed that influenza viruses grow exponentially as a function to their fitness, and compete for the host (clonal interference). The model is able to predict expansion along the genealogical tree trunk (113 out of 121 cases) and loss of branches (clade decline in 51 out of 67 cases) (Figure 5).

An innovative feature compared to the other researches mentioned, Łuksza and Lässig (2014) also test their model with A/H1N1 data to see its applicability to other influenza strains. The data set has larger regional and seasonal biases, potentially weaker antigenic selection (Bhatt et al. 2011) and less background knowledge about epitope positions (Huang et al. 2012). Therefore, the predictions are noisier but are still comparable to the A/H3N2 ones, proving that the model also applies to other influenza viruses.

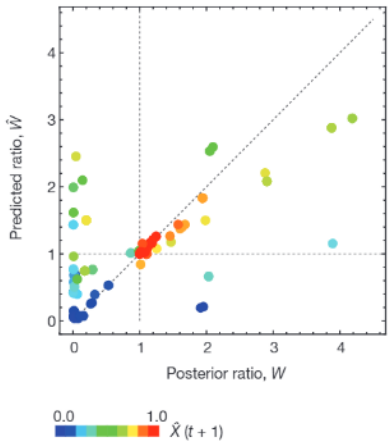

Figure 5. Year-to-year predictions of HA evolution. The predicted frequency ratio is plotted against the observed frequency ratio for 188 influenza HA clades with a specific initial frequency (>0.15) since 1993. The predicted frequency is indicated by color. Good predictions are within the diagonal line, clade growth is correctly predicted in 113 of 121 cases and decline in 51 of 67 cases. Łuksza and Lässig (2014)

Neher et al. (2014) made a comparison between their model's results and those obtained from Łuksza and Lässig (2014)'s model, illustrated in Figure 6. The predictions are comparable in many years and that can be explained by the fact that Łuksza and Lässig's model is specifically designed to capture epistatic interactions, taking into account synonymous mutations of each clade as additional predictors. The tree length, indicator of prediction in Neher et al.'s model, is strongly correlated with the number of synonymous mutations.

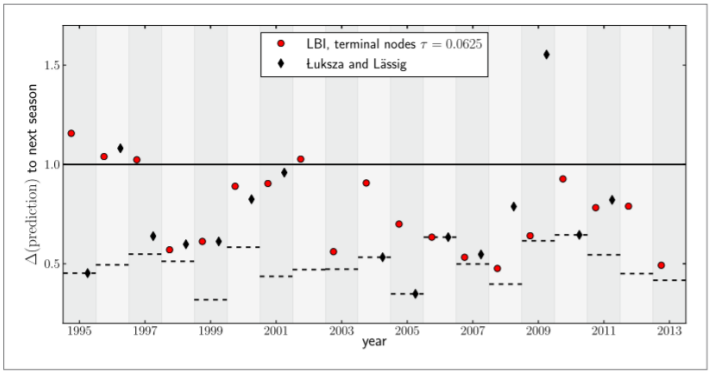

Figure 6. Comparison of the models of Łuksza and Lässig (2014) and Neher et al. (2014). The results are comparable in many years, while also at other one model outperforms the other. Neher et al. (2014)

Box 1. **Fitness measures** reviewed in this Essay

Local Branching Index (LBI):
Fitness measure based on the shape of genealogical trees; it explains that nodes in branches with a long trajectory has higher fitness. It uses HA sequence data and measures clade growth. Implemented in Neher et al. 2014, Huddleston et al. 2020 and Barrat-Charlaix et al. 2021.

Epitope antigenic novelty:
It uses HA sequence data and measures antigenic drift as a fitness category, characterize by changes in the epitope region. Implemented in Łuksza and Lässig, 2014 and Huddleston et al. 2020.

Epitope ancestor:

It is a measure of antigenic drift and measures fitnes from HA sequence data. Different strains can cause cross-immunity and this measure takes that into account. Implemented by Łuksza and Lässig, 2014 and Huddleston et al. 2020.

HI antigenic novelty:
It measures antigenic drift and uses data from serological assays. Implemented in Huddleston et al. 2020.

Mutational load:

It represents a functional constrain from HA sequences. It is characterized by changes in the non-epitope regions. Implemented by Łuksza and Lässig, 2014, Huddleston et al. 2020 and Barrat-Charlaix et al. 2021.

Deep mutational scanning (DMS) mutational effects:
It measures functional constrains and comes from DMS assays data. Implemented in Huddleston et al. 2020 (original form Lee et al. 2018).

Delta frequency:
It measures chance of clade frequency over time (six months). It uses HA sequence data and is a measure of clade growth. Implemented in Huddleston et al. 2020.

The models explained above omit experimental measurements of antigenic or functional phenotypes. To overcome this, more recent models have been designed in which phenotypic data is accounted for.

The study conducted by Barrat-Charlaix et al. 2021 looked into the predictability of **amino acid mutation trajectories** as well as the fixation or loss of amino acid substitutions. The product variable of their analysis is “frequency trajectories” of amino acids at each position in the sequences. Surprisingly, trajectories followed a pattern observed in neutral models of evolution, instead of the expected dominance of selectives sweeps taking over the population in A/H3N2 data. The authors expected that different mutations that affected the fitness of the strain would have a tendency to **fix** (if the mutation is beneficial) or to be **lost** (when detrimental). Nevertheless, even for mutations at epitope positions (which are expected to have a large effect on the fitness) the authors found that the predictability of the trajectories is very limited for A/H3N2, as the probability of fixation is similar to its current frequency. it appears that the dynamics followed by a mutation is not only determined by its fitness but also by the fitness of the genetic background and the fitness of the competing strains (Strelkowa and Lässig 2012). The extent to which these components affect influenza evolution has been reported to affect equally on fixation (Illingworth and Mustonen 2012), consequently, a model that also considers genetic background and relative fitness may improve the predictions.

In contrast, the same analysis for A/H1N1 reveals a consistent upward trend of novel frequency trajectories. For A/H3N2, past dynamics alone is uninformative of future dynamics. Additionally, the authors also performed an analysis for the **predictability of loss or fixation.** In the case of A/H3N2, the expected probability of fixation was very close to the frequency of the mutation in the time, agreeing with neutral expectation. For A/H1N1, the fixation probability was moderately higher than current fixation (excluding synonymous mutations) (Figure 7). One possible explanation for this could be that the immunity of the population for A/H1N1pdm might be less variable as it has only been circulating for 11 years.

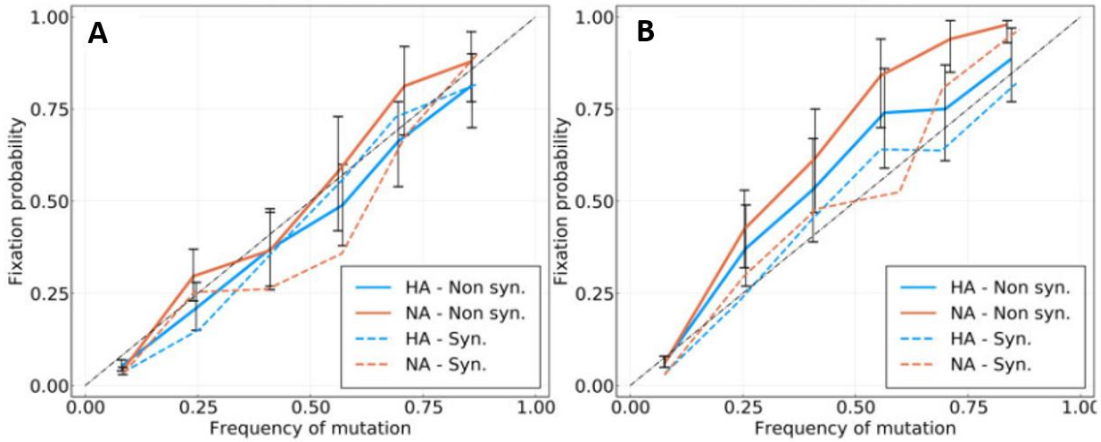

Figure 7. Fixation probabilities as a function of frequency for HA and NA. Only new mutations are considered. The diagonal black dashed line is the expectation from a neutrally evolving population. A) For A/H3N2 HA and NA B) For A/H1N1pdm HA and NA, the fixation probability is a bit higher than the frequency of the mutation. Barrat-Charlaix et al. 2021

In an attempt to search for features that allow prediction beyond frequency, Barrat-Charlaix et al. 2021 considered other predictors based on **LBI, variation in frequency**, **mutational load**, and **serological data**, and two of their combinations. The only effective predictor of fixation was mutational load, nevertheless, other predictors predicted future population composition (but not frequency of strains). After their analysis they also suggested that the accuracy of the predictions concluded by Neher et al. 2014 may be due to the fact that the nodes with the highest LBI are representative of the population and not necessarily reflect fitness, as the **consensus sequence** (defined as the average sequence composed by the most prevalent nucleotide in each position of the initial set of sequences, it does not necessary need to exist among the population) has proven to be a better predictor than LBI in a lot of cases.

In agreement with the difficulty of predicting the rate of change of the frequency of a strain rather than composition there is another study by Huddelston et al. (2020), where instead of accounting for individual mutations, they focus on the fitness of complete amino acid haplotypes. They developed a framework for forecasting influenza where they integrated published sequence-only fitness estimates with phenotypic measures of antigenic drift and functional constraints. They aim to predict sequence composition of the future population, the frequency dynamics of clades, and the virus in the current population that most represents the future population. Alike Łuksza and Lässig (2014) they assumed that viruses grow exponentially as a function of their fitness and that viruses compete between each other through clonal interference.

In their analysis, Huddelston et al. (2020) included different metrics that could explain the fitness of the virus and perform prediction for each of them individually and some in combination. They used epitope antigenic novelty and mutational load (non-epitope mutations), as described in Łuksza and Lässig (2014). As well as other metrics that included Hemagglutination inhibition (HI) antigenic novelty (Neher et al. 2016); deep mutational scanning (DMS) mutational effects (Lee et al. 2018) which measure the effect of single amino acid mutation in HA from the background of a previous vaccine strain, A/pERTH/16/2009 (Lee et al. 2018); local branching index (LBI) (Neher et al. 2014); and change in clade frequency over time (delta frequency), a growth metrics that estimates the success of populations of strains in the last six months. Additionally, after having performed the first analysis, they added another metric, the “epitope ancestor” metric which is defined as a linear model and counted the mutations in the epitope since each strain’s ancestor in the previous season. They applied these metrics to simulated and natural populations.

Some of these metrics were also tested together to check whether they could outperform individual metrics, HI antigenic novelty and mutational load, mutational load and LBI and a combination of the three were fit in a model. The model with the best performance across the validation data was mutational load and LBI; but was the worst in the test data. The probabilistic nature of evolution may explain these results and highlight the importance of identifying models that remain robust to stochastic evolutionary changes. The authors find that antigenic drift and mutational load were the most robust predictors of future success in A/H3N2 populations. Nevertheless, generally, composite models outperformed models based on their individual

coefficients, although efforts need to be made to understand which measures should be combined together.

All in all, the biologically-informed fitness metrics constantly outperformed the naive model (which predicted that the future population was identical to the current one) forecast, therefore providing a strong tool to help selecting the principal strain circulating in one year ahead (Huddelston et al. 2020).

3. Future perspectives and discussion

There are still open questions and room for improvements in the applicability of these models, where different estimates of fitness have been used (Box 1). Which metrics to apply or how to improve the current ones remain one of the biggest challenges and was partly addressed by Huddelston et al. 2020, where they proved that experimental measures of antigenic drift still provided more information about the future population than sequence-only metrics. They also highlight that the combination of different fitness definitions would lead to more accurate predictions. Huddelston and Barrat-Charlaix (Huddleston and Barrat-Charlaix, 2020) already proposed a new distance from consensus metrics that in combination with HI antigenic novelty could potentially provide better estimates of functional constraints and antigenic drift.

Additionally, the integration of more recent experimental historical data may improve the estimates of antigenic drifts; for instance, in Huddleston et al. 2020 a new metric, the focus reduction assays (FRA) antigenic novelty was introduced, however, although promising, there was not enough data to train the model as it only dated back from 2012.

Not only new but more veracious data could improve the predictions. The utility of these explicit models depends on the availability of extensive historical data or a detailed understanding of the influenza virus sequence-to-fitness map. Additionally, as there are vastly different sampling intensities across the globe, the models should account for the geographic distribution.

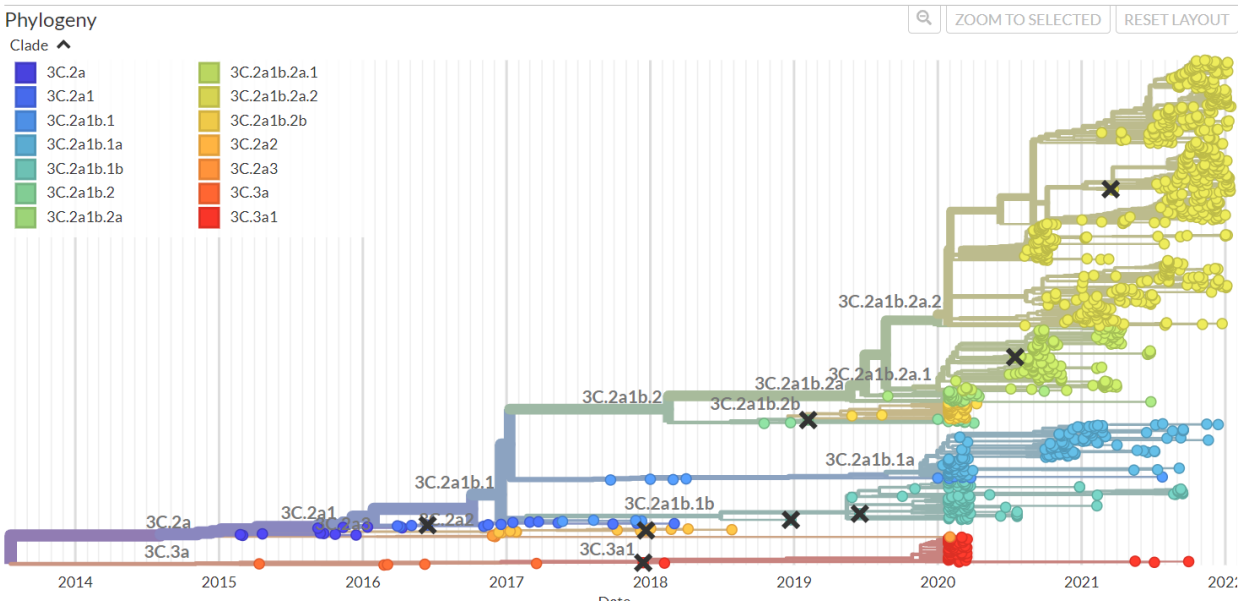

Figure 8. Evolution of the A/H3N2 influenza virus from 2014 until 2022. The platform nextstrain.org gathers data of the influenza (and other viruses) sequences from all over the world. Hadfield et al. (2018)

Interestingly, some models (Łuksza and Lässig, 2014; Neher et al. 2014) fail to predict the accurate frequency of the most predominant clade when evolution is produced in a non step-manner. This outstands the fact that modeling/predicting stochastic changes is a challenge; which also applies to new arising variants from animal reassortment. The latest could be addressed by increasing surveillance in swine and avian influenza.

As a matter of fact, the success of these models can be attributed to the global effort in the surveillance of human influenza, resulting in a big database which allows to track human influenza evolution lively (Figure 8) (Hadfield et al. 2018).

All in all, even though predicting the relative frequencies of all the circulating influenza strains have shown to be hard, most of the models done to this day have been able to predict the strain that will be more prevalent in the next season with the same or better accuracy than the WHO recommendations, which is of most importance in vaccine design. Therefore, proving that predicting influenza evolution can be a great tool for the improvement of vaccine efficiency.

Nowadays, the need to improve prediction tools is crucial; between 1700 and 1889, the average inter-pandemic period ranged from 50-60 years; since 1889, it has been shortened to 10-40 years (Potter, 2001). Vaccines are one of the most powerful tools developed so far, designing them accurately would help a lot in minimizing the impact of the outbreaks; as they are inevitable. Nevertheless, prediction should not be the only approach and the surveillance efforts remain the key point not only to improve prediction accuracy but also to identify completely new antigenic variants (Figure 8, Hadfield et al. 2018). One of the challenges that remains is to reach a consensus on how to analyze and interpret all this data and which models to implement, as well as making them universal for all influenza subtypes.

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