Analyses of Merging Clinical and Viral Genetic Data for Influenza Surveillance

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Abstract
The annual influenza vaccine is one of the most common public health interventions and is universally recommended for all individuals older than six months. Vaccine composition depends on viruses circulating over the past flu season and are estimated to be the most prevalent and representative strains in the current season. Here, we use clinical data outfitted with viral genetics to characterize confirmed influenza cases from the past two flu seasons and genetically compare them to the strains that they were vaccinated against that year. We show that case similarities to vaccine strains differ by geographic region and that the vaccines appear to have different levels of effectiveness by region. This study demonstrates the value of merging viral genetics with clinical data. Further research is needed to formally evaluate whether this improves biosurveillance efforts and enhances efficacy of influenza vaccines.

Introduction
One of the cornerstones of public health improvements over the past several decades is widespread vaccination against potentially threatening diseases such as tetanus, measles, mumps, rubella, and hepatitis. Of particular note, influenza has caused a number of pandemics as a result of novel strains circulating in the general population, such as the 1918 H1N1 Spanish influenza which caused an estimated 20-50 million deaths worldwide¹. More recently, global influenza infection rates are estimated to include 5-10% of all adults and 20-30% of children annually during a non-epidemic year² amounting to 250,000-500,000 deaths according to a 2014 a World Health Organization (WHO) report³. In an effort to curb these rates, programs to vaccinate a large portion of the population are carried out each year in countries such as the United States, resulting in annual vaccination rates falling between 39.4-42.2% of the population from 2009-2014⁴. However, efficacy of these vaccinations can vary significantly year to year by region, demographics, and time of vaccination⁵-⁷.

Influenza vaccines to counter detrimental effects on public health were introduced in the 1940s, initially including at least one influenza A and B strain⁸. Each vaccine uses a dead or weakened strain of virus to promote the body’s immune response and help protect against an actual infection with little to no risk to the patient⁸. The Centers for Disease Control and Prevention (CDC) provides a recommended vaccination schedule for both children⁹ and adults¹⁰. Included in these schedules is the annual influenza vaccine which comes in two forms: inactivated influenza vaccine (IAV) and live, attenuated influenza vaccine (LAIV)¹¹ and are administered depending on the age and status of the patient. These vaccines are generally trivalent and can protect against three of the most common types of influenza: A/H1N1, A/H3N2, and B. It is recommended that all individuals at least six months old receive a vaccination annually¹² in time for the beginning of the flu season, which falls between weeks 40-20 in the Northern Hemisphere. In preparation for this demand, 150-160 million vaccines are annually produced for use in the United States.

The vaccines are created based off of recommendations by the WHO and are a result of a continuous process analyzing data and viruses that circulated during the previous flu season. There are 130 national influenza centers (NIC) in 101 countries¹³, including four in the United States. These centers are tasked with analyzing clinically-confirmed influenza cases by a variety of genetic and antigenic tests and determining the strains that are the most representative or novel. These strains are then forwarded to one of five WHO Collaborating Centers for consultation and further testing¹³. The additional testing involves inoculation into ferrets for antisera analysis, human serology studies, analysis of antiviral resistance properties, and phylogenetic clade analysis. After taking these results into consideration, primary selection of the H1N1, H3N2, and B viruses are made and initial vaccines are created for further analysis; the H1N1 and H3N2 strains are created by classical reassortment while the wild type B virus is used because there is no growth advantage for its reassortment. These vaccines are used to gather ferret antisera once more to ensure that growth properties and antigenic content are sufficient and that the antigens created are identical to those of the wild type viruses. In the event that the antigenic state or growth properties are insufficient, the WHO may decide to try to utilize all wild type viruses...
or may select entirely new strains. Once sufficient vaccines have been created and demonstrated, the WHO will make its recommendations for the upcoming flu season and these recommendations apply to the entire Northern or Southern Hemisphere; however each country generally has a local agency approve the recommendations or choose different, but antigenically similar, strains. In the United States, final approval is provided by the Food and Drug Administration (FDA). Mass production of vaccines begins after final virus selection is made, usually February through May, in order to have the massive amount of required stockpiles ready for the beginning of the vaccination period in October.

The most important takeaway from the selection and production process is that the strains selected for inclusion in the vaccine reflect highly educated guesses of the strains that will be the most representative in the upcoming flu season. Vaccine effectiveness depends on the actual strains circulating when the flu season arrives, the number of individuals that receive the vaccination, and several other factors. Early estimates of the 2014-2015 flu season in the United States indicate overall vaccine effectiveness at just 23% due to genetically drifted H3N2 strains in circulation. The same vaccine is generally distributed throughout the entire United States, with the exception of some quadrivalent vaccines that protect against an additional influenza B virus, and do not take into account locally circulating viruses in real time during the flu season. This enables phenomena like the current flu season where antigenically divergent strains predominate and reduce efficacy of the vaccine. Global geographic dispersal has been implicated as the main mechanism by which the influenza A virus survives and evolves into antigenically divergent strains between seasons. This demonstrates that geography can be a causal factor toward the prevalent strains during a flu season and led us to explore the use of clinical data from confirmed influenza cases to analyze genetic similarities between the cases and the viruses that were vaccinated against. These types of data may be used to help clinicians decide on treatment options vaccines that may be available to administer to patients in their area, and help governing bodies of public health better determine strains to target for vaccination at a more granular level. For instance, this type of analysis would be useful for physicians at the local level to prescribe drugs potentially more effective at treating patients based on the strain of influenza they are infected with. Additionally, given the availability of a complete data sets encompassing both genetic and clinical characteristics of a flu isolate and its associated host, this would allow tracking of effectiveness of vaccines for particular regions in conjunction with trends in strain resistance factors.

The CDC oversees a federal influenza surveillance program that includes participation from state health agencies. The current initiative includes subtyping, antigenic characterization, and antiviral resistance testing. There is also information on influenza-associated hospitalizations and outpatient influenza-like illness. Beyond Federal reporting, some individual healthcare facilities have begun to share both clinical and viral genetic data with the researcher community. For example, the influenza research database (IRD) contains a relatively new feature for facilities to share clinical data from influenza-related hospitalization as well as the sequences of the virus itself. Currently sixteen distinct facilities are participating including Beth Israel Deaconess Medical Center, Children’s Hospital Boston, the University of Rochester Medical Center, and the National Research Center in Egypt.

In this study, we analyze overall and geographic distribution of genetic similarities between the cases and vaccines and demonstrate the potential of combining clinical and viral genetic data to explore trends of influenza epidemics.

**Methods**

We collected clinical data for influenza cases from the IRD. We used the Human Isolates with Clinical Metadata function and searched for all data from Week 40-20 for each of the 2012-2013 and 2013-2014 flu seasons in the United States. Of the returned results, all categories of data were selected and downloaded. These included: GenBank database accession ID, age of the patient, gender, vaccination status and date, presence of fever and other clinical symptoms, as well as genetic data about the virus such as markers for antiviral resistance (adamantine or oseltamivir). We discarded any record that was not influenza A subtype H1N1, H3N2, or type B. Of the remaining cases, we discarded any record that did not contain a specific strain or sequence accession number. We used the accession IDs in our result set to search GenBank and download the hemagglutinin (HA) sequence for each case. We discarded all records containing a partial sequence and this resulted in the final dataset. In Figure 1, we show a flowchart of the data collection and preprocessing.
In order to compare the sequences with those contained within the vaccine, we collected a strain recommended by the WHO for inclusion in the respective flu seasons in the Northern Hemisphere\textsuperscript{21, 22} from the IRD\textsuperscript{23}. The WHO recommends “like-virus” strains (e.g. A/Brisbane/59/2007(H1N1)-like virus) rather than a wild type strain, rather than specific strains, and the strains vaccinated against may differ between vaccine manufacturers. This makes it impossible to conclude exactly which strains each individual is vaccinated against in a set of deidentified data. For this reason, we collected a random HA sequence from GenBank matching the suggested strains within the IRD\textsuperscript{24}. In Table 1, we describe these strains.

![Flowchart A](image1.png)

**Figure 1.** Flowcharts resulting in the cases we used for analysis in this study. A) 2012-2013 flu season with data representing 81.2% of all cases. B) 2013-2014 flu season with data representing 79.9% of all cases.

<table>
<thead>
<tr>
<th>WHO Recommendation</th>
<th>2012-2013 Flu Season</th>
<th>2013-2014 Flu Season</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>H1N1</td>
<td>H3N2</td>
</tr>
<tr>
<td>A/California/07/2009(H1N1)</td>
<td>CY121680</td>
<td>KJ942680</td>
</tr>
<tr>
<td>A/Victoria/361/2011(H3N2)</td>
<td>CY115480</td>
<td>KJ942680</td>
</tr>
<tr>
<td>B/Wisconsin/01/2010</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strain</td>
<td>H1N1</td>
<td>H3N2</td>
</tr>
<tr>
<td>A/California/07/2009(H1N1)</td>
<td>CY121680</td>
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</tr>
<tr>
<td>A/Victoria/361/2011(H3N2)</td>
<td>CY115480</td>
<td>KJ942680</td>
</tr>
<tr>
<td>B/Massachusetts/02/2012</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.** WHO Recommendations for Composition of Influenza Vaccines in the Northern Hemisphere by flu season and their corresponding GenBank Accession ID. These Accession IDs correspond to the specific HA sequence we used for analysis in this study.

We aligned sequences for each virus subtype from each season with the sequences representing the subtype contained in that year’s vaccine using Geneious Pro v.6.1.6 (Biomatters Ltd., Auckland, New Zealand) and MAFFT v7.017. We utilized the default parameters (Algorithm = auto, Scoring Matrix = 200PAM / k = 2, Gap Open Penalty = 1.53, Offset Value = 0.123). Post-alignment, we measured genetic distance in the number of single nucleotide polymorphisms...
(SNPs) from each sequence to the vaccine and translated the aligned sequences with Geneious to determine the number of nonsynonymous SNPs. We appended these distances to our clinical data file. In order to quantify the impact of geography among the cases, we appended a column in the clinical data file with the United States Department of Health and Human Services (HHS) Region corresponding to the state from which the case was isolated. There are ten HHS Regions\textsuperscript{25} in total representing each of the 50 United States and several territories including American Samoa, Guam, and others.

**Results**
The results we obtained focus on genetic variation among confirmed flu cases and the vaccinated strains as well as a metric of vaccine effectiveness among cases in both the regional and overall scopes. In Figure 2, we show the number of SNPs compared to the strain in which it was vaccinated against.

![Figure 2. The number of post-alignment SNPs in our cleaned set of cases compared to the strain in which it was vaccinated against (Table 1). The blue and orange bars represent the average synonymous and nonsynonymous SNPs per virus per year, respectively, with error bars as standard deviations for n cases, listed above.](image)

After eliminating cases with unknown vaccination status, we determined that 49.2\% and 50.2\% of our cases were vaccinated for the 2012-2013 and 2013-2014 flu seasons, respectively. The lack of control individuals here makes calculation of vaccine effectiveness and efficacy\textsuperscript{26} impossible. We summarize these data in Table 3.

<table>
<thead>
<tr>
<th>Vaccination Status</th>
<th>2012-2013 Flu Season</th>
<th>2013-2014 Flu Season</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H1N1</td>
<td>H3N2</td>
</tr>
<tr>
<td>Yes</td>
<td>44.0%</td>
<td>24.6%</td>
</tr>
<tr>
<td>No</td>
<td>28.0%</td>
<td>22.7%</td>
</tr>
<tr>
<td>Unknown</td>
<td>28.0%</td>
<td>52.7%</td>
</tr>
<tr>
<td>C</td>
<td>VS+,Y</td>
<td>61.1%</td>
</tr>
</tbody>
</table>

Table 2. Overall vaccination rates among analyzed cases. Unknown includes cases with unknown or missing statuses. C|VS+,Y can be read as “cases with a vaccination status of yes given a known vaccination status”.

Also of interest was the variation of vaccination status among the HHS Regions in the United States in order to capture
geographic trends and compare them with genetic features. We summarize the vaccination statuses of our cases by region and year in Table 3. We visualize these data in Figures 3 and 4 by showing the average number of SNPs per case, separated by strain, for each region. Background color of the regions indicates the C\(VS^+,Y\) column shown in Table 3.

| Region | 2012-2013 Flu Season | | 2013-2014 Flu Season |
|--------|----------------------|----------------------|
|        | Cases | Yes | No | UN | C\(VS^+,Y\) | Cases | Yes | No | UN | C\(VS^+,Y\) |
| 1      | 127   | 45  | 52 | 30 | 46.4%     | 5     | 4   | 1  | 0  | 80.0% |
| 2      | 141   | 44  | 26 | 71 | 62.9%     | 9     | 3   | 0  | 6  | 100.0%|
| 3      | 31    | 10  | 7  | 14 | 58.8%     | 5     | 3   | 2  | 0  | 60.0% |
| 4      | 89    | 10  | 7  | 58 | 32.3%     | 46    | 20  | 25 | 1  | 44.4% |
| 5      | 23    | 3   | 9  | 11 | 25.0%     | 5     | 1   | 4  | 0  | 20.0% |
| 6      | 43    | 9   | 15 | 19 | 37.5%     | 71    | 37  | 29 | 5  | 56.1% |
| 7      | 5     | 2   | 0  | 3  | 100.0%    | 3     | 1   | 2  | 0  | 33.3% |
| 8      | 22    | 3   | 2  | 17 | 60.0%     | 51    | 26  | 25 | 0  | 51.0% |
| 9      | 28    | 9   | 6  | 13 | 60.0%     | 42    | 19  | 21 | 2  | 47.5% |
| 10     | 19    | 3   | 4  | 12 | 42.9%     | 13    | 4   | 8  | 1  | 33.3% |

Table 3. Summary statistics of vaccination status of each patient by HHS Region. UN refers to individuals with a status of “unknown” or “N/A”. C\(VS^+,Y\) is the proportion of cases where the individual was vaccinated to the number of cases with a known vaccination status, expressed as a percent (previously defined in Table 2).

Figure 3. 2012-2013 flu season by HHS Region, indicated in boldface. Background color is represented by column C\(VS^+,Y\) in Table 3 and genetic variation is represented in number of SNPs compared to strain vaccinated against.
Influenza B and H3N2 consisted of the bulk of cases for the 2012-2013 flu season. Region 1 had the most cases for H3N2 with \( n = 126 \) and showed an average variation of \( 20.4 \pm 4.2 \) SNPs (51% nonsynonymous), while variation was largest in Region 5 with \( 28.7 \pm 7.4 \) SNPs (45% nonsynonymous). For influenza B, the most cases and most variation, excluding Region 7 that had just two cases, was seen in Region 4, with an average of \( 96.3 \pm 73.6 \) SNPs (23% nonsynonymous) for \( n = 28 \) cases and smallest in Region 6 with \( n = 27 \) cases and an average variation of \( 50.7 \pm 39.3 \) SNPs (25% nonsynonymous). Genetic variation from the H1N1 strain vaccinated against was largest in Region 2 with \( n = 5 \) cases and an average of \( 29.6 \pm 0.9 \) SNPs (41% nonsynonymous) and was smallest in Region 3 with an average of \( 25.7 \pm 1.2 \) SNPs (47% nonsynonymous) in \( n = 3 \) cases. During this flu season the overall amount of nonsynonymous mutations for H1N1, H3N2, and influenza B were 46%, 49%, and 24%, respectively.

**Figure 4.** 2013-2014 flu season by HHS Region, indicated in boldface. Background color is represented by column C|VS+,Y in Table 3 and genetic variation is represented in number of SNPs compared to strain vaccinated against.

H1N1 was the predominant strain in our 2013-2014 flu season dataset. The most cases were in Region 6, with \( n = 66 \) cases and an average of \( 29.8 \pm 2.2 \) SNPs (44% nonsynonymous) from the strain vaccinated against. Variation peaked for H1N1 in Region 3 with \( n = 4 \) cases and an average of \( 30.8 \pm 2.8 \) SNPs and was smallest in Region 4 with an average of \( 28.8 \pm 2.1 \) SNPs in \( n = 42 \) cases. Only Regions 3, 4, and 6 had any H3N2 cases, with \( n = 1 \), \( n = 2 \), and \( n = 3 \), respectively and only Regions 4, 6, and 9 had any influenza B cases with \( n = 2 \), \( n = 2 \), and \( n = 5 \), respectively. The average variation in Region 9 for influenza B was, on average, \( 153.2 \pm 58.8 \) SNPs, 21% of which were nonsynonymous. During this flu season the overall amount of nonsynonymous mutations for H1N1, H3N2, and influenza B were 45%, 53%, and 21%, respectively.

In addition to the HA gene, some clinical records also contained GenBank accession IDs for each gene in the genome of the influenza specimen that infected the patient. For the 2012-2013 flu season, 134 of the 470 records contained an accession ID for the neuraminidase (NA) gene, another surface protein on influenza viruses in addition to HA. Three
of these records were from H1N1 cases, 129 were from H3N2 cases, and two were from influenza B cases. We also compared the NA strains with the corresponding NA reference sequences: NC_026434.1, KJ942682, and CY115185 for H1N1, H3N2, and B, respectively. Overall, H1N1 showed an average of 14.0 ± 1.0 SNPs (40% nonsynonymous), H3N2 showed 11.0 ± 3.9 SNPs (26% nonsynonymous), and influenza B showed 28.5 ± 2.1 SNPs (39% nonsynonymous) from their respective reference sequences. We did not further analyze the NA gene by HHS Region due to the limited samples.

Discussion

Our analysis has implications for a number topics that overlap from both the public and clinical health perspective. For example, understanding trends in vaccination efficacy based on strain, emerging drug resistance, and patient drug response given their infection type are a few of many possible applications. However, making such inferences remain difficult given the lack of integration between clinical and viral genetic data. As seen in Figure 2, the genetic variation between cases and the strains vaccinated against was highest in the 2012-2013 influenza B cases and the 2013-2014 influenza A/H3N2 cases; however only seven cases exist for the latter, making the former, with 132 cases, a far more interesting sample. In Figure 3, we can see that influenza B variation is high per HHS Region (Region 1 had no influenza B cases for that season), which is consistent with the overall trend in high variation. Genetic variation among 2012-2013 A/H3N2 and 2013-2014 A/H1N1 cases was quite small, surprising given the large number of cases per year for those strains (n = 313 and n = 225, respectively). Unfortunately, 52.3% of all cases for the former (Table 2) had an unknown vaccination status, making it difficult to match a trend to these data. Reporting of vaccination status was greatly improved between the 2012-2013 and 2013-2014 flu seasons, with the percentage of unknown cases falling from 40.4% to just 6% between the seasons.

This improved reporting of vaccination status made it possible to reliably compare the statuses with genetic variation for the 2013-2014 season. As seen in Figure 4, the H1N1 virus has variation in the northern regions than in the southern ones, while no type B cases are observed in the northern regions, although data was sparse for this and the H3N2 viruses (Figure 1). Unfortunately, it is difficult to compare trends between the two seasons we analyzed because the reported number of cases per virus per year decreased drastically for H3N2 and B but increased drastically for H1N1, as seen in Figure 1. Regardless, the visualization of these trends indicate that perhaps a bold step such as regionalization of vaccines to better capture and protect against representative virus strains in circulation may prove beneficial to millions of individuals. We will consider this for future work, perhaps culminated by a meta-analysis of vaccination efforts, as completed by Osterholm et al.\textsuperscript{16}. This could be combined with a viral genetic analysis like we have shown here.

There are several notable amino acid changes in the HA gene that have shown to enhance transmissibility of H1N1 viruses, including the simultaneous 222G and 163E mutations\textsuperscript{27} as well as an independent 219K mutation\textsuperscript{28}. None of these mutations were found in the samples from either flu season analyzed. A genetic motif (RERRRKKR) that has been linked to HA cleavage and a potential target for drugs against H5N1 influenza\textsuperscript{29} was an available column in the clinical metadata file but was generally left blank. No sequence was found to contain this motif, perhaps an indication that it is conserved within H5 viral clades. The analysis of the NA gene was also critical because the main pharmaceutical treatment of influenza, oseltamivir, is a neuraminidase inhibitor and several mutations in this gene have been shown to cause oseltamivir resistance, including 119V, 292K, and 274Y\textsuperscript{30}. None of these mutations were observed in any of the 129 NA sequences. According to the CDC, oseltamivir resistance was seen in 0.7% (n = 687), 0.2% (n = 2,440), and 0.0% (n = 1,044) of tested H1N1, H3N2, and influenza B samples tested during the 2012-2013 flu season\textsuperscript{13}. It is possible that our lack of similar findings is simply a result of the drastically smaller sample size.

The authors recognize several limitations of this study. These include the lack of control data that could have been used to measure true efficacy and effectiveness of the vaccines rather than our C|VS+,Y representation. This could have led to more confident assessments of potential regionalization of vaccine production. It is unknown whether control cases were intentionally excluded from these data sources or there were simply no control cases to report (i.e. no negative tests for influenza at the reporting clinics). Furthermore, the clinical data we obtained and used via IRD was hardly complete and many columns were entirely devoid of data. Examples of columns that were generally blank.
included transmission properties such as “Increased Virulence” and “Enhanced Transmission to Human”, and pre-
visit and post-visit medications prescribed to the patients. While we were able to gather useful information from the
columns where data was entered, it shows that we were only able to represent a small portion of the potential of these
data. Having information about virus properties, particularly that of resistance, could have enabled us to
geographically visualize these trends as well, similar to Figures 3 and 4, but with perhaps more significance due to the
ever present concerns over enhanced mutation and transmissibility. Other limitations include the aforementioned lack
of specific information regarding vaccine contents, forcing the use of a random HA sample for each of the strains
vaccinated against. Data regarding where the patients were vaccinated and the specific vaccine that they received
would have been invaluable for geographic accuracy and reliability of this study. Patient symptoms were listed for
nearly every case, but we did not explore possible ways to capture severity of the cases based on these symptoms.

An innovative combination of viral genetic data with clinical records would certainly fall under the umbrella of the
Precision Medicine Initiative32, especially in relation to resistance of oseltamivir and other antiviral drugs. If these
clinical records were outfitted with human genomic data as well as the viral sequences, it could highlight biomarkers,
genes, and SNPs associated with susceptibility to antiviral resistance. This would not strictly be limited to influenza
surveillance and could easily apply to concerning clinical bacterial infections like methicillin-resistant Staphylococcus
aureus (MRSA) or emerging zoonotic pathogens like Middle East respiratory syndrome coronavirus (MERS-CoV).
While this paper constituted a review of one application of linking clinical records with genetics of an infectious agent,
the potential for an innovative informatics pipeline to identify medication-resistant motifs and associate them with
clinical symptoms, human genomic markers, and geography is a challenge yet to be adequately addressed. In order
for this to occur, however, it appears that more clinics would need to be willing to begin outfitting their records with
links to pathogen DNA.

In this study, we demonstrate the value of integrating clinical data with viral genetics. While most clinical
microbiology laboratories do not have the financial or personnel resources to generate full genomic viral sequences
for each clinical case, our work highlights the usefulness of how even a sample of cases can provide valuable insight
into geographic disparities to seasonal epidemics and vaccination effectiveness. While our geographic representations
are not unlike FluView by the CDC33, we have shown that individual clinics or research labs could create such maps
in real time as sequences become available. Clinics should be interested in these types of reports because data
demonstrating a differentiated diffusion of strains with particular mutations that are indicated for antiviral resistance
could inform physicians of optimal treatments. Furthermore, these data could show expected trends in viral properties
among patients entering the health centers with acute respiratory illnesses, increasing efficacy of care and reducing
healthcare costs to both the individuals and clinics. While ultimately the integration of human genomic data into health
records remains the ideal gold standard, accomplishing an additional step like the incorporation of virulence could
prove to be a valuable resource for reducing morbidity and mortality and improving overall public health and
biosurveillance of infectious diseases.

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