

Vaccination has minimal impact on the intrahost diversity of H3N2 influenza viruses

Running Title: Vaccination and influenza diversity

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1 **Abstract**

2 While influenza virus diversity and antigenic drift have been well characterized on a global scale,
3 the factors that influence the virus' rapid evolution within and between human hosts are less
4 clear. Given the modest effectiveness of seasonal vaccination, vaccine-induced antibody
5 responses could serve as a potent selective pressure for novel influenza variants at the
6 individual or community level. We used next generation sequencing of patient-derived viruses
7 from a randomized, placebo-controlled trial of vaccine efficacy to characterize the diversity of
8 influenza A virus and to define the impact of vaccine-induced immunity on within-host
9 populations. Importantly, this study design allowed us to isolate the impact of vaccination while
10 still studying natural infection. We used pre-season hemagglutination inhibition and
11 neuraminidase inhibition titers to quantify vaccine-induced immunity directly and to assess its
12 impact on intrahost populations. We identified 166 cases of H3N2 influenza over 3 seasons and
13 5119 person-years. We obtained whole genome sequence data for 119 samples and used a
14 stringent and empirically validated analysis pipeline to identify intrahost single nucleotide
15 variants at $\geq 1\%$ frequency. Phylogenetic analysis of consensus hemagglutinin and
16 neuraminidase sequences showed no stratification by pre-season HAI and NAI titer,
17 respectively. In our study population, we found that the vast majority of intrahost single
18 nucleotide variants were rare and that very few were found in more than one individual. Most
19 samples had fewer than 15 single nucleotide variants across the entire genome, and the level of
20 diversity did not significantly vary with day of sampling, vaccination status, or pre-season
21 antibody titer. Contrary to what has been suggested in experimental systems, our data indicate
22 that seasonal influenza vaccination has little impact on intrahost diversity in natural infection and
23 that vaccine-induced immunity may be only a minor contributor to antigenic drift at local scales.

24

25 Key Words – influenza, vaccine, next generation sequencing, diversity, evolution

26

27 **Introduction**

28 Despite recommendations for universal influenza vaccination and the ample availability of
29 vaccines in the United States, influenza continues to cause significant morbidity and mortality [1].
30 This is, in part, a result of the modest effectiveness of current vaccines, so that considerable
31 numbers of vaccine failures occur each year. Within individuals, influenza populations exist as a
32 collection of closely related, and at times antigenically distinct, variants that may exhibit diverse
33 phenotypes [2-6]. Intra-host single nucleotide variants (iSNV) can be transmitted as part of the
34 infecting population [5,7-10] or generated over the course of an infection due to the virus' low
35 replication fidelity [11,12]. The evolutionary forces that shape the genetic structure of viral
36 populations within hosts and ultimately give rise to novel antigenic variants at the host
37 population level are poorly understood. A clear understanding of the intra-host diversity of
38 influenza virus populations and its impact on influenza virus evolution is central to many
39 questions of direct clinical and public health relevance [13].

40
41 Influenza vaccines are considered for reformulation each year to counter the viral antigenic drift
42 that enables escape from the previous year's vaccine [14]. Annual influenza vaccine
43 effectiveness is 60% on average, and can be much lower during antigenically unmatched years
44 [15,16]. While antigenic drift is monitored annually on a global scale, the source of antigenic
45 variation is ultimately at the level of the individual host. Phylogenetic studies of whole genome
46 sequences from cities and smaller communities have demonstrated that multiple lineages
47 circulate over the course of a single influenza season [2,17], and individual hosts may harbor
48 mixed infections that include antigenically novel variants [3,4]. While human hosts could be
49 preferentially infected with one lineage over another based on pre-infection immune status, the
50 degree to which circulating escape variants contribute to vaccine failure is currently unknown.

51

52 Host immune selection is a major driver of influenza virus evolution on the global scale. Both
53 phylogenetic analysis and antigenic cartography have demonstrated that antibodies exert
54 positive selective pressure on the viral hemagglutinin (HA) and neuraminidase (NA) proteins
55 (23). Vaccination and natural influenza infection often lead to partial, or non-sterilizing immunity,
56 and post-vaccination antibody titers are only a moderate predictor of subsequent protection
57 [18,19]. Previous work has demonstrated that sub-neutralizing concentrations of immune sera
58 can promote the generation of antigenic variants, and some have suggested that vaccination
59 can accelerate the process of antigenic drift [20]. A recent study in vaccinated people suggested
60 that novel antigenic variants could be present at low frequencies [21]. Importantly, humans often
61 differ in their prior exposure to influenza viruses and vaccines, and pre-existing immunity may
62 confound such studies [22]. Therefore, the extent to which partial immunity selects for
63 antigenically relevant variants during natural infection in humans is unclear.

64
65 By necessity, most of the available data on vaccination and intrahost evolution have come from
66 analyses of HA sequences in large animal models of infection, including horses, pigs, and dogs.
67 These studies have suggested that intrahost populations include a number of somewhat rare
68 single nucleotide variants that increase and decrease in frequency over the course an infection
69 with sporadic fixation events occurring in some animals. The overall impact of vaccination on
70 antigenic diversification was not clear [7,23-25]. Furthermore, some studies found distinct
71 differences in population structure and diversity between experimentally and naturally infected
72 animals, perhaps due to differences in the infecting strain, the size of the inoculum, and
73 background immunity [7,8,24]. These differences are not unique to animal models and are likely
74 to be an issue in extrapolating results from human experimental challenge models to natural
75 infection [26].

76

77 Some have suggested that high intrahost diversity reflects increased viral fitness, and
78 mechanisms that alter intrahost diversity may impact evolutionary trajectories [27-29]. If the
79 transmission bottleneck is sufficiently wide, low frequency variants that arise within a host can
80 plausibly be transmitted and spread through host populations [5,10]. Work in animal models and
81 at least one study in humans support the existence of a loose transmission bottleneck [5,8,24].
82 Bottleneck size appears to be smaller in vaccinated animals [25], and vaccinated pigs and
83 ferrets shed less virus than immunologically naïve animals [24,30]. Understanding how intrahost
84 diversity is generated and maintained and the extent to which host immune status impacts this
85 diversity may be important for defining influenza virus' larger evolutionary patterns [21,24,25].

86
87 Here we used next generation sequencing to define the impact of vaccine-induced immunity on
88 the intrahost diversity of influenza virus during natural infection. We specifically asked: (i)
89 whether influenza viruses in vaccinated individuals represent escape variants, (ii) whether non-
90 sterilizing antibody responses select for novel antigenic variants within hosts, and (iii) the
91 degree to which vaccine-induced immunity impacts the overall diversity of intrahost populations.
92 Because we analyzed influenza populations from individuals enrolled in a randomized, double-
93 blind, placebo-controlled trial of influenza vaccine efficacy [31-33], we were uniquely positioned
94 to define this aspect of immunity to natural infection.

95

96 **Methods**

97 *Subjects and specimens*

98 We characterized host-derived influenza populations archived from a randomized, double-blind,
99 placebo-controlled, clinical trial of influenza vaccine efficacy that ran from the 2004-2005
100 through the 2007-2008 influenza seasons (ClinicalTrials.gov number, NCT00133523, [31-33]).
101 Each year, healthy adults, ages 18-49, were randomized to receive trivalent inactivated
102 influenza vaccine (IIV), live attenuated influenza vaccine (LAIV), or placebo. The study was

103 approved by the Institutional Review Board of the University of Michigan Medical School, and all
104 human subjects provided informed consent. Throat swab specimens were collected from
105 individuals with influenza-like illness within 7 days of onset; residual specimen material was
106 stored in veal infusion broth (VIB) at -80°C. Viral RNA was extracted from 140µl of VIB using the
107 QIAamp viral RNA mini kit (Qiagen 52906), eluted in 50µl buffer, and stored at -80°C.
108 Hemagglutination inhibition (HAI) and Neuraminidase agglutination inhibition (NAI) titers for
109 subjects in this study were previously measured and reported in [18,34].

110

111 *Determination of genome copy number*

112 Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was performed on 5µl
113 RNA from each sample using CDC RT-PCR primers InfA Forward, InfA Reverse, and InfA
114 probe, which bind to a portion of the influenza M gene (CDC protocol, 28 April 2009). Each
115 reaction contained 5.4µl nuclease-free water, 0.5µl each primer/probe, 0.5µl SuperScript III
116 RT/Platinum Taq mix (Invitrogen 111732) 12.5µl PCR Master Mix, 0.1µl ROX, 5µl RNA. The
117 PCR master mix was thawed and stored at 4°C, 24 hours before reaction set-up. A standard
118 curve relating copy number to Ct values was generated based on 10-fold dilutions of a control
119 plasmid run in duplicate.

120

121 *Illumina library preparation and sequencing*

122 We amplified cDNA corresponding to all 8 genomic segments from 3µl of the viral RNA using
123 the SuperScript III One-Step RT-PCR Platinum Taq HiFi Kit (Invitrogen 12574). Reactions
124 consisted of 0.5µl Superscript III Platinum Taq Mix, 12.5µl 2x reaction buffer, 8µl DEPC water,
125 and 0.2µl of 10µM Uni12/Inf1, 0.3µl of 10µM Uni12/Inf3, and 0.5µl of 10µM Uni13/Inf1 universal
126 influenza A primers [35]. The thermocycler protocol was: 42°C for 60 min then 94°C for 2 min
127 then 5 cycles of 94°C for 30 sec, 44°C for 30 sec, 68°C for 3 min, then 28 cycles of 94°C for 30

128 sec, 57°C for 30 sec, 68°C for 3 min. Amplification of all 8 segments was confirmed by gel
129 electrophoresis, and 750ng of each cDNA mixture were sheared to an average size of 300 to
130 400bp using a Covaris S220 focused ultrasonicator. Sequencing libraries were prepared using
131 the NEBNext Ultra DNA library prep kit (NEB E7370L), Agencourt AMPure XP beads (Beckman
132 Coulter A63881), and NEBNext multiplex oligonucleotides for Illumina (NEB E7600S). The final
133 concentration of each barcoded library was determined by Quanti PicoGreen dsDNA
134 quantification (ThermoFisher Scientific), and equal nanomolar concentrations were pooled.
135 Residual primer dimers were removed by gel isolation of a 300-500bp band, which was purified
136 using a GeneJet Gel Extraction Kit (ThermoFisher Scientific). Purified library pools were
137 sequenced on an Illumina HiSeq 2500 with 2x125 nucleotide paired end reads. All raw
138 sequence data have been deposited at the NCBI sequence read archive (BioProject submission
139 ID: SUB1907046)

140

141 *Variant detection*

142 Sequencing reads that passed standard Illumina quality control filters were binned by index and
143 aligned to the reference genome using Bowtie [36]. Single nucleotide variants (SNV) were
144 identified and analyzed using DeepSNV [37], which relies on a clonal control to estimate the
145 local error rate within a given sequence context and to identify strand bias in base calling. The
146 clonal control was a library prepared in an identical fashion from 8 plasmids containing the
147 genome for the respective circulating reference strain and sequenced in the same flow cell to
148 control for batch effects. True positive SNV were identified from the raw output tables by
149 applying the following filtering criteria in R: (i) Bonferonni corrected p value <0.01, (ii) average
150 MapQ score on variant reads >30, (iii) average phred score on variant positions >35, (iv)
151 average position of variant call on a read >32 and <94, (v) variant frequency >0.01. We only
152 considered SNV identified in a single RT-PCR reaction and sequencing library for samples with
153 copy number $\geq 10^5$ genomes/ μ l transport media or in two separate RT-PCR reactions and

154 sequencing libraries for samples with copy number 10^3 - 10^5 genomes per μl . Our strategy for
155 variant calling is described in [6] and all code can be found at
156 https://github.com/lauringlab/variant_pipeline.

157
158 *Phylogenetic analysis*
159 Consensus nucleotide sequences for the HA and NA proteins were aligned using MUSCLE [38].
160 The best-fit models for nucleotide substitution was identified using jModelTest v2.1.10 [39].
161 Maximum likelihood phylogenetic trees were generated using RAxML v8 [40] with a
162 GTRGAMMA model, Genbank sequences for vaccine strains as outgroups, and 1000
163 bootstraps. Trees were visualized and annotated using FigTree (v1.4.2).

164
165 *Data analysis and statistics*
166 All statistical analyses were performed using Prism 6 and R. Description of the analysis and
167 annotated code are available at https://github.com/lauringlab/fluvsacs_paper. HA structural
168 models were generated and visualized with PyMol.

169
170 **Results**
171 *Study subjects and specimens*
172 We utilized influenza A positive samples from a randomized, double-blind, placebo-controlled
173 study of vaccine efficacy that took place during the 2004-2008 influenza seasons at six study
174 sites in Michigan. This trial measured vaccine efficacy of both the trivalent inactivated (IIV) and
175 live attenuated influenza vaccine (LAIV) compared to placebo and each other. We sequenced
176 patient-derived influenza populations without culturing from three seasons: 2004-2005, 2005-
177 2006, and 2007-2008. The 2006-2007 influenza season did not have enough influenza-positive
178 samples for our study (total $n=16$). Influenza A (H3N2) strains dominated the other three
179 seasons, and the circulating 2004-2005 virus was considered at the time to be only a modest

180 mismatch with the vaccine strain. The other seasons were antigenically matched. The number
181 of subjects each year was as follows: 2004-2005 season, 522 IIV, 519 LAIV, and 206 placebo
182 [31]; 2005-2006 season, 867 IIV, 853 LAIV, and 338 placebo [32]; 2007-2008 season, 813 IIV,
183 814 LAIV, and 325 placebo [33]. Over these 5119 person-years of observation, 165 individuals
184 had culture or RT-PCR confirmed influenza A (H3N2) infection and specimens available for
185 analysis. Of these, 80 individuals had received LAIV, 42 had received IIV, and 43 had received
186 placebo. For 2004-2005, flu-positive samples were available for 28 subjects: 7 IIV, 12 LAIV, and
187 9 placebo, and for 2005-2006, 32 samples were available to study: 13 IIV, 14 LAIV, and 5
188 placebo. During the 2007-2008 season, 105 flu-positive samples were available: 22 IIV, 54 LAIV,
189 and 29 placebo. We were able to amplify and quantify genomes for 119 of the 165 influenza-
190 positive samples (Table 1). The average age of this sequenced cohort from all years was 24.5,
191 indicating that participants were generally young and likely shared similar influenza pre-
192 exposure histories particularly after randomization. The age, sex and race of the cohort were
193 similar to that of the overall study cohort for each of the 3 seasons.

194
195 In the 2007-2008 season, roughly 40% of individuals in the larger cohort reported having ever
196 received a prior influenza vaccination. Despite subject randomization, differences in pre-existing
197 immunity due to prior vaccination or influenza infection could impact results. To control for this
198 possibility, we obtained pre-season antibody titers by hemagglutination inhibition (HAI) and
199 neuraminidase inhibition (NAI) assays for all study participants against that season's vaccine
200 strain (Supplementary Figure 1). Vaccine-induced antibody titers and overall vaccine efficacy
201 are generally stable for a single flu season [41]. Therefore, our pre-season HAI and NAI titers
202 are likely to be similar to titers at the time of infection. Pre-season (post-vaccination) titers for
203 individuals in the IIV group were above the geometric mean for the entire sequenced cohort,
204 LAIV subjects had titers spanning the mean, and those in the placebo group were generally
205 below the mean for all seasons. These data demonstrate that in the IIV group, and to a lesser

206 degree, the LAIV group, individuals had strain-specific antibody levels sufficient to apply
207 selective pressure against the infecting virus.

208

209 *Viral load across groups*

210 We have previously shown that viral load influences the sensitivity and specificity of iSNV
211 detection [6]. In order to determine whether viral load was different among the IIV, LAIV, and
212 placebo samples that we sequenced, we measured genome copy number by RT-qPCR for the
213 2004-2005, 2005-2006, and 2007-2008 seasons. For the 2007-2008 season, which had the
214 most samples, there were no significant differences in copy number by vaccination group
215 (Figure 1A). In agreement with the 2007-2008 data, we did not detect differences in copy
216 number by vaccination group for the 2004-2005 and 2005-2006 seasons (Supplementary Figure
217 2). Since copy number is dependent on time from illness onset [42,43], we analyzed the data
218 based on sample collection day (Figure 1B). Using days 2-4, for which there were at least 5
219 data points for each treatment group, we did not find any significant differences ($p=0.24-0.57$ for
220 days 2-4, non-parametric one way ANOVA). We divided the larger group of 2007-2008 subjects
221 into groups based on pre-season HAI and NAI titers ≥ 40 or < 40 against that season's strain, as
222 an HAI titer of 40 is typically considered to be associated with 50% protection given exposure
223 [18,19,44]. This cutoff was identical to the HAI and NAI geometric mean titers for our sequenced
224 cohort (61.9 and 34.5, respectively), given the dilutions used. We did not detect differences in
225 copy number based on HAI or NAI titer (Figure 1C-D), even when accounting for day of
226 symptom onset ($p=0.25$ for HAI, $p=0.97$ for NAI, Mann-Whitney U test). Because we only
227 measured copy number in the subset of virus populations that were amplified and sequenced,
228 these data should not be interpreted in the context of vaccination and overall shedding [45].

229

230 *Deep sequencing of intrahost influenza populations*

231 We used the Illumina platform to determine the whole genome consensus sequence and to
232 identify intrahost single nucleotide variants for each patient-derived sample. Importantly, we
233 have developed and rigorously benchmarked a variant calling pipeline that maintains high
234 sensitivity for rare iSNV detection while dramatically reducing false positive variant calls [6]
235 (Supplementary Table 1). We have found that the number of false positive iSNV calls is much
236 higher in samples with genome copy numbers $<10^3$ per μl of transport media; therefore, we only
237 report iSNV from a high quality dataset that includes 64 samples from the 2007-2008 season.
238 High quality iSNV data for the 2004-2005 and 2005-2006 influenza seasons are included in
239 Supplementary Figures 4, 6, and Supplementary Table 2. Our libraries yielded an average
240 coverage above 20,000 reads per base with even coverage across the coding region of all
241 segments for each season (Supplementary Figure 3).

242

243 *HA and NA sequences do not cluster by vaccination status or pre-season antibody titer*

244 Given the frequent observation of community-level diversity in circulating influenza viruses
245 [2,17], we asked whether vaccinated individuals were infected with distinct strains relative to the
246 placebo group. If vaccine failures were due to infection with an antigenically distinct variant, we
247 would expect to see evidence of clustering by vaccination- or sero-status in HA and NA
248 phylogenetic trees. We therefore analyzed HA and NA consensus sequences from all 87
249 individuals in the 2007-2008 season (Figure 2). There was very little diversity in either gene, and
250 we found that sequences from individuals in each treatment group (e.g. IIV, LAIV, or placebo)
251 were dispersed throughout the tree. More importantly, we found no evidence for clustering
252 based on pre-season HAI and NAI titer (by colors in Figure 2). We obtained similar results from
253 the 2004-2005 and 2005-2006 seasons, albeit with fewer sequenced samples (Supplementary
254 Figure 4). These data suggest that within-season and within-host antigenic drift due to high
255 levels of vaccine-induced antibodies are not major determinants of vaccine failure.

256

257 *Intrahost diversity in vaccinated and unvaccinated individuals*

258 We next analyzed the iSNVs present in 64 of our samples from the 2007-2008 season. We
259 identified 369 minority variants across the entire genome, most of which were present at a
260 frequency of <0.1 (Figure 3A). The vast majority of iSNV were only found once (Figure 3B). We
261 also evaluated whether partial immunity impacts viral diversity by comparing the number of
262 iSNVs per sample in the HA and NA genes based on HAI and NAI titers, respectively. We did
263 not observe a difference in iSNV count based on HAI and NAI titers for either HA or NA (Figure
264 3C-D, $p=0.20$ for HA, $p=0.26$ for NA, Mann Whitney U test). The average number of iSNV per
265 sample was similar across the genome regardless of host treatment group (Table 2, $p=0.38$,
266 non-parametric one way ANOVA) or HAI and NAI titers (Supplementary Figure 5, $p=0.12$ for
267 HAI, $p=0.22$ for NAI, Mann Whitney U test).

268
269 Most iSNV from the 2004-2005 and 2005-2006 seasons were also found in only one sample
270 each and at frequencies <0.1 (Supplementary Figure 6). The number of iSNV did not differ
271 based on treatment group or HAI and NAI titers (Supplementary Table 2). We did not identify
272 any variants specifically associated with vaccination group or HAI and NAI titers for any of the
273 influenza seasons analyzed. Unlike experimental challenge studies, we only had one sample
274 per person and could not evaluate changes in diversity at the level of the individual host.
275 However, we did not identify any significant differences in diversity by day of infection across the
276 cohort by vaccination status (Figure 4, $p=0.15-0.79$ for days 2-4, non-parametric one way
277 ANOVA) or antibody titer. We identified only a marginal difference in the number iSNV across
278 the genome based on HAI titer on day 2 (uncorrected p -value 0.02 with >6 comparisons, Mann
279 Whitney U test). These data suggest that our results are unlikely to be confounded by temporal
280 sampling issues.

281

282 *Evidence for low antigenic diversity within hosts*

283 Some have suggested that vaccine-induced immunity will select for novel antigenic variants
284 within hosts [21]. We therefore compared iSNV in the HA gene by vaccination group and
285 serostatus. Of the 17 variants we identified that resulted in nonsynonymous changes within HA,
286 11 were in HA1 and 6 were in HA2 (Figure 5, Supplementary Table 3). Five antigenic sites,
287 comprising 131 amino acid positions, have been described in HA1 for H3N2 viruses [46-48]. Six
288 of the HA1 variants identified in our study were located in antigenic sites C and D (Figure 5B),
289 one of which was found in two samples (Supplementary Table 3). Of these potential antigenic
290 variants, three were found in vaccinated individuals (1 IIV, 2 LAIV) and four were found in those
291 in the placebo group. When grouped by HAI titer, four were found in samples from individuals
292 with titers ≥ 40 , while three were found in individuals with titers < 40 . No variants were specific to
293 vaccinated individuals, and antigenic diversity was similar across all groups. None of the
294 identified mutations were observed in subsequent circulating H3N2 strains.

295

296 **Discussion**

297 We set out to define the relationship between vaccine-induced immunity and the intrahost
298 diversity of influenza virus. We hypothesized that non-sterilizing immunity could potentially
299 select for novel antigenic variants and contribute to larger scale patterns of influenza evolution.
300 We were able to definitively address these questions using samples derived from a randomized,
301 placebo-controlled vaccine trial in healthy, young adults who likely had similar prior exposure to
302 influenza viruses [31,33]. The availability of post-vaccination, preseason HAI and NAI titers
303 allowed us to examine directly the impact of measured serologic antibody pressure rather than
304 using vaccination alone as a surrogate marker. Because all individuals were infected naturally,
305 our data provide a rare view of within-host influenza virus diversity in humans. We directly
306 sequenced the samples without passage in cell culture, eliminating the possibility of culture-
307 adapted mutations and employed a well-benchmarked variant calling pipeline [6] that
308 dramatically reduces the false positive iSNV calls that often plague next generation sequencing

309 studies. In this exhaustive and well-controlled study, we found no differences in intrahost
310 influenza diversity based on vaccination status or HAI and NAI titers.

311
312 Our findings in a natural infection system are concordant with an equine influenza virus
313 evolution study in vaccinated horses. Intrahost variation was similar between naïve and
314 vaccinated horses, regardless of whether they were infected naturally or experimentally [25].
315 However, not all experimental infection studies mirror results seen during natural infection. A
316 study investigating swine influenza virus found discrepancies in intrahost variation based on
317 whether animals encountered natural infection or were experimentally infected [24]. Our data is
318 in contrast with a study of experimentally-infected dogs that uncovered differences in intrahost
319 diversity and evolution in antigenic sites based on vaccination status [23]. Two other studies of
320 equine influenza virus found mixed infections of multiple influenza lineages during natural
321 infection, which would not be seen in an experimental model but may be relevant to the
322 transmission and spread of novel variants [7,8].

323
324 We did not detect phylogenetic clustering of HA and NA sequences based on vaccination status,
325 type of administered vaccine, or pre-season HAI and NAI titers. These results are consistent
326 with those of Dinis et al., who found no segregation of HA sequences based on vaccination
327 status in a case test-negative study of vaccine effectiveness [21]. Together, these data suggest
328 that vaccinated and unvaccinated individuals are infected with similar strains and that within
329 season antigenic drift is not a major contributor to reduced vaccine efficacy. Because we also
330 stratified our analysis by pre-season HAI and NAI titer, we can similarly exclude viral escape
331 from a non-sterilizing antibody response. Our data further suggest that pre-existing antibody
332 against circulating strains does not apply sufficiently strong selective pressure to drive the
333 emergence of antigenically distinct strains within a given host.

334

335 High intrahost diversity may be an important factor in viral evolution, since it increases the
336 number of novel variants on which natural selection can act. Some have proposed that the
337 intrahost diversity of RNA viruses is linked to virulence [27,28], suggesting that processes that
338 act to restrict or enhance intrahost diversity may alter disease phenotypes. We found that within
339 host diversity of influenza virus was quite low. Most iSNV were present at frequencies of less
340 than 0.1, which means they would only plausibly be spread between hosts if the transmission
341 bottleneck were reasonably large [5]. The number of iSNV in a given host was similar between
342 vaccine and placebo groups, both across the genome and on the segments coding for HA and
343 NA. Furthermore, there were no significant differences in diversity when samples were grouped
344 by HAI and NAI titer, and we did not find evidence of treatment-specific iSNV. Together our data
345 suggest that vaccine-induced immunity does not significantly influence intrahost diversity and is
346 a relatively weak selective pressure at the level of the individual host.

347
348 While the number of iSNV was similar in all groups, we considered it possible that partial
349 immunity may drive the emergence of specific antigenic variants. Vaccination and natural
350 infection can induce a wide range of immune responses depending on host and viral factors.
351 These can range from complete protection to leaky responses that allow infection, but influence
352 disease severity or duration. Non-sterilizing immune responses have the potential to select for
353 escape variants within each host. In the setting of current recommendations for universal
354 vaccination, a highly vaccinated population could potentially select for antigenically evolved
355 viruses more quickly than the spread of natural infection. For this reason, it is important to tease
356 apart the role of vaccination on influenza evolution. We did find a number of variants in antigenic
357 sites, but these were no more frequent than in other regions of the genome and did not vary
358 with vaccination status or HAI and NAI titer. Importantly, our results are in conflict with earlier
359 work in several model systems that demonstrate the rapid selection of antigenic variants in the
360 presence of sub-neutralizing antibody or in experimentally infected animals [24,25,49-51]. This

361 discrepancy may be due to differences in infectious dose, host genetic background, history of
362 prior influenza infection, immune correlates not captured by HAI and NAI titers, or the strains
363 tested. Animal studies are often performed in immunologically naïve or genetically identical
364 animals, whereas humans have complex genetic backgrounds and immunological histories that
365 could play a role in mediating population-wide immunity.

366
367 While global patterns of influenza transmission and evolution are complex, our study provides
368 important insights regarding influenza evolution on intra- and interhost scales. We were able to
369 define aspects of intrahost evolution within a geographically-constrained and relatively young
370 cohort with similar vaccination histories and previous influenza exposures across groups,
371 limiting potential confounding factors. Still, we acknowledge that by reducing these confounders,
372 we may be missing important determinants of intrahost evolution. For example, intrahost
373 diversity may be different in children, older adults, or those with high-risk conditions. Our study
374 included 5119 person-years of observations to yield a dataset of 165 viruses, 119 of which were
375 sequenced. Large, placebo-controlled, randomized influenza vaccine trials involving thousands
376 of people are unlikely to be conducted in the future due to the recommendation for universal
377 vaccination. Therefore, our sample set likely represents the best chance to directly assess the
378 impact of vaccination on influenza evolution in the context of natural human infection.

379
380 Despite our well-controlled study, we found little evidence for vaccine-driven evolution in the
381 context of the community that was sampled. None of the low frequency variants identified in the
382 antigenic sites were found in subsequent seasons. While we cannot rule out the possibility that
383 evolutionary patterns would be different in other geographic regions, different influenza seasons,
384 or with other subtypes, we did not uncover differences between vaccinated and unvaccinated
385 populations with respect to genome-wide or antigenic diversity in three H3N2 seasons. We were
386 not able to evaluate the impact of vaccination on H1N1 or subtype B viruses. Furthermore, our

387 sample set cannot discern whether there are differences in evolutionary pressure based on
388 vaccine match/mismatch, as the vast majority of our samples were from antigenically matched
389 seasons. A major limitation of our study was that only one sample was available for each
390 participant, so we could not track changes in diversity or mutation accumulation within each
391 individual over the course of their infection. However, we did not observe significant differences
392 in the number of mutations over the first 6 days of infection, which is consistent with previous
393 work in horses [25] and a deep sequencing study of 7 humans in an experimental challenge
394 model [52]. Together with these works, our data suggest that within host dynamics are
395 dominated by purifying selection with the transient appearance of minority variants and little
396 sustained fixation. Selection and transmission of antigenically and epidemiologically important
397 variants is likely to be a rare event when studied at this scale. Given that our data are derived
398 from 165 incident infections in 5119 season-years of observation, detection of such events in
399 the course of a natural infection would require an unrealistic sample size.

400

401 We evaluated the potential for vaccine-induced immunity to drive intrahost evolution, as this is
402 an important issue in light of the current recommendation for universal influenza vaccination.
403 We did not find evidence for vaccine-induced pressure on the intrahost or consensus level,
404 despite employing several methods used to study evolutionary processes. Our study is larger
405 than other reports and involves extensive analysis of both placebo and vaccination groups.
406 While randomization, placebo control, and reliance on a young healthy population allowed us to
407 address this question in a rigorous manner, these factors may have lessened person-to-person
408 variation. By better defining the intrahost evolutionary mechanisms and how they impact
409 population-wide influenza evolution, we hope to address questions of import to clinicians and
410 public health workers, improve vaccine design, and develop more efficient epidemiological
411 control measures.

412 **References**

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568

569

570 **Figure Legends**

571 Figure 1: Viral shedding by vaccination status. Genome copy number per μl of transport media
572 was determined by RT-qPCR all samples from the 2007-2008 season. (A) Copy number by
573 vaccination status. IIV, inactivated influenza vaccine; LAIV, live attenuated influenza vaccine.
574 (B) Copy number by day of infection (onset of symptoms is day 0) and vaccination status. Circle,
575 IIV; Square, LAIV; Triangle, Placebo. (C) Copy number by HAI titer (D) Copy number by NAI
576 titer. There were no differences among any of the groups by one-way ANOVA with Bonferroni
577 correction.

578 Figure 2: Phylogenetic trees of HA and NA consensus sequences from the 2007-2008 season.
579 Maximum likelihood trees of HA (left) and NA (right) with tips coded by vaccine status and pre-
580 season HAI (left; blue >40, magenta <40) or NAI (right; blue >40, magenta <40) titer. Outgroups
581 are HA (EU103823.1) and NA (CY114383.1) for the vaccine strain A/Wisconsin/67/2005.
582 Bootstrap values (n=1000 bootstraps) are shown and nodes with bootstrap values <50 are
583 collapsed for easier visualization.

584 Figure 3: Intra-host diversity in samples from the 2007-2008 season. (A) Histogram of the
585 number of iSNV at a given frequency. Bin width = 0.01. (B) Histogram of the number of samples
586 in which each iSNV is found. Arrows indicate bars with one SNV, which are hard to discern in
587 the histogram. These polymorphic SNV, at PB2 position 900 and PA position 515 respectively,
588 were found at 4-6% frequency within hosts and in similar numbers of individuals across
589 vaccination groups. (C) Number of HA iSNV per sample stratified by pre-season HAI titer. >40 =
590 serologically immune, <40 = not serologically immune. (D) Number of NA iSNV per sample
591 stratified by pre-season NAI titer. >40 = serologically immune, <40 = not serologically immune.

592 Figure 4. Temporal patterns of intra-host diversity. Number of genome-wide iSNV per sample (y-
593 axis) by day of symptoms (x-axis) stratified by (A) recipients of IIV, magenta; LAIV, blue;

594 placebo, white (B) HAI >40, magenta; HAI <40, white (C) NAI >40, magenta; NAI <40, white.

595 Mean number of iSNV in each group is indicated (bar).

596 Figure 5: Structural mapping of HA variants. A homology model of the A/Brisbane/10/2007

597 (H3N2) HA trimer is shown, with each monomer represented by a different color (purple, grey,

598 and teal) and HA1 and HA2 designated by lighter and darker shades of the same color,

599 respectively. (A) A side view of HA. All identified non-synonymous mutations and known

600 antigenic amino acid positions are shown as balls on the grey monomer. Those variants colored

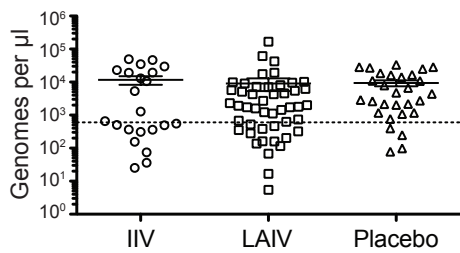
601 red are within known antigenic sites, while light orange mutations are not. (B) A side and top

602 view of HA. All amino acid positions within known antigenic sites are displayed as balls, with the

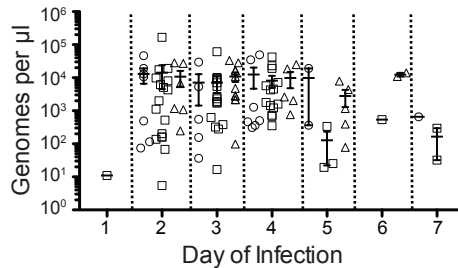
603 antigenic mutations identified here shown in red on all three monomers.

Figure 1

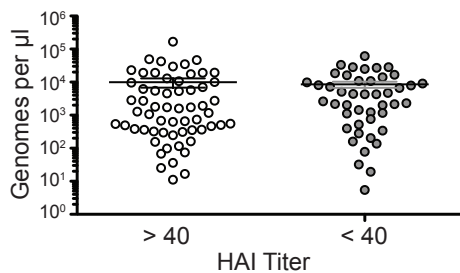
A



B



C



D

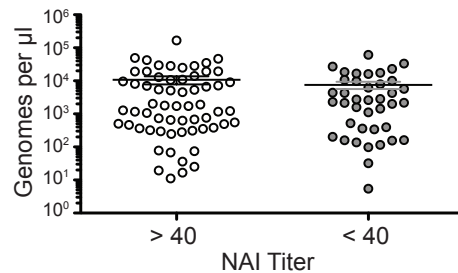
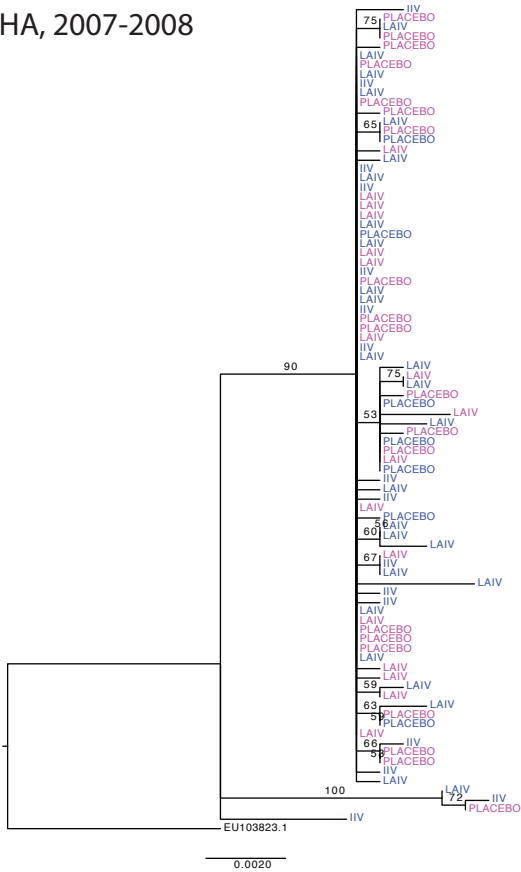


Figure 2

HA, 2007-2008



NA, 2007-2008

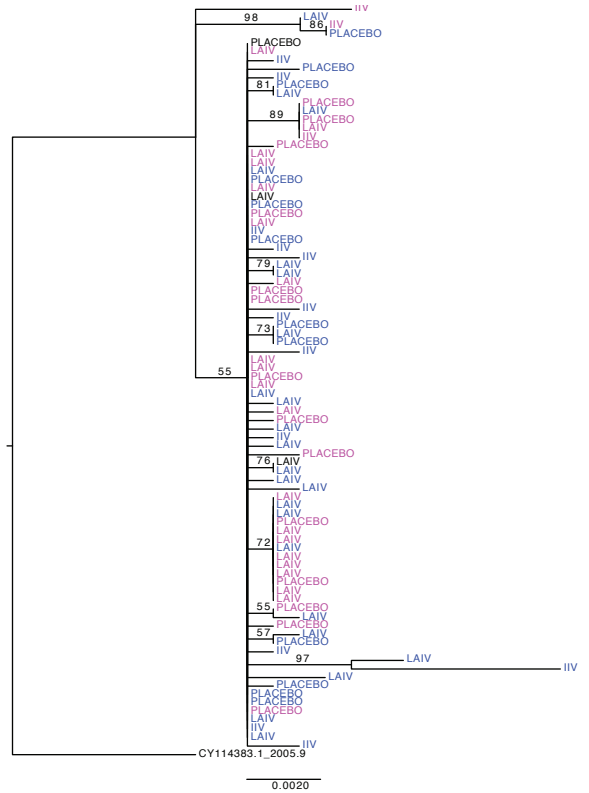


Figure 3

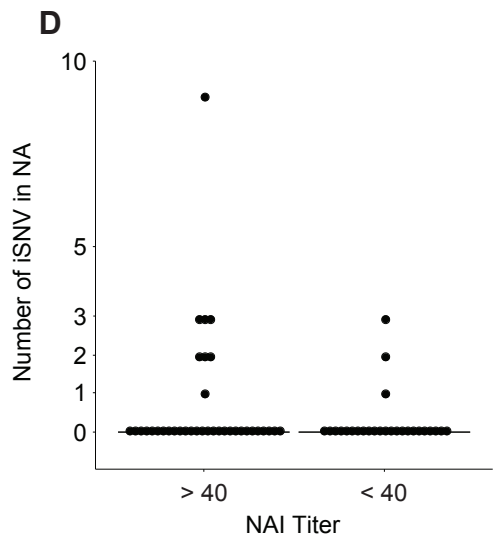
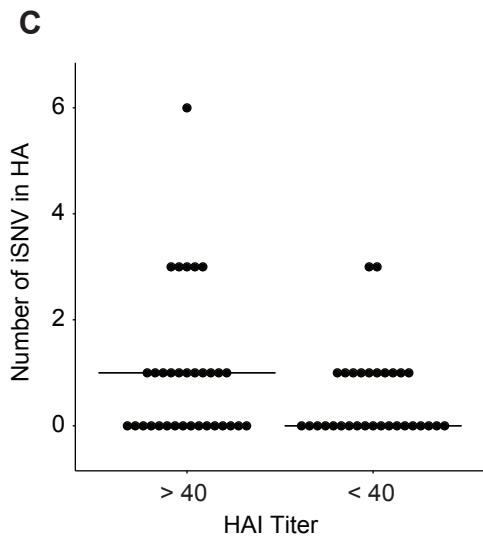
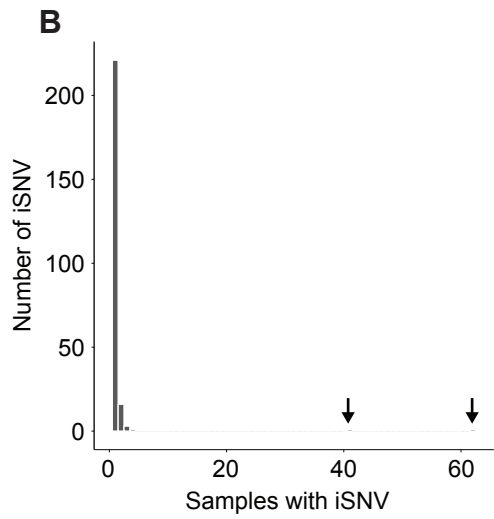
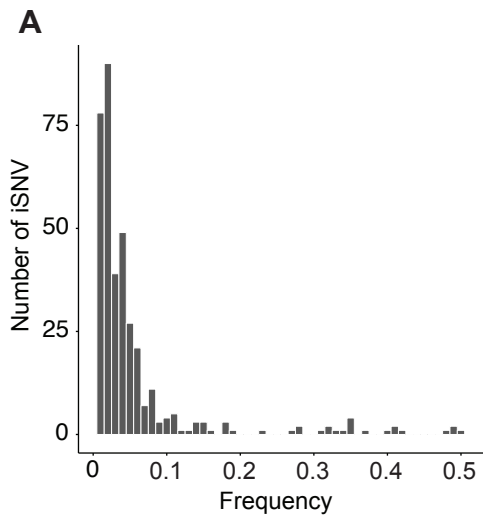
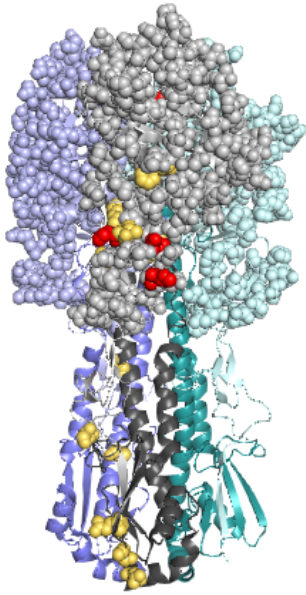


Figure 5

A



B

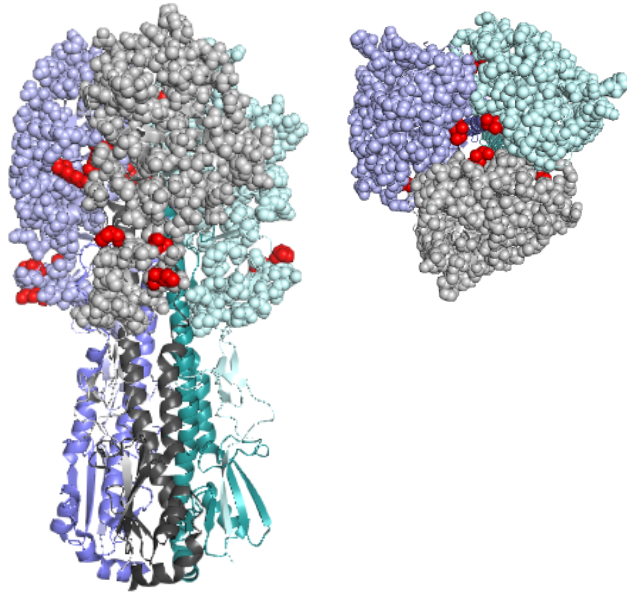


Table 1: Samples analyzed over three FLU-VACS Seasons

	2004-2005	2005-2006	2007-2008
H3N2 Vaccine Strain	Wyoming/3/2003	California/7/2004	Wisconsin/67/2005
Circulating Strain(s)	Wyoming/3/2003 California/7/2004	California/7/2004 Wisconsin/67/2005	Wisconsin/67/2005 Brisbane/10/2007
Demographics			
Average Age (Years)	30.0	28.8	22.8
Sex (% Female)	71.4%	83.3%	70.1%
Race (% White)	92.9%	94.4%	86.2%
Sequenced Samples			
IIV	4	9	16
LAIV	5	6	44
Placebo	5	3	27
Intrahost SNV Data			
IIV	3	6	11
LAIV	4	1	30
Placebo	3	2	23

Table 2: Number of iSNV (mean \pm interquartile range) by segment and treatment group for samples from the 2007-2008 season.

Segment	IIV (n=11)	LAIV (n=30)	Placebo (n=23)
1 (PB2)	2.1 \pm 2	1.78 \pm 1	1.89 \pm 1
2 (PB1)	1.8 \pm 1	1.46 \pm 1	1.45 \pm 1
3 (PA)	1.8 \pm 0.75	1.7 \pm 1	1.65 \pm 1
4 (HA)	1.43 \pm 1	1.27 \pm 0.5	1.27 \pm 0
5 (NP)	1.75 \pm 1.25	1.33 \pm 0.75	1.4 \pm 1
6 (NA)	1 \pm 0	1.17 \pm 0	1.25 \pm 0.25
7 (M)	1 \pm 0	1.14 \pm 0	1 \pm 0
8 (NS)	1 \pm 0	1.33 \pm 0.75	1.29 \pm 0.5