Deep Sequencing Reveals Potential Antigenic Variants at Low Frequencies in Influenza A Virus-Infected Humans

Jorge M. Dinis,^a Nicholas W. Florek,^a Omolayo O. Fatola,^b Louise H. Moncla,^a James P. Mutschler,^{a,d} Olivia K. Charlier,^a Jennifer K. Meece,^c Edward A. Belongia,^c Thomas C. Friedrich^{a,d}

Department of Pathobiological Sciences, University of Wisconsin School of Veterinary Medicine, Madison, Wisconsin, USA^a; Department of Biological Sciences, University of Maryland, Baltimore, Maryland, USA^b; Center for Clinical Epidemiology and Population Health, Marshfield Clinic Research Foundation, Marshfield, Wisconsin, USA^c; Wisconsin National Primate Research Center, Madison, Wisconsin, USA^d

ABSTRACT

Influenza vaccines must be frequently reformulated to account for antigenic changes in the viral envelope protein, hemagglutinin (HA). The rapid evolution of influenza virus under immune pressure is likely enhanced by the virus's genetic diversity within a host, although antigenic change has rarely been investigated on the level of individual infected humans. We used deep sequencing to characterize the between- and within-host genetic diversity of influenza viruses in a cohort of patients that included individuals who were vaccinated and then infected in the same season. We characterized influenza HA segments from the predominant circulating influenza A subtypes during the 2012–2013 (H3N2) and 2013–2014 (pandemic H1N1; H1N1pdm) flu seasons. We found that HA consensus sequences were similar in nonvaccinated and vaccinated subjects. In both groups, purifying selection was the dominant force shaping HA genetic diversity. Interestingly, viruses from multiple individuals harbored low-frequency mutations encoding amino acid substitutions in HA antigenic sites at or near the receptor-binding domain. These mutations included two substitutions in H1N1pdm viruses, G158K and N159K, which were recently found to confer escape from virus-specific antibodies. These findings raise the possibility that influenza antigenic diversity can be generated within individual human hosts but may not become fixed in the viral population even when they would be expected to have a strong fitness advantage. Understanding constraints on influenza antigenic evolution within individual hosts may elucidate potential future pathways of antigenic evolution at the population level.

IMPORTANCE

Influenza vaccines must be frequently reformulated due to the virus's rapid evolution rate. We know that influenza viruses exist within each infected host as a “swarm” of genetically distinct viruses, but the role of this within-host diversity in the antigenic evolution of influenza has been unclear. We characterized here the genetic and potential antigenic diversity of influenza viruses infecting humans, some of whom became infected despite recent vaccination. Influenza virus between- and within-host genetic diversity was not significantly different in nonvaccinated and vaccinated humans, suggesting that vaccine-induced immunity does not exert strong selective pressure on viruses replicating in individual people. We found low-frequency mutations, below the detection threshold of traditional surveillance methods, in nonvaccinated and vaccinated humans that were recently associated with antibody escape. Interestingly, these potential antigenic variants did not reach fixation in infected people, suggesting that other evolutionary factors may be hindering their emergence in individual humans.

Seasonal influenza A epidemics cause an estimated 3 to 5 million cases of severe respiratory illness each year, resulting in approximately 250,000 to 500,000 deaths worldwide (1). Available influenza vaccines only provide moderate protection against infection and illness. Vaccine effectiveness can vary greatly by season, but the average effectiveness at preventing illness has been estimated to be 59% (2). Even when there is good antigenic match between circulating viruses and vaccine strains, people can become infected despite being vaccinated earlier in the same season, a situation known as vaccine failure (3–5).

Viral antigenicity is influenced by multiple factors, including the accumulation of point mutations in the gene encoding the viral attachment protein hemagglutinin (HA), the specificity and avidity of HA for its receptor, and epistatic interactions within HA and between gene segments (6, 7). The HA protein has five classically defined antigenic sites that are important for the recognition of neutralizing antibodies; amino acid substitutions in these sites can significantly change viral antigenicity (8, 9). For example, “antigenic cartography” studies demonstrated that H3N2 strains cir-

culating from 1968 to 2003 formed 11 distinct “antigenic clusters” (10). Transitions between antigenic clusters occur as variant viruses emerge unpredictably in the population and replace older viruses in circulation (11). Recently, Koel et al. found that single amino acid substitutions in one of seven residues near the HA receptor-binding domain were responsible for the majority of previously observed H3N2 antigenic cluster transitions; these

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Address correspondence to Thomas C. Friedrich, thomasf@primate.wisc.edu.

J.M.D. and N.W.F. contributed equally to this article.

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seven residues have been said to form an “antigenic ridge” on HA (12). Similarly, eight residues were identified in H1N1pdm HA that could lead to escape from neutralizing antibodies (13).

While the molecular basis of antigenic change in influenza is becoming increasingly well understood, the evolutionary processes by which antigenic variants emerge are less clear, particularly on the scale of the individual host. In a host, influenza viruses exist as a diverse collection of genetically similar variants that arise due to low-fidelity genome replication, rapid replication kinetics, and high viral loads (14, 15). The level of within-host genetic diversity is determined through random mutation, and the action of natural selection, which can either promote genetic diversification or purify deleterious mutations from the virus population. High within-host diversity is thought to enable influenza viruses to rapidly adapt to changing environments (16, 17). However, until recently it was difficult to study the degree to which viral genetic diversity encodes antigenic diversity and contributes to the emergence of antigenic variants within individual hosts. Traditional surveillance methods based on Sanger sequencing cannot resolve variants present below 20% of the viral population (18). In contrast, deep sequencing can detect variants present at much lower frequency, revealing for example how low-frequency drug-resistant variants can rapidly emerge and become dominant after oseltamivir treatment in immunocompromised individuals (19). Moreover, we recently showed that HA variants present below the detection limit of Sanger sequencing could be transmitted via respiratory droplets (20), suggesting viral variants present at low frequency in individual hosts may have a previously unappreciated role in influenza virus biology.

We used deep sequencing to determine how viral variants potentially encoding antigenic change are generated and selected during human infection, using respiratory samples collected from patients presenting to clinics with acute influenza infections during two recent influenza seasons. Our patient population included subjects with vaccine failure, affording us the opportunity to determine whether within-host viral populations differed between vaccinated and nonvaccinated subjects. We sequenced viruses directly from clinical samples without isolation in eggs or passage in tissue culture. Viral populations did not differ at the consensus level between vaccinated and nonvaccinated subjects. Within-host HA segment genetic diversity varied considerably among humans, although most viral polymorphisms were typically present either at or near fixation (90 to 100% of viral sequences) or at low frequency (1 to 10%) relative to the relevant vaccine strain sequence. Patterns of HA segment diversity were consistent with viruses undergoing purifying selection to maintain fit viral populations. Interestingly, we detected nonsynonymous mutations that had previously been shown to confer escape from neutralizing antibodies in both H3N2 and H1N1pdm viruses infecting both nonvaccinated and vaccinated subjects. These mutations were always detected at low frequencies in the viral population. Together, our data suggest that putative influenza antigenic escape variants are generated in influenza virus-infected humans but often do not become fixed in the virus population.

MATERIALS AND METHODS

Study population. The source population for this study included residents of the Marshfield Epidemiologic Study Area (MESA), a population-based cohort of approximately 54,000 people in a 14-zip-code area surrounding the Marshfield Clinic campus in Marshfield, Wisconsin (21). In

the 2012–13 and 2013–14 seasons, most community-dwelling MESA residents aged ≥ 6 months were eligible to be recruited for a separate study of influenza vaccine effectiveness during an outpatient encounter for acute respiratory illness with cough. Patients meeting symptom criteria were eligible if the duration of illness was ≤ 7 days. Participants completed an interview to assess illness onset and symptoms. Individuals were considered vaccinated if they had received an influenza vaccine for the current influenza season ≥ 14 days prior to illness onset. We obtained nasal and oropharyngeal swabs from nonvaccinated and vaccinated participants aged ≥ 9 years; swabs were combined in viral transport media for testing. Influenza infection was confirmed using quantitative reverse transcription-PCR (qRT-PCR) (22). During the 2012–13 and 2013–14 seasons, subtypes H3N2 and 2009 pandemic H1N1 (H1N1pdm), respectively, were dominant in the United States (4, 23); for this reason, we only had access to H3N2 during the 2012–13 season and H1N1pdm viruses during the 2013–14 season.

The Marshfield Clinic Institutional Review Board approved study procedures. Informed consent was obtained from all adults and parents/guardians of children. Additional details on the vaccine effectiveness studies for which these human samples were originally collected have been published (4, 23).

Template preparation. Total nucleic acids were extracted from clinical specimens using the RNeasy minikit (Qiagen, USA). Influenza vRNAs were reverse transcribed using the Uni12-F (AGCAAAAGCAGG) primer (24) with Superscript III reverse transcriptase (Invitrogen, USA) according to the manufacturer’s instructions. Single-stranded cDNA was used as a template for PCR amplification to amplify the HA genes using either the primers H3N2-F (AGCAAAAGCAGGGGATAATTC) and H3N2-R (AGTAGAAACAAGGGTGTTTT) or the primers H1N1pdm-F (ATATACGCGTAGCGAAAGCAGGGGAA) and H1N1pdm-R (ATATACGCGTAGAAACAAGGTGT) with high-fidelity Phusion 2 $\times$ master mix (New England BioLabs, Inc., USA). PCR was performed by incubating the reaction mixtures at 98°C for 20 s, followed by 35 cycles of 98°C for 10 s, 57°C for 10 s, and 72°C for 1 min, followed by a final extension step at 72°C for 10 min. PCR products were separated by electrophoresis on a 1% agarose gel. The band corresponding to the full-length HA gene segment was excised, and the DNA was recovered using a QIAquick gel extraction kit (Qiagen).

Deep sequencing. Gel-purified PCR products were quantified using the Qubit dsDNA high-sensitivity kit (Invitrogen). Purified PCR products were made compatible for deep sequencing using the Nextera XT DNA sample preparation kit (Illumina, USA). Each sample was enzymatically fragmented and tagged with short oligonucleotide adapters, followed by 14 cycles of PCR for template barcoding. Barcoded H3N2 and H1N1pdm libraries were pooled to a final concentration of 2 nM, and 10 μ l of the 2 nM pooled library was denatured in 10 μ l of 0.2 N NaOH for 5 min. Denatured pooled libraries were diluted to a final concentration of 12 pM with a PhiX-derived control library accounting for 1% of total DNA. Next, 600 μ l of diluted-denatured template was added to a 500-cycle reagent cartridge. Illumina MiSeq run settings were entered into the sample sheet by using Illumina Experiment Manager Software v1.3.66 (workflow [FASTQ]; assay [Nextera XT]; chemistry [Amplicon]; reads, 250 $\times$ 250). Sequence reads were imported into CLC Genomics Workbench, version 7.5.2 (CLC bio, Denmark). Sequence reads were trimmed using a quality score cutoff of Q30 (i.e., 1 error per 1,000 sequenced bases). For each sample, we mapped trimmed sequence reads against a vaccine strain HA reference sequence: A/Victoria/361/2011 (H3N2; [KC306165](#)) or A/California/07/2009 (H1N1pdm; [CY121680](#)).

Between-host evolutionary analysis. For each reference-based mapping, we extracted a consensus sequence that consists of the majority nucleotide at each nucleotide site. Consensus sequences were imported into Geneious Version 5.6.3 (Biomatters, Ltd., New Zealand). In Geneious, we created individual alignments using the MUSCLE alignment algorithm (25) for H3N2 and H1N1pdm HA gene sequences. Phylogenetic trees were constructed from alignments in PHYLIP version 3.0 (26,

TABLE 1 Descriptive characteristics of influenza A cases by season

Characteristic	2012-13 (n = 68)	2013-14 (n = 46)	Total (n = 114)
Age group (yrs)			
<9	18	9	27
9–17	5	5	10
18–49	20	17	37
≥50	25	15	40
Vaccination status			
Nonvaccinated	37	32	69
Vaccinated	31	14	45
IIV	24	10	34
LAIV	3	4	7
HD	2	0	2
Vaccinated, unknown type	2	0	2
Interval from onset to swab (days)			
<3	34	29	63
3–4	22	8	30
5–7	12	9	21
Swab collection (mo)			
December	13	22	35
January	37	22	59
February	17	2	19
March	1	0	1
Influenza A virus subtype			
H3N2	68	0	68
H1N1pdm	0	46	46

27) using the HKY85 substitution model with 1,000 bootstrap replicates to assess statistical confidence. The consensus HA gene segments has been deposited in NCBI GenBank under accession numbers [KR611199](#) to [KR611312](#).

Within-host evolutionary analysis. Single-nucleotide polymorphisms (SNPs) were detected, at nucleotide positions with coverage of at least 100 sequence reads and a central base quality score of $\geq Q30$, relative to a vaccine strain reference sequence (A/Victoria/361/2011 H3N2 or A/California/07/2009 H1N1pdm) using CLC Genomics Workbench version 7.5.2. SNPs were only considered for subsequent analysis if detected at a cutoff frequency of $\geq 1\%$, a validated threshold that reduces the carryover of errors introduced by reverse transcription, amplification, or basecalling (20). The nucleotide diversity statistics π_N and π_S were calculated in PoPoolation version 1.2.2 (28) using subsampled sequence mappings containing 1,000 randomly chosen sequences per nucleotide position to minimize potential coverage bias. HA protein structures were created with MacPymol (<http://www.pymol.org/>). Mature H3N2 numbering is used throughout the manuscript for H3N2 and H1N1pdm viruses.

RESULTS

Participant characteristics. Samples used for the present study represent a cross-sectional sampling of combined nasal and oropharyngeal swabs collected as part of a separate study of influenza vaccine effectiveness from 114 human subjects aged ≥ 9 years with medically attended acute respiratory illness (MAARI) who tested positive for influenza A virus by qRT-PCR (Table 1). Unfortunately, neither time-serial respiratory samples nor sera were available from these subjects. As a result, we were not able to assess antibody titers in these subjects. The mean intervals $\pm$ the standard deviations (SD) from illness onset to sample collection were

2.7 ± 1.5 and 2.7 ± 1.8 days, respectively, for subjects who tested positive for H3N2 and H1N1pdm infection. The samples assessed here were collected from 68 individuals infected with H3N2 during the 2012-13 season and from 46 individuals infected with H1N1pdm during the 2013-14 season. In total, 45 of these subjects were vaccinated in the season in which they were infected, and 69 were nonvaccinated. As shown in Table 1, 34 vaccinated subjects received an inactivated influenza vaccine (IIV), 7 received a live attenuated influenza vaccine (LAIV), and 2 received high-dose IIV (HD); the vaccine type was not recorded for an additional 2 subjects (unknown [UN]).

Deep-sequencing HA segments. Because mutations near the receptor-binding pocket can be introduced during propagation of clinical specimens in eggs (29–31), we sequenced PCR-amplified viral nucleic acids extracted directly from swabs. Amplified HA genes were prepared for deep sequencing using the Illumina MiSeq because of its high throughput and low error rate (32, 33). The mean sequence coverages $\pm$ the SD of HA were $16,370.91 \pm 10,887.82$ (H3N2) and $13,786.92 \pm 5,711.98$ (H1N1pdm) sequences per nucleotide site.

Vaccine failure is not associated with particular HA sequences. We first sought to determine whether vaccine failure might be associated with selection for HA variants in vaccinated individuals. We constructed maximum-likelihood phylogenetic trees using nearly full-length nucleotide HA consensus sequences (Fig. 1). The phylogenies yielded topologies consistent with established relationships among influenza strains circulating during the 2012-13 and 2013-14 seasons (34, 35). All H1N1pdm viruses infecting our subjects belonged to genetic subgroup 6 (46/46). Almost all H3N2 viruses belonged to clade 3C, with the exception of two viruses belonging to clade 6. Currently circulating H3N2 clade 3C viruses can be further resolved into multiple subgroups with differing degrees of antigenic relatedness (35). Within our subjects, 72.7% (48/66) of clade 3C H3N2 viruses belonged to subgroup 3C.2 and 27.3% (18/66) belonged to subgroup 3C.3.

We examined our phylogenies for monophyletic clades consisting primarily of HA consensus sequences from vaccinated subjects, which we would expect to observe if in either season, antibodies boosted by vaccination selected for variant viruses detectable at the consensus level in subjects with vaccine failure. Instead of this monophyletic clustering, sequences from vaccinated and nonvaccinated individuals were generally interspersed throughout the trees (Fig. 1). We did detect one monophyletic clade consisting of HA sequences entirely from vaccinated individuals ($n = 2$; H3N2 genetic subgroup 6), but no sequences of subgroup 6 viruses from nonvaccinated subjects were available for comparison. Together, these data indicate that vaccine failure was not associated with mutations in the HA gene detectable at the consensus level in either season studied.

Action of natural selection on HA genes in human infections. We considered the possibility that vaccine-induced antibodies could act to eliminate some variants from the infecting viral “swarm” without altering the consensus sequence, which might be expected if they attenuate the infecting dose but fail to block infection. In this case, within-host HA genetic diversity might be reduced in cases of vaccine failure relative to virus populations in nonvaccinated individuals. To estimate within-host HA nucleotide diversity we calculated the statistic π . π_N , or nonsynonymous diversity, describes the frequency of pairwise sequence differences within a virus population that encode an amino acid change. Sim-

FIG 1 Phylogenetic relationships of influenza A viruses infecting nonvaccinated and vaccinated subjects in Marshfield, WI. (a) H3N2 phylogenetic tree constructed using 68 hemagglutinin genes from subjects infected during the 2012-13 season and 11 outgroup sequences representing WHO-recommended vaccine strains and representative H3N2 taxonomic clades (alignment length, 1,641 nucleotides). (b) H1N1pdm phylogenetic tree constructed using 46 hemagglutinin genes from subjects infected during the 2013-14 season and nine outgroup sequences representing WHO-recommended vaccine strains and representative H1N1pdm taxonomic clades (alignment length, 1,698 nucleotides). Bootstrap values were determined from 1,000 replicates and are indicated above the corresponding nodes when values are above 50%.

ilarly, π_S , describes the frequency of silent mutations within a virus population. Note that these statistics describe the genetic diversity of viral populations within a single host and do not rely on an external reference sequence. Comparing the values of these statistics for a given gene provides information about the “direction” of natural selection. Generally, a π_N/π_S ratio of >1 indicates that positive selection is favoring genetic diversification. In contrast, a π_N/π_S ratio of <1 indicates that purifying (or negative) selection is acting to maintain a fit virus population by removing deleterious mutations. Comparing HA π_N and π_S in vaccinated and nonvaccinated subjects thus allows us to determine whether overall HA genetic diversity is different in these two groups and also may indicate whether differences in diversity are due to nat-

ural selection acting on HA. We computed π_N and π_S for the full-length HA coding region and also for HA1, HA2, and the receptor-binding domain (RBD) to account for the possibility that selection could act differently at these discrete functional domains of the HA gene.

We computed the overall mean π_N and π_S values for H3N2 ($n = 68$) and H1N1pdm ($n = 46$) viruses. Mean π_N and π_S values for each region varied widely (Table 2). For example, individual means of π_N for full-length HA genes ranged from 0.00040 to 0.00281 substitutions per site for H3N2 viruses and from 0.00035 to 0.00108 substitutions per site for H1N1pdm. We found that overall mean π_N was lower than π_S at each HA domain for all viruses (Tables 2 and 3; two-tailed $P < 0.0001$ to 0.05; Student t

TABLE 2 Estimates of nonsynonymous and synonymous nucleotide diversity for hemagglutinin genes of influenza A viruses^a

Subtype and domain	π_N			π_S			π_N vs π_S (<i>P</i>)
	π_N (10^{-3})	SEM	Range (10^{-3})	π_S (10^{-3})	SEM	Range (10^{-3})	
H3N2							
HA	0.69	0.04	0.40–2.81	1.24	0.19	0.51–13.48	0.0053
HA1	0.70	0.04	0.39–2.61	1.16	0.15	0.32–9.68	0.0036
HA2	0.68	0.05	0.39–3.42	1.47	0.38	0.36–26.42	0.0412
RBD	0.76	0.06	0.36–4.44	1.38	0.21	0.29–13.33	0.0052
H1N1pdm							
HA	0.60	0.02	0.35–1.08	1.07	0.11	0.33–3.82	0.0001
HA1	0.61	0.03	0.32–1.35	0.96	0.10	0.28–3.15	0.0012
HA2	0.59	0.03	0.33–1.06	1.25	0.17	0.34–4.99	0.0002
RBD	0.56	0.03	0.26–1.00	0.99	0.12	0.24–3.92	0.0008

^a π_N , nonsynonymous nucleotide diversity; π_S , synonymous nucleotide diversity. *P* values correspond to comparisons of mean π_N and π_S values at each HA gene domain. Statistical analysis performed using a Student *t* test. Values for π_N , π_S , and ranges are presented as coefficients $\times 10^{-3}$ substitutions per site; e.g., a listed value of 0.69 equals 6.9×10^{-4} substitutions per site.

tests), showing that HA genes are generally subject to purifying selection in these individuals.

We did not detect significant differences in nucleotide diversity between nonvaccinated and vaccinated subjects. The one exception to this trend was in the RBD domain of H1N1pdm viruses, in which π_N was slightly but significantly greater in vaccinated than in nonvaccinated subjects ($P = 0.016$; Table 3). One might predict that within-host HA diversity would increase with time after in-

fection, as random mutations accumulate, and potentially as antibody pressure favors the outgrowth of antigenic variants. In this case, we would expect that π_N and/or π_S values would increase with time after infection. However, we found no association between π_N or π_S values and the number of days after symptom onset for either H3N2 or H1N1pdm viruses (data not shown), suggesting that the day subjects presented to the clinic did not bias our observations. Overall, our data indicate that purifying selection was strong on both full-length HA segments and individual HA functional domains in both nonvaccinated and vaccinated subjects and that there was no significant reduction in HA genetic diversity in vaccinated versus nonvaccinated subjects.

Deep sequencing reveals HA genetic and potential antigenic diversity in humans. Finally, we asked whether any of the HA mutations we observed had the potential to encode antigenic diversity. To do this, we enumerated HA SNPs relative to various reference sequences (Fig. 2). We only considered SNPs if they were detected above our experimentally defined cutoff of $\geq 1\%$ of sequence reads in a sample (20). In total, we detected 695 nonsynonymous and 875 synonymous SNPs from individuals infected with H3N2 viruses; in H1N1pdm-infected individuals, we detected 700 nonsynonymous SNPs and 735 synonymous SNPs (Table 4). The SNP frequency spectrum had a bimodal distribution, with most mutations detected either at low frequency (1 to 10% of sequences) or at high frequency (90 to 100% of sequences, Fig. 2). Overall, the average number of SNPs relative to vaccine strains in each host was significantly greater for H1N1pdm viruses (nonsynonymous SNPs, 14.8 ± 3.2 ; synonymous SNPs, 16.0 ± 3.1) than H3N2 viruses (nonsynonymous SNPs, 10.2 ± 2.4 ; synonymous SNPs, 12.9 ± 3.8 [two-tailed $P > 0.0001$, Student *t* test]). This

TABLE 3 Estimates of nonsynonymous and synonymous nucleotide diversity in nonvaccinated and vaccinated subjects^a

Subtype and domain	Nonvaccinated			Vaccinated			<i>P</i>
	π_N (10^{-3})	SEM	Range (10^{-3})	π_N (10^{-3})	SEM	Range (10^{-3})	
H3N2							
HA	0.72	0.06	0.40–2.81	0.65	0.03	0.40–1.14	0.329
HA1	0.72	0.06	0.41–2.61	0.68	0.03	0.39–1.25	0.576
HA2	0.73	0.08	0.39–3.42	0.62	0.03	0.42–1.44	0.235
RBD	0.81	0.11	0.43–4.44	0.70	0.04	0.36–1.17	0.385
H1N1pdm							
HA	0.58	0.03	0.35–1.08	0.67	0.04	0.38–0.90	0.094
HA1	0.59	0.04	0.32–1.35	0.66	0.05	0.36–0.94	0.316
HA2	0.56	0.03	0.33–0.96	0.67	0.05	0.36–1.06	0.056
RBD	0.52	0.03	0.26–1.00	0.66	0.05	0.42–0.97	0.016
Subtype and domain	π_S (10^{-3})			π_S (10^{-3})			<i>P</i>
	π_S (10^{-3})	SEM	Range (10^{-3})	π_S (10^{-3})	SEM	Range (10^{-3})	
H3N2							
HA	1.39	0.35	0.51–13.48	1.06	0.10	0.65–3.20	0.405
HA1	1.25	0.25	0.32–9.68	1.06	0.11	0.62–3.05	0.517
HA2	1.80	0.69	0.36–26.42	1.08	0.14	0.64–4.01	0.350
RBD	1.58	0.37	0.29–13.33	1.13	0.15	0.46–5.18	0.296
H1N1pdm							
HA	1.10	0.16	0.33–3.82	0.99	0.10	0.47–1.91	0.665
HA1	0.99	0.14	0.28–3.15	0.88	0.06	0.41–1.28	0.614
HA2	1.28	0.23	0.34–4.99	1.17	0.23	0.43–3.48	0.774
RBD	1.01	0.17	0.24–3.92	0.95	0.13	0.34–2.28	0.827

^a π_N , nonsynonymous nucleotide diversity; π_S , synonymous nucleotide diversity. *P* values correspond to comparisons of mean π_N and π_N or of π_S and π_S values for each HA gene domain (values were compared horizontally). Statistical analysis performed using a Student *t* test.

FIG 2 Deep sequencing reveals sequence variation in hemagglutinin genes during human infection. We used deep sequencing to reveal the within-host viral variation from nonvaccinated and vaccinated subjects. Displayed are SNPs detected in H3N2-infected (a, c, and e) and H1N1pdm-infected (b, d, and f) subjects. All sequence reads were mapped against three reference sequences: a vaccine strain, the most abundantly detected HA gene sequence, and against each sample's own autologous consensus sequence. Black circles represent nonsynonymous mutations, and gray squares represent synonymous mutations. The x axis represents the nucleotide position, and the y axis represents the frequency for which each mutation was detected from human biological samples. Above each SNP frequency plot is a cartoon depiction of the linear HA gene with shaded functional domains: HA1 (dark gray), HA2 (light gray), and the receptor-binding domain (RBD, black). Density plots indicate the likelihood for a nonsynonymous (black) or synonymous (gray) SNP to occur, regardless of frequency, in a given position across the HA gene.

greater genetic distance between vaccine and circulating strains for H1N1pdm viruses may be due to the fact that ~5 years elapsed between emergence of the CA07 vaccine strain in 2009 and the 2013-14 influenza season evaluated here, whereas only ~1 year separated the emergence of Vic361 and the 2012-13 season. Consistent with this observation, the density of nonsynonymous SNPs was greatest in the HA RBD for both H1N1pdm and H3N2 viruses (Fig. 2a and b, top panels). The elevated density of nonsynonymous variants in RBD is only observed when vaccine strains are used as reference sequences, and disappears when SNPs are defined with respect to consensus sequences from the relevant season or from within our subjects (Fig. 2c to f, top panels). Note that our analyses of diversity and selection presented in Tables 2 and 3 above did not use reference sequences and are therefore not affected by these observations.

To identify mutations with the potential to alter influenza antigenicity, we queried SNP data for mutations that lay in previ-

TABLE 4 Summary of nonsynonymous and synonymous mutations detected in our study^a

Mutation frequency (%)	No. of mutations					
	H3N2-2012/13 (n = 68)			H1N1pdm-2013/14 (n = 46)		
	N	S	N + S	N	S	N + S
1–10	97	84	181	100	64	164
10–20	6	6	12	9	6	15
20–30	2	6	8	1	2	3
30–40	3	0	3	1	0	1
40–50	0	0	0	0	0	0
50–60	0	0	0	0	0	0
60–70	2	6	8	0	0	0
70–80	2	1	3	0	2	2
80–90	0	0	0	1	7	8
90–100	583	772	1,355	588	654	1,242
Total	695	875	1,570	700	735	1,435

^a N, nonsynonymous; S, synonymous.

TABLE 5 Single-nucleotide polymorphisms detected in antigenicity-associated HA positions from infected humans^a

Subtype and nucleotide position	Nucleotide change	H3 amino acid residue no.	Amino acid change	Antigenic site	No. of subjects	Frequency range (%)	Detected in 2014-15 influenza season ^b
H3N2							
205	G→A	53	D→N	C	2	100	0/155
472	A→G	142	R→G	A	19	2.1–100	31/155
472	AG→GA	143	R→E	A	1	2.0	0/155
515	AA→GC	156	Q→R	B	1	3.3	0/155
516	A→C	156	Q→H	B	68	96.1–100	155/155
668	A→C	207	K→T	D	1	3.7	0/155
670	A→G	208	R→G	D	1	1.2	10/155
706	A→G	220	R→G	D	2	2.9–3.5	0/155
882	T→G	278	N→K	C	66	100	150/155
H1N1pdm							
283	C→A	80	S→Y	Cb	1	1.4	NA
283	C→T	80	S→F	Cb	1	2.2	NA
292	GC→AT	82b	S→N	Cb	1	100	NA
490	G→A	143	G→E	Ca2	1	1.1	NA
535	G→A	158	G→E	Sa	2	2.3–2.4	NA
539	T→A	159	N→K	Sb	3	1.4–5.8	NA
550	A→G	163	K→R	Sa	2	1.0–2.5	NA
558	A→C	183	K→Q	Ca1	45	100	NA

^a Antigenic sites refer to defined HA protein sites important for the recognition of neutralizing antibodies (7, 8). The number of subjects signifies the total number of individuals (nonvaccinated and vaccinated) for which a particular nucleotide change was observed (H3N2, $n = 68$; H1N1pdm, $n = 46$). The frequency range indicates the minimum and maximum frequency that a particular nucleotide change was observed in our cohort.

^b That is, the proportion of individual “antigenic ridge” mutations found in publically available H3N2 HA sequences from the 2014–15 influenza season in the United States. No H1N1pdm HA sequences were deposited for the 2014–15 influenza seasons from the United States (indicated as not applicable [NA]). A complete summary of each amino acid polymorphisms is available upon request.

ously defined antigenic sites. Nonsynonymous mutations occurred in our subjects in 9 HA amino acid positions in H3N2 and 8 HA amino acid positions in H1N1pdm located at or near the receptor binding pocket, which were previously found to affect viral antigenicity (Table 5 and Fig. 3). Notably, we found substitutions in HA amino acid position 156 in H3N2 viruses (Q156H/R) and positions 158 and 159 of H1N1pdm viruses (G158E and N159K), all of which lie in the “antigenic ridge” of the HA protein (H3 numbering used throughout). The antigenic ridge is a span of residues on the periphery of the receptor-binding site previously shown to be responsible for antigenic change in H3N2 (12) and H1N1pdm viruses (13). An A→G SNP at nucleotide 515 encoding a glutamine-to-histidine change at amino acid position 156 was detected at or near fixation all H3N2-infected subjects. This Q156H substitution was associated with vaccine mismatch during the 2003–2004 influenza season and has been found in HA consensus sequences of circulating H3N2 viruses ever since (36). The A→C SNP encoding the glutamine-to-arginine substitution at amino acid position 156 was detected in a single vaccinated subject (V130083) in 3.3% of viral sequences. The Q156R mutation was previously linked to egg adaptation of the 2012–2014 Vic361 vaccine virus (37). The Q156R mutation was not found in any of 155 publically available consensus sequences generated from the 2014–15 influenza season in the United States (Table 5). Outside the antigenic ridge, we detected an A→G SNP at nucleotide position 472 that encoded an arginine-to-glutamate substitution at amino acid position 142. If detected, the R142G mutation was found at or near fixation ($\geq 98\%$) in the virus population. However, we did detect the R142G substitution in 2.1% of viral sequences in subject U130482. Our data do not allow us to determine why R142G is present at such low

frequency in this individual, when it is the dominant variant in viruses infecting other subjects in the cohort. Mutations at position 142 have been documented to confer changes to antibody binding (38), and in recent years codon 142 has toggled between encoding arginine and glycine in circulating H3N2 viruses (39). We therefore speculate that this site is likely varying under immune pressure.

In H1N1pdm viruses, we detected a G→A SNP at nucleotide 535 encoding a glycine-to-glutamate substitution at amino acid position 158 in 2.3 and 2.4% of viral sequences in U140225 and V140227, respectively. G158E is detected in consensus H1N1pdm HA sequences in the Influenza Research Database (40) but only in 0.65% ($n = 4,001$) of samples. Interestingly, viruses with the G158E substitution replicated *in vitro* in the presence of H1N1pdm-specific ferret antibodies (41). A T→A SNP encoding an asparagine-to-lysine substitution at position 159 was detected in three H1N1pdm-infected subjects at low frequencies: V14022 at 1.4%, V140172 at 2.2%, and U14106 at 5.7% of viral sequences. In a previous study, H1N1pdm viruses bearing the N159K substitution (N156K in H1 numbering) outcompeted wild-type viruses in tissue culture, replicated efficiently in H1N1pdm immunized ferrets, and were efficiently transmitted among animals (42). Interestingly, despite causing escape from H1N1pdm-specific antibodies *in vitro* and *in vivo* in ferret studies, this mutation is found at the consensus level in a minority of viruses that previously circulated in humans (0.1% of sequences; $n = 4,001$). Taken together, these data show that mutations with the potential to encode antigenic variation are generated in humans naturally infected with influenza viruses. Unfortunately, because serum samples from these subjects were not available, we were not able to assess the impact of these mutations on autologous antibody rec-

FIG 3 Localization of amino acid substitutions identified in this study on the HA structure. The structure of an A/Aichi/2/1968 HA trimer (Protein Data Bank accession 5HMG) (53) is shown. The three monomers are shown in black and gray with the receptor-binding site in light brown, and positions previously defined as responsible for antigenic cluster transitions are shown in dark brown (12). Mutations detected in human subjects infected with H3N2 and H1N1pdm viruses are shown in red. All mutations are shown in H3 numbering. (a) H3N2 antigenic sites, as defined by Wiley et al. (8); antigenic sites A to E are shown in blue, green, magenta, orange, and yellow, respectively. (b and c) Mutations detected in subjects infected with H3N2 viruses. (d) H1N1 antigenic sites, as defined by Caton et al. (9); antigenic sites Sa, Sb, Ca1, Ca2, and Cb are shown in blue, green, magenta, orange and yellow, respectively. (e and f) Mutations detected in subjects infected with H1N1pdm viruses. Images were created with MacPyMol (<http://www.pymol.org/>).

ognition. These mutations exist well below the detection limit of Sanger sequencing in our subjects and are only rarely detected at the consensus level in humans.

DISCUSSION

Although influenza viruses are known to exist as a genetically diverse population within an infected host, the potential impact of this diversity on viral adaptation to immune selection is not well understood. Here, we characterized influenza HA genes directly from human clinical specimens and evaluated the contribution of HA within-host genetic variation to vaccine failure, as well as the potential ways in which vaccine-induced immunity shapes within- and between-host diversity. Influenza viruses infecting non-vaccinated and vaccinated subjects in our study were genetically similar and did not display patterns of sequence diversity consistent with the selection of antigenic variants within any individual host. Within-host HA genetic diversity in both nonvaccinated and vaccinated subjects consists of more synonymous diversity than nonsynonymous diversity, suggesting that most mutations are deleterious and purifying selection is the dominant evolutionary force affecting HA in this cohort. Interestingly, we observed several nonsynonymous mutations below the detection threshold of Sanger sequencing in both H3N2 and H1N1pdm viruses located in positions that can cause significant antigenic change. Moreover, several low-frequency nonsynonymous mutations were detected reproducibly in multiple subjects, in both nonvaccinated and vaccinated groups. This observation raises the possibility that antigenic variants are frequently generated during influenza virus infection in humans, but that they often do not become fixed in the

viral population even when they would be expected to have a strong fitness advantage.

There are important caveats to the interpretation of our results. First, we initially compared HA gene sequences from nonvaccinated and vaccinated subjects to identify potential mutations associated with vaccine failure. Humans have highly variable preexisting immune repertoires against influenza, which are shaped by individual infection and vaccination histories, as well as by age, comorbidities, and host genetics (41, 43–45). Grouping virus sequences purely by same-season vaccine status may not adequately capture important biological differences among these subjects. When possible, it would be desirable to use immunological characteristics (e.g., hemagglutination inhibition titers) to stratify subjects into more biologically relevant groups; unfortunately, that was not possible for our cohort. Second, because autologous serum was not available, we do not know whether putative antigenic variants specifically escape antibodies present in the subjects in which the variants were detected. However, past reports have shown that Q156H/R (H3N2) impacts antigenicity of vaccine strains, and G158E and N159K (H1N1pdm) confer escape from H1N1pdm-specific antibodies *in vitro* and *in vivo*.

Our findings indicated that within-host HA genetic diversity and HA consensus sequences were similar between nonvaccinated and vaccinated subjects. It is likely that virtually all of these subjects had some degree of preexisting immunity to influenza due to previous infection and/or vaccination, and as a result, mutations that confer escape from antibodies in experimental animals may not be as effective in humans. To the extent that the mutations we

describe here represent bona fide antigenic changes, we cannot explain why antibody escape mutants have not emerged at the consensus level using sequence data alone. However, we speculate that for antigenic variants to reach fixation in the virus population, compensatory mutations may be needed to enhance viral replicative fitness before the antigenic variant is lost by purifying selection. In accord with our observations, a previous study showed that prior immunity had little effect on the level and structure of genetic diversity of influenza viruses infecting vaccinated horses (46). In that study, Murcia et al. speculated that purifying selection (i.e., more synonymous diversity relative to nonsynonymous diversity) is the dominant signal of influenza within individual hosts (46); this is consistent with the idea that influenza viruses are well adapted to replication and transmission in their natural hosts, and therefore most mutations are likely to be deleterious. Indeed, mutations that alter HA antigenicity may frequently exact a fitness cost that requires compensatory mutations to restore viral replicative capacity (47). Finally, because each subject was sampled only once, we could not follow the changing frequencies of SNPs with time to detect potential positive selection at individual sites. Approaches using time-series samples enable better detection of site-specific positive selection (48).

From 1968 to 2011 14 antigenic clusters of H3N2 viruses have emerged, each cluster being replaced by viruses with distinct antigenic characteristics (11). Single amino acid substitutions, restricted to seven positions (145, 155 to 159, 189, and 193) on the HA protein, were responsible for antigenic cluster transitions during the evolution of H3N2 viruses from 1968 to 2003 (13). One mutation detected in H3N2 viruses in our study, encoding Q156H, fell within this defined “antigenic ridge.” A Q156H substitution was responsible for the Sydney-1997 to Fujian-2002 antigenic cluster transition during the 2003-04 season, resulting in vaccine mismatch (12, 36). Acquisition of a glutamine (Q) at HA position 156 during egg passage of vaccine seed stocks was also associated with low vaccine effectiveness against H3N2 viruses in the 2012-13 season (49). Contemporary H3N2 strains in circulation possess a histidine at HA position 156 in publically available consensus sequences; however, we found that 3.3% of sequences within a single vaccinated case (V130083) possessed a Q156R substitution. Acquisition of arginine at position 156 has been previously linked to egg adaptation of the initial egg-grown A/Victoria/361/2011 wild-type virus for the production of the 2012-13 and 2013-14 vaccines (37). However, the impact of Q156R on recognition of antibodies raised by vaccines encoding glutamine at position 156 has yet to be evaluated *in vitro* or in animal models.

The overwhelming majority of isolated H1N1pdm viruses remain antigenically similar to the prototypical A/California/07/2007 vaccine virus (50). In the HA protein of H1N1pdm viruses, substitutions in amino acid positions 154 to 162 (H3 numbering) located adjacent to the receptor-binding site can cause escape from human infant and ferret antibodies after H1N1pdm virus infection (i.e., ≥ 4 -fold reduction in HI titer) (13). We detected a G158E substitution in a nonvaccinated case and a vaccinated case in 2.3 and 2.4% of sequences, respectively. The G158E substitution considerably alters the electrostatic properties which may be important for surface interactions between the HA protein and antibodies (41), changing the antigenic properties of H1N1pdm viruses as mapped by human monoclonal antibodies (13, 51) and by polyclonal antibodies in ferrets (13, 41, 42). In three humans, one nonvaccinated and two vaccinated, we detected an N159K

substitution at a low frequency. The N159K mutation has been described as affecting the local electrostatic charge potential of the HA protein (42). In a ferret model, the N159K mutation was found to confer escape from H1N1pdm-specific antibodies elicited by suboptimal vaccination (42). Viruses with the N159K mutation remained fit in a ferret transmission model and outcompeted the wild-type virus. During replication in ferrets, N159K variant viruses acquired additional mutations in HA and other gene segments. Interestingly, when the N159K mutant virus was generated by reverse genetics using backbones from A/Puerto Rico/8/1934 (H1N1) and A/Perth/261/2009 (H1N1pdm), it could not be rescued as a pure population, demonstrating that additional mutations not in the reverse genetic virus must be acquired to restore fitness. Furthermore, because a pure population of N159K virus was not recovered, escape from adult human antisera could not be tested (42). In another study, Koel et al. introduced different substitutions at position 159 (N→D, N→G, N→Y, and N→S) and found that this position was important for antigenic change of H1N1pdm viruses, as measured against both human and ferret sera (13).

The ways in which influenza viruses evolve antigenically at the population level have been the subject of intense study in the past decade (10–12, 52). However, new antigenic variants are initially generated and selected at the level of individual infected hosts. Here, we showed that influenza virus populations infecting individual humans possess potential antigenic variation at low frequency. We previously showed that even very low-frequency influenza viral variants can pass through selective bottlenecks and be transmitted to new hosts via the airborne route. It is therefore possible that low-frequency putative antigenic variants we observed here could be transmitted via respiratory droplets. The use of Sanger sequencing for influenza surveillance typically defines consensus sequences and cannot resolve minority variants present below 20% of the viral population (18). Traditional Sanger sequencing could therefore miss potentially antigenic variants that occur in nature. The evolutionary mechanism by which antigenic variants are generated and selected for within individual hosts remains unclear. Our findings suggest that generating antigenic variants through mutation alone may not be sufficient for the emergence of new antigenic variants within a host. Instead, antigenic variants likely need compensatory mutations to restore replicative fitness, to avoid loss by genetic drift, and to be transmitted to new hosts to successfully emerge in nature. Additional studies are needed to more fully define the molecular basis of antigenic change and, perhaps more importantly, to determine the impact of antigenic changes on viral fitness and the evolutionary pathways by which fitness is restored in antigenic variants.

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