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Directed selection of influenza virus produces antigenic variants that match circulating human virus isolates and escape from vaccine-mediated immune protection

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Summary

Influenza vaccination does not provide 100% protection from infection, partly due to antigenic drift of the haemagglutinin (HA) protein. Low serum antibody titres increase the risk of infection. To determine whether there were additional correlates of risk, we examined the relationship between human serum immunity and antigenic variation in seasonal H3N2 influenza viruses. Seasonal H3N2 vaccine strains grown in the presence of heterogeneous human or mono-specific ferret antisera selected variants with mutations in the HA antigenic sites. Surprisingly, circulating strains infecting human subjects in the same seasons displayed mutations in the same positions, although only in one case did the change correspond to the same amino acid. Serum antibody titres were lower against both the *in vitro* selected and clinical isolates compared with the vaccine strains, suggesting that the mutations are relevant to vaccine failure. Antibody titres were also significantly lower in sera from infected subjects than in non-infected subjects, suggesting relatively poor responses to vaccination in the infected subjects. Collectively, the data suggest that risk from influenza infection is a result of poor response to vaccination, as well as encounter with drifted seasonal influenza virus antigenic variants. The results also show that directed selection under human immune pressure could reveal antigenic variants relevant to real-world drifted viruses, helping in annual vaccine re-formulation.

Keywords: antigenic drift; circulating strains; haemagglutinin; human influenza virus; influenza-specific antibodies; influenza virus vaccination; vaccine strain.

Introduction

Despite efforts to vaccinate, influenza A (IAV) and B (IBV) viruses are two of the most common causes of respiratory virus infections in humans.[1] IAVs are classified in subtypes, according to the major two surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). In humans, the most frequent seasonal subtypes of IAVs are the H3N2 and the H1N1, and two lineages of IBVs. Accordingly, the seasonal influenza vaccines include three or four viral strains (H1N1, H3N2, and one or two IBVs).[2]

Influenza virus vaccines confer protection mainly through the induction of neutralizing antibodies that block HA attachment to sialic acid-bearing proteins on the cell surface,[3,4] and to a lesser extent to the NA, which can inhibit the ability of the NA to facilitate virus release from infected cells.[5,6] The influenza HA receptor is a trimer consisting of HA1 and HA2 subunits. HA1 forms the globular head domain containing the receptor-binding site, and is the least conserved influenza virus protein. HA2 together with some HA1 residues forms a stalk-like domain, which includes the transmembrane region and is highly conserved. Antibodies directed against the head of the HA are the most abundant of the neutralizing antibodies after vaccination, although antibodies directed against the more conserved HA-stalk domain have been found in humans.[7–12]

Since 1968, H3N2 IAVs have been continually circulating in humans and undergoing antigenic drift.[13] This process has been more extensively studied for the HA protein, but it has been shown that the NA can also undergo antigenic drift.[14,15] Antigenic drift necessitates periodic updates to the virus strains contained in the seasonal vaccine to maintain a good match with circulating viruses.[16–18] Drift is believed to occur due to immune pressure resulting in selection of viruses that can escape. Many studies have demonstrated the selection of escape mutations in the HA, and to a lower extent in the NA, in the presence of monoclonal antibodies[19] or immune animal sera.[20,21] However, few studies have directly demonstrated the selection of mutations in the HA protein using human sera,[22] a process that could aid in the selection of the strains used for vaccination.

In most years, the strains of influenza in the vaccine and those circulating match well, but every so often the vaccines poorly match the predominant circulating viruses, and protection is further reduced.[23–26] Even when the vaccine is a good match, protection is < 100%,[27] raising the question of why some individuals are not protected in spite of recent immunization. One explanation is that the viruses that are circulating are in fact antigenically distinct enough from the vaccine strains that they can escape the vaccine-induced immunity. The second possibility is that the vaccine fails to elicit a protective antibody response in some people in terms of amount or specificity. However, few studies have compared the reactivity of immune sera obtained from flu-infected patients against the infecting and vaccine viruses.

In this study, we evaluated the relationships between human immunity to influenza and selection of *in vitro* selected and naturally occurring antigenic variants. We observed a close match between the artificially selected and natural clinical variants, suggesting a method to reveal potential mutations likely to occur in seasonal viruses that contribute to antibody evasion. This could improve strategies to predict emerging seasonal variants and update the strain vaccine accordingly.

Materials and methods

Study design and human subjects

Human subjects were enrolled as part of either an 'acute influenza' surveillance protocol, or from a prospective 'family flu' surveillance study of families with at least one child in the household < 4 years of age (denoted with an 'F' after the subject number; see Supplementary material, Table S1). In both cohorts, subjects reporting influenza-like illness (fever, cough, rhinitis) were asked to visit the University of Rochester Vaccine Research Unit for sampling by nasal wash and nasopharyngeal swab (combined). Sera from subjects enrolled in the acute influenza surveillance protocol were obtained at the acute visit (2–3 days post-infection), and around 28 days later. For the 'family flu' surveillance protocol, sera from subjects were obtained before the start of the flu season, and around 28 days after the acute illness visit. Subjects with a positive RT-PCR for influenza A or B were subsequently asked to return to the Vaccine Research Unit for additional sampling. In the families, close contacts of the infected subjects were also sampled by nasal wash and nasopharyngeal swab and blood draw. Families were followed for at least 1 week for evidence of infection. Some of these people received influenza vaccination at the beginning of the season (H3 component A/Perth/16/2009 and A/Victoria/361/2011, for seasons 2010/11 and 2012/13, respectively; see Supplementary material, Tables S1 and S2). Each study was approved by the University of Rochester Human Research Subjects Review Board (protocol numbers 09-0034 and 07-0046). Informed written individual or parental consent was obtained for each participant.

Cells and viruses

Madin–Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection (CCL-34; ATCC, Manassas, VA). MDCK cells were grown in Dulbecco's modified minimal essential medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco) and 100 units/ml penicillin, 0·1 mg/ml streptomycin and 50 μg/ml gentamicin (Gibco).

The influenza vaccine strains A/Victoria/361/2011 and A/Perth/16/2009, grown in eggs, were obtained from BEI Resources, Manassas, VA (NR-44022) and Influenza Reagent Resource, Manassas, VA (FR-370), respectively.

Virus isolation and titrations

Starting with nasal washes, MDCK cells were inoculated and observed for cytopathic effect. Supernatants were collected and used to infect fresh cells. In the case that no cytopathic effect was observed, supernatants were collected at 72 hr post-infection, and used to infect fresh cells up to three serial passages. For virus titrations, confluent MDCK cell monolayers were infected with 10-fold serial dilutions, monolayers were overlaid with agar and incubated for 3 days at 37°. Viral plaques were visualized 3 days post-infection fixing the cells with 10% formaldehyde and staining them with crystal violet. All the infections were performed in the presence of 1 μg/ml of TPCK trypsin (Sigma, St Louis, MO).

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Virus passages

Viruses A/Perth/16/2009 and A/Victoria/361/2011 and viruses isolated from patients were incubated for 1 hr at room temperature with limiting neutralizing dilutions of sera from patients (acute visit), and ferret sera, or in the absence of sera. Then, sera–virus mixtures were used to infect MDCK cells. When cytopathic effect was evident, total RNA was collected, an RT-PCR was performed, and the PCR products were sequenced, using the protocol described above. Supernatants were collected and passaged in the presence of sera, up to 10 times.

Competition experiments

The viruses obtained after growing the reference vaccine strain A/Victoria/361/2011 in the presence of immune sera were mixed with the original A/Victoria/361/2011 virus in a proportion of 1 : 10. The mixtures were incubated with the patient sera for 1 hr at room temperature, and the mixtures were used to co-infect MDCK cell monolayers. When cytopathic effect was 10%, cellular extracts were collected, and the HA protein was sequenced as described above.

HA sequencing

RNA was obtained from 300 μl of patient nasal washes using the QIAamp viral RNA extraction kit (Qiagen, Hilden, Germany) or from cell culture extracts using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Reverse transcription reactions were performed for 2 hr at 37° using the High-capacity cDNA reverse transcription kit (Life Technologies, Carlsbad, CA), and the primers HA-A(H3)-5′-VS (5′-GCAAAAG CAGGGGATAATTCTATTAACCATG-3′) and HA-A-3′-RS (5′-CGTCTCGTATTAGTAGAAACAAGGGTG-3′), complementary to nucleotides 2–32 of HA open reading frame and nucleotides 1761–1787 of the HA gene, untranslated region, respectively. The cDNAs were amplified in two HA overlapping regions by using the Pfx polymerase (Life Technologies), and the primers HA-A (H3)-5′-VS and HA-A-1039-RS (5′-GCCTCTAGTTT GTTTCTCTGGTACATTTCG-3′) (for region 1), and H3-F (5′-AAGCATTCCYAATGACAAACC-3′) and HA-A-3′-RS (for region 2). The VS and RS primers used for each PCR were used for Sanger sequencing. Starting from nasal washes/swabs, the complete HA sequences of 14 clinical samples from the 2010/11 season, and 13 clinical samples from the 2012/13 season, were obtained (GenBank: KR611819, KR611821, KR611822, KR611824, KR611826-28, KR611830-35, KR611837, KR611839-41, KR611843, KR611844, KR611846, KR611848-52, KR869978, KR869979), and compared with the sequence of the viruses A/Perth/16/2009 (GenBank: GQ293081.1) and A/Victoria/361/2011 (GenBank: KC306165.1) used for vaccination in each season, respectively. Four of 14 and six of 13 viruses from the 2010/11 and 2012/13 seasons, respectively, were successfully isolated. The HA protein sequences of virus stocks were sequenced (GenBank: KR611820, KR611823, KR611825, KR611829, KR611836, KR611838, KR611842, KR611845, KR611847, and KR861153).

Publically available HA sequences

Complete segment HA protein sequences for viruses that circulated in the USA during the 2010/11 (216) and 2012/13 (402) seasons were obtained from the Influenza Resource Database (www.fludb.org). Only sequences with 566 amino acids were used (616) and those that were missing amino acids where removed (552).

Clade classification and phylogenetic tree

Isolated viruses were aligned with H3N2 clade reference strains (http://ecdc.europa.eu/en/publications/Publications/influenza-virus-characterisation-April-2015.pdf), using the MUSCLE algorithm. Phylogenetic trees were created using PhyML analysis[28]

Principal component analysis for displaying antigenic relatedness between influenza viruses

The amino acid HA sequences were aligned using the Clustal multiple alignment tool. To determine antigenic relatedness, the amino acids were restricted to the five neutralizing epitopes (A, B, C, D and E) of the HA protein.[29] The percentage of amino acids differing between a set of viruses was calculated for each epitope and a distance matrix was constructed. To account for differences in neutralizing efficiency difference between epitopes, each epitope's distance matrix was weighted using the distance from the centroid of the atomic positions of amino acids found in each epitope and the centroid of atomic positions of the receptor-binding site.[30] The distance matrices for each of the epitopes were then summed and principal component analysis was performed using the factominer r package.

HA inhibition and virus microneutralization assays

Sera from ferrets infected with influenza viruses A/Perth/16/2009 and A/Victoria/361/2011 were obtained from the Influenza Reagent Resource (FR-446 and FR-1079, respectively). For HA inhibition (HAI) assays, twofold dilutions of human or ferret sera treated with receptor destroying

enzyme (RDE; Denka Seiken, Campbell, CA) were mixed with 8 HA Units/50 μl of each virus. The HA proteins of vaccine strain viruses used in these assays were identical to the ones deposited in GenBank (accession numbers GQ293081.1 and KC306165.1, for A/Perth/16/2009, and A/Victoria/361/2011, respectively). The mixtures were incubated with 0·7% guinea pig red blood cells (RBCs; Lampire Biological Laboratories, Pipersville, PA), for 60 min to allow haemagglutination of RBCs. The HAI assays were performed in the presence of 20 nM oseltamivir carboxylate. The HAI titre was defined as the highest dilution of serum that inhibits the haemagglutination of RBCs. For microneutralization (MN) assays, two-fold dilutions of human or ferret sera treated with RDE (Denka Seiken) were mixed with 200 plaque-forming units of each virus, and incubated for 60 min at room temperature to allow the binding of the antibodies to the viruses. The serum–virus samples were then transferred to MDCK cells in M96 plate wells. After an absorption period of 60 min, the inocula were removed, and the cells were cultured for 3 days in Dulbecco's modified minimal essential medium containing 10% fetal bovine serum and 1 μg/ml of TPCK trypsin (Sigma). Then, a haemagglutination assay was performed to analyse virus production. The MN titre was defined as the highest dilution of serum that completely abolishes the growth of influenza virus. When HAI and MN titres were below the limit of detection (< 10), an arbitrary value of 2 was considered.

Avidity assays

Guinea pig RBCs (Lampire Biological Laboratories) were treated with different amounts (from 25 000 to 0 μg/ml) of RDE (Denka Seizen) during 90 min at 37°. The RBCs were then washed with PBS, and 0·7% (volume/volume) RBC solutions were made in PBS; 50 μl of RBC solution was mixed with 8 HA units of virus (as determined in non-RDE treated RBCs), and the mixtures were incubated for 2 hr at 4°, before agglutination was measured.

Statistical analysis

Differences were examined by the Wilcoxon rank sum test for between-group comparisons and by the Paired *t*-test for paired-sample comparisons. For HAI or MN titres below the limit of detection,[10] an arbitrary value of 2 was assigned.

Results

Selection of HA variants by growing influenza viruses in the presence of ferret and human immune sera

At least five antigenic sites (A–E) have been assigned to IAV of H3N2 subtype.[31,32] It has been shown that after influenza virus exposure, human and ferret antisera contain antibodies that differ in the antigenic sites recognized.[2,33] To determine whether these differences can be used to interrogate mutability in seasonal vaccine strains of the virus, vaccine and circulating influenza strains from matching seasons were grown in the presence of different human and ferret antisera. The vaccine strain A/Victoria/361/2011 (from the 2012/13 season), was passaged in the presence of sera from the acute visits of six subjects (numbers 55, 58, 65, 87, 90 and 91), or in the presence of sera from ferrets infected with A/Victoria/361/2011. In addition, the viruses isolated from three subjects (numbers 58, 85 and 91), were passaged in the presence of the sera from each corresponding subject and in the presence of A/Victoria/361/2011 ferret antisera. As expected, no mutations of known antigenic sites were found in any virus passaged in the absence of sera and no mutations in antigenic sites were observed for the clinical isolates passaged in matched serum (Table 1 and data not shown), demonstrating that the matched serum–virus combination does not select for additional antigenic variation. Interestingly, human immune sera selected A/Victoria/361/2011-derived viruses incorporating non-silent mutations in known antigenic sites, but not elsewhere (Table 1). Sera from patients 55, 58 and 91 selected the same three-point mutations (Q172R, D206E, Y235S, in antigenic sites B, B and D, respectively). In addition, sera from subject 58 selected the mutation F175L, in antigenic site B. Sera from patients 65, 90 and 87 selected viruses with mutations T144N (site B), I242N (site D) and I156R: N142D (site A), respectively. Similarly, immune ferret antisera selected a virus with a mutation I242S (site D). Silent mutations were not found in any virus, suggesting that immune sera exert non-random positive pressure in the viruses. These results indicate that viruses presenting mutations in antigenic sites are selected in the presence of immune sera. Further, different serum pools selected different antigenic mutations, demonstrating variability in immunity among the human subjects distinct from ferret antisera.

To test whether the variants selected in the HA protein contribute to immune evasion, HAI assays were performed with sera from ferrets infected with the vaccine strain A/Victoria/361/2011, and the viruses from passage 10 grown in the absence of sera, or in the presence of sera from patients 55 and 91 (incorporating mutations Q172R, D206E, Y235S), 58 (incorporating mutations Q172R, F175L, D206E, Y235S), 65 (incorporating mutation T144N), 87 (incorporating mutations N142D and I156R) and 90 (incorporating mutation I242N). It has been shown that MDCK-grown H3N2 viruses can acquire mutations in the NA protein, which can facilitate the agglutination of red blood cells through the NA protein.[34,35] All the HAI assays were therefore performed in the presence of oseltamivir, a compound that binds in the sialic acid binding site of NA protein.[36] Ferret sera antibody titres for virus A/Victoria/361/2011 passaged in

Table 1. Mutations found in the haemagglutinin (HA) protein after growing the vaccine strain A/Victoria/361/2011 in the presence of human (acute visit) and ferret sera. HA inhibition (HAI) titres using sera from ferrets infected with A/Victoria/361/2011

Sera	Mutations (antigenic site)	HAI titer
55	Q172R (B)	40
	D206E (B)	
	Y235S (D)	
58	Q172R (B)	20
	F175L (B)	
	D206E (B)	
	Y235S (D)	
65	T144N (B)	80
87	N142D (A)	80
	I156R (A)	
90	I242N (D)	40
91	Q172R (B)	40
	D206E (B)	
	Y235S (D)	
Ferret	I242S (D)	80
None	P237T (–)	80

the presence of sera from patients 58 and 91, and from patients 55 and 90 were, respectively, four-fold and two-fold lower than those against the virus passaged in the absence of sera (Table 1), suggesting that the mutations selected affect antibody recognition.

To analyse whether the antigenic variants have any growth advantages in the presence of human sera, the vaccine virus A/Victoria/361/2011 and the virus incorporating mutations Q172R, D206E, Y235S, were mixed at a proportion of 10 : 1, incubated in the presence or absence of immune sera from the acute visit of patient 55, and the mixture was used to co-infect cells. In the cultures without sera, as expected, the predominant virus produced was the vaccine strain virus (Fig. 1). However, in the cells co-infected with the viruses incubated in the presence of immune sera, the antigenic variant presented a selective advantage, even in a one-step infection (Fig. 1). These data further suggest that mutations that allow the viruses to evade immune pressure provide a selective growth advantage in immune subjects.

To analyse the possibility that the escape antigenic mutations revert when the immune pressure is removed, the viruses from passage 10 grown in the presence of sera from patients 55 (incorporating mutations Q172R, D206E, Y235S), 65 (incorporating mutation T144N), 87 (incorporating mutations N142D and I156R) and 90 (incorporating mutation I242N) were passaged six times in MDCK cells in the absence of sera. No reversion was observed for any HA protein mutation (data not shown), suggesting that the mutations selected under immune pressure remain stable.

To determine whether selection of these escape mutants was unique to A/Victoria/361/2011, the vaccine strain A/

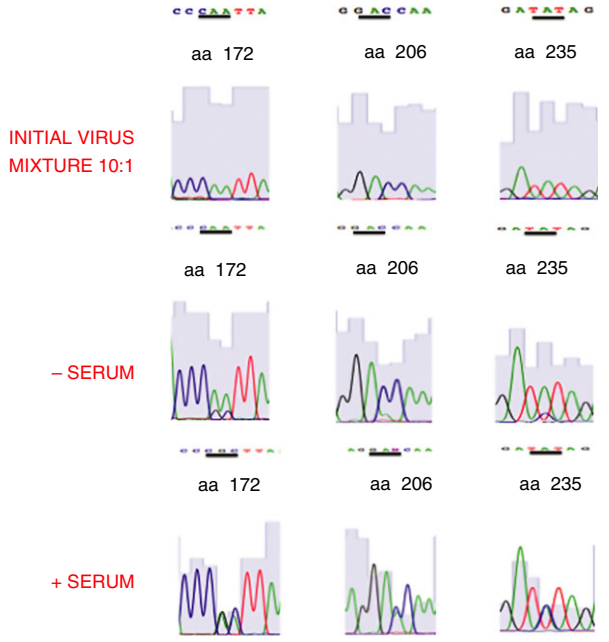

Figure 1. Co-infection of vaccine and human sera-passaged influenza virus strains. The vaccine virus A/Victoria/361/2011, and the virus that emerged after passage in the presence of human sera from subject 55 (incorporating mutations Q172R, D206E and Y235S) were used to co-infect cells at a ratio 10 : 1 in the presence (+ serum) or absence (– serum) of sera. When cytopathic effect was evident, cellular RNA from infected cells was extracted and the haemagglutinin (HA) sequence was obtained. Chromatograms showing the sequences for amino acids 172, 206 and 235 before (initial virus mixture), and after infection (– and + serum) are shown. Underlined are the codons encoding for the amino acids 172, 206 and 235.

Table 2. Mutations found in the haemagglutinin protein after growing the vaccine strain A/Perth/16/2009 in the presence of human sera (acute visit)

Sera	Mutations (antigenic site)
21	Y211H (B)
23	S278N (E)
38	K108N (E)
40	–
47	–
NONE	D505E (–)

Perth/16/2009 was passaged in the presence or absence of sera from the acute visits of five patients (numbers 21, 23, 38, 40 and 47). No mutations in antigenic sites were observed for the viruses passaged in the absence of sera, though one silent mutation at nucleotide 1326 was observed. Sera from patients 21, 23 and 38 selected viruses with the mutations Y211H (site B), S278N (site E) and K108N (site E), respectively (Table 2). No differences in HAI titres were observed between the original A/Perth/16/2009 and the viruses incorporating the mutations specified in Table 2 (data not shown); however, as these changes are

located at previously described antigenic site positions, additional binding assays should be used to detect minor differences in the antigenic character of the viruses. Collectively, these data indicate that viruses incorporating mutations in antigenic sites can be selected in the presence of immune sera, and that mutations selected can vary depending on the serum used and the strain under selection.

Comparison of naturally occurring HA protein antigenic variation to the *in vitro* selected viruses

To analyse whether the mutations found after growing the viruses in the presence of immune sera match those found in circulating strains, the complete HA sequences of 14 clinical samples from the 2010/11 season, and 13 clinical samples from the 2012/13 season, were obtained, and compared with the sequence of the viruses A/Perth/16/2009 and A/Victoria/361/2011 used for vaccination in each season, respectively, and to the sequences of the sera-grown viruses. The viruses from the 2010/11 season had incorporated mutations in four (two viruses) or five (12 viruses) antigenic sites, with six to ten unique amino acid changes (Tables 3 and 4).[26,30] Viruses from the 2012/13 season incorporated mutations in three (six viruses), four (six viruses), or five (one virus) antigenic sites, with five to eight unique amino acid changes (Tables 3 and 4).[26,30] In addition to these previously described positions for antigenic sites, mutations I239V, M239V, N505D and N519K (2010/11 season) and Y9H, R49Q, M363V and N505D (2012/13 season) were found. Mutations E78K (site E), N160K (site A), A228T (site D), I230S (site D) (season 2010/11) and mutations R49Q, H172Q (site B), G202V (site B), S235Y (site D) and K294N (site C) (season 2012/13) were common for all the clinical isolates in each season (Table 3). The antigenic site differences between the HA protein of circulating viruses (from subjects 85 and F46) and vaccine strains showing the most prevalent mutations are illustrated (Fig. 2). From the 2010/11 season, 7·7% of the viruses corresponded to clade 3A, 46·1% of the viruses corresponded to clade 3C, 38·5% of the viruses corresponded to clade 5, and 7·7% of the viruses corresponded to clade 6 (see Supplementary material, Table S2 and Fig. S1). From 2012/13, all the viruses corresponded to clade 3C, as did the vaccine strain A/Victoria/361/2011 (see Supplementary material, Table S2, and Fig. S1).

Interestingly, amino acid changes in positions 144, 156, 172, 235 and 242, found when passing the vaccine strain A/Victoria/361/2011 in the presence of human and ferret sera, were found in viruses isolated from patients in this study (Table 1). Similarly, an amino acid change in position 108, found in the human sera-grown A/Perth/16/2009 virus, was found in circulating strains. However, only in the case of mutation S235Y, the amino acid that changed was exactly the same (Table 3). These data suggest that passing virus under contemporary immune pressure could reveal information about the most likely potential mutations leading to escape and could be useful in assessing virus risk and inform annual vaccine updates.

Sequence-based antigenic cartography and serological distance measures

To look at the antigenic relatedness of the variant viruses and the vaccine strains, a principal component analysis was performed comparing the HA antigenic site sequences from the 2010/11 and 2012/13 circulating viruses obtained in this work, those reported from the same seasons circulating in USA in the Influenza Research Database (IRD), and those of the vaccine strains from the 2004/05 to 2014/15 seasons. Principal component analysis showed that the circulating viruses were genetically distinct from the vaccine strain viruses in the antigenic sites (Fig. 3). Interestingly, viruses isolated from patients F46, F49, F192, F193 and F292 (2010/11 season), were more similar to the strain A/Victoria/361/2011 used for vaccination in the 2012/13 and 2013/14 seasons, than to the strain A/Perth/16/2009 (2010/11 season). Similarly, the viruses isolated from patients 73, 85 and 87 (2012/13 season), were more similar to strain A/Switzerland/9715293/2013 used for vaccination in the southern hemisphere in the 2014/15 season, than to the strain A/Victoria/361/2011 used in the 2012/13 and 2013/14 seasons.

To analyse the relevance of the identified mutations in evading the host immune response, HA protein sequences of virus stocks were sequenced and compared with the sequence obtained directly from the nasal washes. From the ten isolated viruses, two viruses presented two amino acid changes, and two viruses presented one point change (see Supplementary material, Table S3). However, none of these changes were located in known antigenic sites. The NA protein was sequenced as well to analyse changes in this protein among the viruses present in the nasal washes/swabs and the MDCK cell-grown viruses. All of the MDCK cell-grown stocks presented a polymorphism in the codon for residue 151, with the presence of D or N at residue 151. It has been observed that the mutation D151N facilitates the NA protein-mediated binding to RBCs,[34,37] so all the HAI assays were performed in the presence of oseltamivir, a compound that binds in the sialic acid binding site of NA protein.[36]

The isolated and vaccine strain viruses were used in HAI and MN assays with sera from ferrets infected with each vaccine reference strain. In both assays, antibody titres were 2- to 16-fold lower for all the isolated viruses than for the vaccine strain virus (Fig. 4). These data indicated that the mutations found in the HA may contribute to the failure of antibodies to inhibit haemagglutination and to neutralize the viruses.

Table 3. Antigenic site mutations in the haemagglutinins from influenza A/H3N2 circulating during 2010/11 and 2012/13 seasons

	Mutations in HA																										
Ag site	A				B							C						D					E				
HA position	156	158	160	161	144	172	173	202	208	214	215	61	64	69	294	296	328	224	228	230	235	246	73	78	108	110	277
2010/11 season																											
Perth	I	R	K	N	T	H	L	G	I	A	S	S	T	D	N	E	N	R	T	S	S	I	Q	K	K	Y	R
21	I	R	N	N	T	H	L	G	T	A	S	S	T	N	N	A	N	R	A	I	S	V	Q	E	K	H	R
38	I	R	N	N	T	H	L	G	T	A	S	S	T	N	N	A	N	R	A	I	S	V	Q	E	K	H	R
40	I	R	N	N	T	H	L	G	I	S	S	N	T	D	N	E	N	R	A	I	S	I	Q	E	K	Y	R
44	I	R	N	N	T	H	L	G	I	A	A	S	T	N	N	A	N	R	A	I	S	V	Q	E	K	H	R
47	M	R	N	N	T	H	L	G	I	A	S	S	T	N	N	A	N	R	A	I	S	V	Q	E	K	H	R
F46	I	R	N	N	T	H	S	G	I	S	S	N	I	D	K	E	S	R	A	I	S	I	Q	E	K	Y	R
F47	I	R	N	N	T	H	L	G	T	A	S	S	T	N	N	A	N	R	A	I	S	V	Q	E	K	H	R
F49	I	R	N	N	T	H	S	G	I	S	S	N	T	D	K	E	S	R	A	I	S	I	Q	E	K	Y	R
F75	I	R	N	N	T	H	L	G	T	A	S	S	T	N	N	A	N	R	A	I	S	V	Q	E	K	H	R
F153	I	R	N	N	T	H	L	G	I	A	S	S	A	D	N	E	S	R	A	I	S	I	H	E	R	Y	R
F192	I	R	N	N	T	H	S	G	I	S	S	N	K	D	K	E	S	R	A	I	S	I	Q	E	K	Y	R
F193	I	R	N	N	T	H	S	G	I	S	S	N	K	D	K	E	S	R	A	I	S	I	Q	E	K	Y	R
F292	I	R	N	N	T	H	S	G	I	S	S	N	K	D	K	E	S	R	A	I	S	I	Q	E	K	Y	R
F294	I	R	N	N	T	H	L	G	I	S	S	N	T	D	N	E	N	R	A	I	S	I	Q	E	K	Y	R
2012/13 season																											
Victoria	I	R	N	N	T	Q	L	V	I	S	S	N	I	D	N	E	S	R	A	I	Y	I	Q	E	K	Y	R
55	I	R	N	S	T	H	L	G	I	S	S	N	I	D	K	E	S	R	A	I	S	I	Q	E	K	Y	R
56	I	R	N	N	T	H	S	G	T	S	S	N	I	D	K	E	S	R	A	I	S	I	Q	E	K	Y	R
58	I	R	N	N	T	H	S	G	I	S	S	N	I	D	K	E	S	R	A	I	S	I	Q	E	K	Y	R
64	I	R	N	N	T	H	S	G	I	S	S	N	I	D	K	E	S	R	A	I	S	I	Q	E	K	Y	R
65	I	R	N	N	T	H	S	G	I	S	S	N	I	D	K	E	S	R	A	I	S	I	Q	E	K	Y	R
68	I	R	N	S	T	H	S	G	I	S	S	N	I	D	K	E	S	R	A	I	S	I	Q	E	K	Y	R
72	I	R	N	N	T	H	S	G	T	S	S	N	I	D	K	E	S	R	A	I	S	I	Q	E	K	Y	R
73	I	G	N	S	A	H	L	G	I	S	S	N	I	D	K	E	S	R	A	I	S	I	Q	E	K	Y	R
74	I	R	N	N	T	H	S	G	T	S	S	N	I	D	K	E	S	R	A	I	S	I	Q	E	K	Y	R
85	I	G	N	S	A	H	L	G	I	S	S	N	I	D	K	E	S	R	A	I	S	I	Q	E	K	Y	Q
87	I	G	N	S	A	H	L	G	I	S	S	N	I	D	K	E	S	R	A	I	S	I	Q	E	K	Y	R
90	I	R	N	S	T	H	L	G	I	S	S	N	I	D	K	E	S	I	A	I	S	I	Q	E	K	Y	R
91	I	R	N	S	T	H	L	G	I	S	S	N	I	D	K	E	S	I	A	I	S	I	Q	E	K	Y	R

Table 4. Mutations in antigenic sites in haemagglutinins from influenza A/H3N2 viruses circulating during 2012/11 and 2012/13 seasons

	Mutations in haemagglutinin				
Antigenic site	A	B	C	D	E
2010/11 season					
21	160	208	69, 296	228, 230, 246	78, 110
38	160	208	69, 296	228, 230, 246	78, 110
40	160	214	61	228, 230	78
44	160	215	69, 296	228, 230, 246	78, 110
47	156, 160	–	69, 296	228, 230, 246	78, 110
F46	160	173, 214	61, 64, 294, 328	228, 230	78
F47	160	208	69, 296	228, 230, 246	78, 110
F49	160	173, 214	61, 294, 328	228, 230	78
F75	160	208	69, 296	228, 230, 246	78, 110
F153	160	–	64	228,230	73, 78, 108
F192	160	173, 214	61, 64, 294, 328	228, 230	78
F193	160	173, 214	61, 64, 294, 328	228, 230	78
F292	160	173, 214	61, 64, 294, 328	228, 230	78
F294	160	214	61	228, 230	78
2012/13 season					
55	161	172, 202	294	235	–
56	–	172, 173, 202	294	235	–
58	–	172, 173, 202	294	235	–
64	–	172, 173, 202	294	235	–
65	–	172, 173, 202	294	235	–
68	161	172, 173, 202	294	235	–
72	–	172, 173, 202	294	235	–
73	158, 161	144,172, 202	294	235	–
74	–	172, 173, 202	294	235	–
85	158, 161	144,172, 202	294	235	277
87	158, 161	144, 172, 202	294	235	–
90	161	172, 202	294	224, 235	–
91	161	172, 202	294	224, 235	–

As discussed previously, human sera differ from ferret sera, and are more complex in terms of antibody repertoire, probably because of different histories of vaccination and infection.[33] HAI and MN assays were performed using the sera obtained from the subjects at the time of the acute illness visit (approximately 2–3 days after infection), and again approximately 28 days after. In both assays, antibody titres against the infecting viruses were 2- to 16-fold lower than those observed for the virus used for vaccination at either time-point (Fig. 5). In all the subjects, virus-specific serum antibody titres increased by the post-illness visit. The data indicate that there was slightly higher fold increase in HAI and MN titres against the vaccine virus compared with the infecting virus for the 2010/11 season, but not the 2012/13 season, when the fold increase to the infecting viruses was much greater. The theory of antigenic distance[29] would suggest that in 2010/11, the infecting viruses were antigenically too close to the vaccine virus to effectively boost antibody responses, whereas the 2012/13 infecting viruses were distant enough to not compete with the vaccine elicited antibodies and B cells.

It has been proposed that under immune pressure, the HA proteins of influenza viruses incorporate mutations in the globular domain that increase or decrease the avidity of binding to sialic acids,[38–40] leading to lower or higher HAI titres, respectively. To be certain that decreased antibody titres were not due to increased binding of HA to its receptors, HA assays were performed using RBCs treated with different concentrations of RDE in the presence of oseltamivir. No significant differences were observed when comparing HA binding avidities of the virus A/Victoria/361/2011, the viruses incorporating mutations after growing them in the presence of ferret and human sera (Table 1) or viruses isolated from patients 55, 58, 65, 85, 90 and 91 (data not shown). In the case of viruses isolated from patients 21, 38, 44 and 47, four-, four-, four- and two-fold decreases in binding avidities, respectively, compared with virus A/Perth/16/2009 were detected (data not shown). These data indicate that the HA proteins encoded in the viruses isolated do not display increased binding avidities to the sialic acid cell surface receptors, and therefore, the lower antibody titres compared with the vaccine strains are most probably the result of antigenic changes.

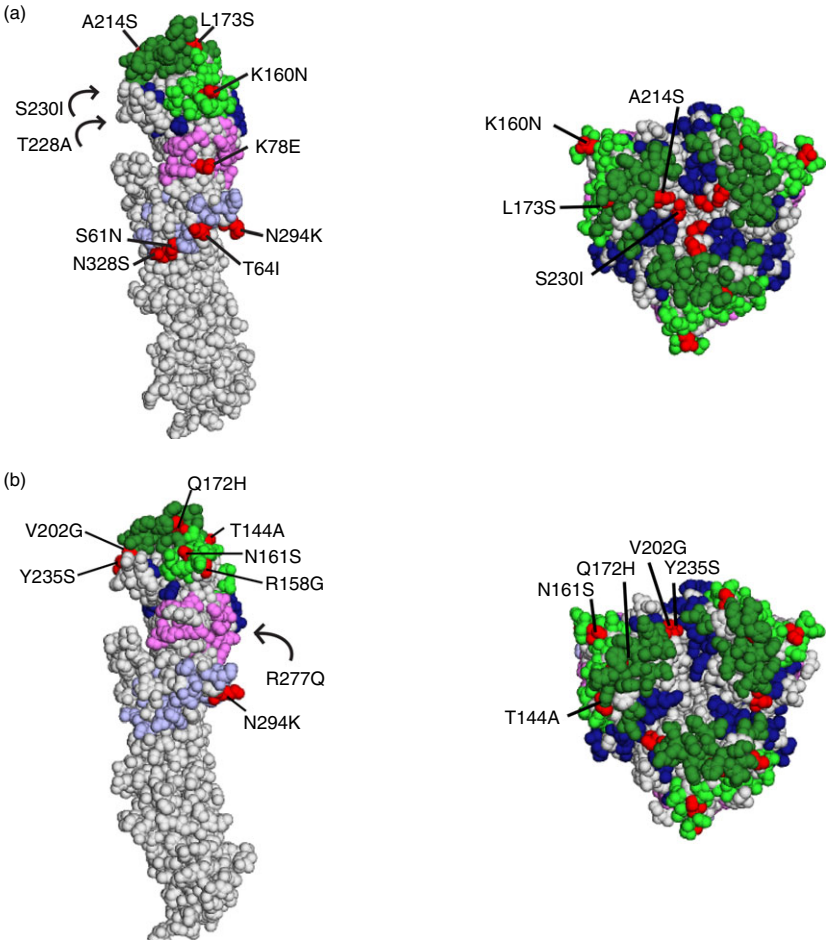

Figure 2. Three-dimensional model of haemagglutinin (HA) antigenic site-related mutations between circulating viruses, and the viruses used for vaccination. Viral HA sequences were derived from viruses infecting subjects F46 (a) and 85 (b) in seasons 2010/11 and 2012/13, respectively. One monomer (left picture) and the trimer conforming the receptor-binding site viewed from the top (right picture), are shown. The antigenic sites A, B, C, D and E are coloured in light green, dark green, purple, blue and pink, respectively. The amino acid changes between circulating and vaccine viruses are shown in red.

To demonstrate whether subjects with no evidence of infection have generally higher antibody titres, suggesting that they are at lower risk for infection, sera from 24 subjects collected before the 2010/11 flu season with no evidence of infection, and sera from 12 subjects who were infected (see Supplementary material, Tables S4 and S5, designated with an 'F', and sera from subjects 21, 38, 44 and 47), were evaluated in HAI assays using the virus A/Perth/16/2009, and the virus isolated from patient 47 in 2010/11. As in Fig. 4, serum titres were higher against the vaccine strain than against the infecting virus (Fig. 6). Interestingly, with few exceptions, serum titres from infected people were lower against the two viruses than in sera from non-infected people (Fig. 6), which is consistent with the conclusion that lower antibody titres correlate with a higher risk of influenza virus infection.

Vaccination decreases the risk of infection by raising specific antibody titres. To analyse whether the titres of antibodies specific for influenza HA protein are higher in subjects vaccinated in the 2010/11 season, than in non-vaccinated subjects, sera from 23 vaccinated subjects, and sera from 12 non-vaccinated subjects (see Supplementary material, Tables S4 and S5), were evaluated in HAI assays using the virus A/Perth/16/2009. As expected, sera HAI titres were higher in vaccinated, than in non-vaccinated people (Fig. 7), confirming that influenza vaccination increases HA antibody titres, and therefore decreases the risk of getting influenza virus infection.

Discussion

Influenza vaccination does not produce 100% protection from infection,[27] and although it is generally known that circulating viruses are antigenically variable, and that low antibody titres are correlated with increased risk from infection, studies that link the two phenomena are limited. Studies of human serum titres tend to focus on the prototypical vaccine strains, and do not often take into account variability in the infecting viruses as an additional component of risk.

Antigenic drift in the circulating strains results in annual re-evaluation of the strains chosen for the annual vaccine. Predicting the vaccine strains currently relies primarily on measuring antigenic variation in the circulating strains using panels of standardized ferret antisera raised against prototypical vaccine strains.[2] Sequence variation is

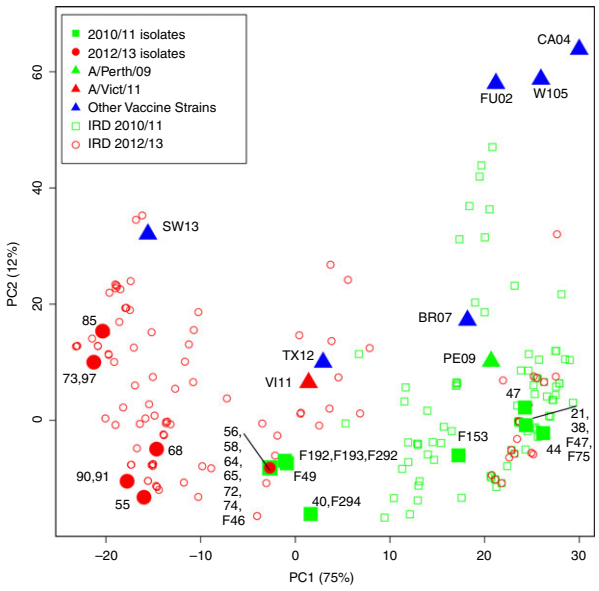

Figure 3. Principal component analysis showing antigenic relatedness for haemagglutinin (HA) proteins. Viruses sequenced in this work circulating during the 2010/11 and 2012/13 seasons are represented in large solid green squares and red circles, respectively (the last two or three numbers of the patient ID are shown, in the case of family flu study and 'F' is preceding the numbers). Green triangles and red triangles represent the viruses A/Perth/16/2009 and A/Victoria/361/2011, used for vaccination in the 2010/11 and 2012/13 seasons, respectively. Solid blue triangles represent the viruses used for vaccination in other seasons. Fu02, virus A/Fujian/411/2002, used for vaccination in 2004/05 season; CA04, virus A/California/17/2004, used for vaccination in the 2005/06 season; WI05, virus A/Wisconsin/67/2005, used for vaccination in the 2006/07, and 2007/08 seasons; BI07, virus A/Brisbane/10/2007, used for vaccination in the 2008/09 and 2009/10 seasons; PE09, virus A/Perth/16/2009, used for vaccination in the 2010/11 and 2011/12 seasons; Vi11, virus A/Victoria/361/2011, used for vaccination in the 2012/13 and 2013/14 seasons; TE12, virus a/Texas/50/2012, used for vaccination in the 2014/15 season (northern hemisphere); SW13, virus Switzerland/9715293/2013, used for vaccination in the 2014/15 season (southern hemisphere). Small open green squares and small open red circles represent the sequences from viruses obtained from the Influenza Research Database, circulating in the USA during the 2010/11 and 2012/13 seasons, respectively.

also considered, but it is not clear how the different measures of variation are weighted in the analysis and choice of strains to include in the vaccine. Adding more knowledge to the decision-making process could possibly improve the choice of vaccine strains.

Directed selection could provide a means to assess how human immunity to a given seasonal strain is most likely to drive antigenic drift. Vaccine strain viruses passaged in the presence of different human and ferret sera selected mutations in the HA proteins matching the mutated sites found in circulating strains. We chose to use different combinations of patient sera with the rationale that protective antibodies would vary in different subjects. Interestingly, not all the patient sera selected for the same mutations, confirming that individuals have different antibody repertoires and pressures depending on the region of HA bound by the antibodies. Accordingly, a study using sera from different patients revealed variations in the binding of antibodies to different regions of HA proteins.[41] Similarly to our study, when an H3N2 strain was grown in the presence of three different patient sera, the mutations selected in the HA protein antigenic sites were not always the same for the three different sera.[22]

To analyse whether the mutations selected after growing the viruses under immune pressure match those found in viruses infecting patients, the HA sequences of influenza A/H3N2 viruses from different isolates circulating in humans in the Rochester, NY area have been compared with the vaccine strains in each season, and with the sera-grown viruses. Compared with the A/Victoria/361/2011 vaccine strain, the mutations A144T (23%), G158R (23%), S161N (54%), H172Q (100%), S173L (54%), G202V (100%), S235Y (100%), K294N (100%), localized to antigenic sites in the 2012/13 isolates (Tables 3 and 4), have also been found in samples obtained from subjects in Canada.[26] Similarly, among the 2010/11 isolates, the mutations N69D (43%), E78K (100%), H110Y (36%), M156I (7%), N160K (100%), T208I (28%), A215S (7%), A228T (100%), I230S (100%), V246I (36%), K294N (36%) and A296E (43%), were found using samples from Ontario, Canada.[42] However, in this previous study, mutations N61S (50%), I64T (7%), K64T (21%), A64T (7%), S173L (36%) and S214A (50%), were not described. The mutations N61S, I64T, E78K, N160K, S214A, A228T, I230S, I239V, found in the viruses from the 2010/11 season, were present in the viruses from the 2012/13 season, and were changed in the vaccine strain used for the 2012/13 season vaccination. These data reinforce the importance of sequencing viral isolates to inform vaccine updates. The strong similarities between *in vitro* selected viruses and the ones observed in circulation suggest that simulating immune pressure *in vitro* produces meaningful functional changes in the viruses that escape.

Of note, three different human sera selected the same three mutations in the vaccine strain A/Victoria/361/2011 HA protein (Q172R, D206E, Y235S, in antigenic sites B, B and D, respectively). Compared with the strain A/Victoria/361/2011, amino acid changes in two out of these three positions (172 and 235) were found in viruses isolated from patients in this study (Tables 3 and 4), in a previous study[26] and in 100% (amino acid 172) and 99·7% (amino acid 235) of the HA sequences deposited in the IRD belonging to the 2012/13 circulating viruses in the USA. Sera from three other patients selected viruses with changes T144N (site B), I242N (site D) and I156R:

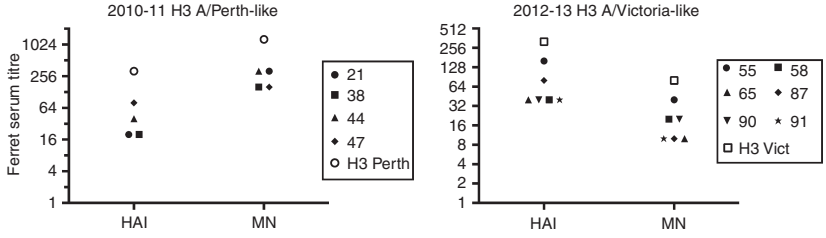

Figure 4. Haemagglutinin inhibition (HAI) and microneutralization (MN) assays using ferret antisera. Standardized antisera from ferrets infected with either the 2010/11 A/Perth/16/2009 or the 2012/13 A/Victoria/361/2011 vaccine viruses were titred using HAI or MN assays against viruses successfully isolated from the nasal washes/swabs of infected subjects, or the vaccine viruses A/Perth/16/2009 or 2012/13 A/Victoria/361/2011. Symbols in the legend correspond to the individual subject numbers from which the viruses were isolated. Experiments were repeated three times, showing reproducible data.

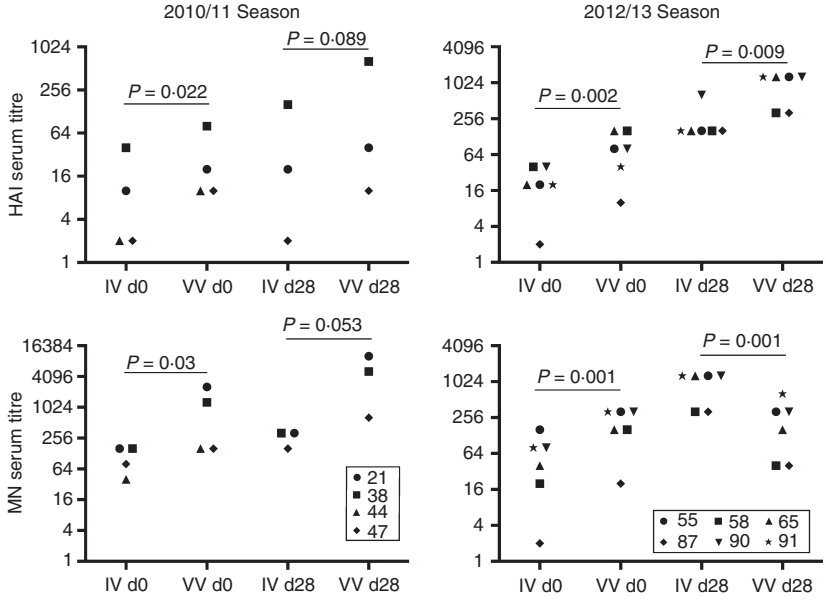

Figure 5. Haemagglutinin inhibition (HAI) and microneutralization (MN) assays using patient sera. Sera collected from infected subjects at the time of illness presentation (d0) or 28 days after presentation (d28) were tested for HAI and MN activity against matched infecting viruses (IV) or vaccine viruses (VV) corresponding to that season's H3 vaccine strain (2010/11 A/Perth/16/2009 or 2012/13 A/Victoria/361/2011). Symbols correspond to individual subject numbers. Differences in titres against vaccine strains (both A/Perth/16/2009 and A/Victoria/361/2011) and against infecting viruses from two seasons were statistically significant using the paired *t*-test (P = 0·022, 0·089, 0·030, 0·053 for HAI days 0, and 28, and MN days 0, and 28, respectively, 2010/11 season, and P = 0·002, 0·009, 0·001, 0·001, for HAI days 0, and 28, and MN days 0, and 28, respectively, 2012/13 season). Experiments were repeated three times, showing reproducible data.

N142D (site A), respectively. The ferret sera selected one change in antigenic site D (I242S). Again, mutations in three of these four positions (144, 156 and 242) were found in viruses from this and other[26] studies, and in 31·5% (amino acid 144), 1·3% (amino acid 156), and 0·5% (amino acid 242) of the HA sequences deposited in IRD within this season within the USA. Remarkably, amino acids at positions 144, 172 and 235, which were mutated using patient sera, were present in the strain A/Texas/50/2012 used for vaccination in the 2014/15 season (http://www.cdc.gov/flu/professionals/vaccination/virusqa.htm).

The similarities we produced by *in vitro* selection were not unique to A/Victoria/361/2011. After growing A/Perth/16/2009 under immune pressure, three different patient sera selected for three different mutations (Y211H, S278N and K108N, in antigenic sites B, E and E, respectively). Similar to the results with the strain A/Victoria/361/2011, changes in residue 108 have been found in this study, and changes in residues 108 (20%) and 278 (99·5%) have been found in the HA sequences deposited in IRD for viruses circulating during the 2010/11 season in the USA. Collectively, comparison of known variation and that introduced by directed

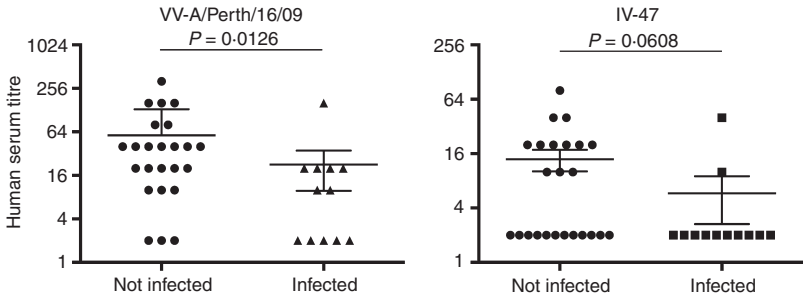

Figure 6. Haemagglutinin inhibition (HAI) assays using sera from infected and non-infected subjects. Sera were collected pre-season from 12 infected and 24 non-infected subjects in the 2010/11 season [designated as an F in Tables S4 and S5 (see Supplementary material), and sera from subjects 21, 38, 44 and 47]. The titres of HAI antibodies against the vaccine strain A/Perth/16/2009 (VV), and against the infecting viruses isolated from patient 47 (IV-47) were measured. *P*-values were calculated using a Wilcoxon rank sum test, comparing the sera from non-infected and infected subjects against each virus. Experiments were repeated three times, showing reproducible data.

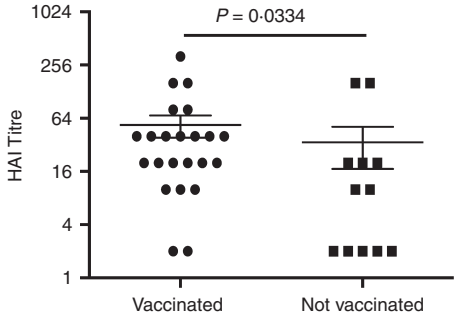

Figure 7. Haemagglutinin inhibition (HAI) assays using sera from vaccinated and non-vaccinated subjects. Sera collected from 12 non-vaccinated subjects and from 23 vaccinated subjects were collected in the autumn of 2010 [designated as an F in Tables S4 and S5 (see Supplementary material), and sera from subjects 21, 38, 44 and 47]. The titres of HAI antibodies against the vaccine strain A/Perth/16/2009. The *P*-value using the Wilcoxon rank sum test, comparing the sera from non-infected and infected subjects against each virus, is indicated. The mean and standard deviation are also plotted. Experiments were repeated three times, showing reproducible data.

selection *in vitro* using antisera reveals strong similarities that could inform the decisions to select vaccine components.

The HA antibody titres of the pre-season sera of people with no evidence of infection in the previous year were significantly higher than in the infected subjects (Fig. 6), suggesting that low antibody titres correlate with susceptibility to infection. Accordingly, in other studies, low titres of antibodies were predictive of fatal outcome following influenza A H1N1 2009 infection.[43] But why were the serum titres uniformly lower to the infecting variants? One possibility is that susceptible subjects have limitations on which antigenic variants they can respond to, perhaps due to limitations in their B-cell repertoire that reflect past exposures. It is well established, for example, that we remain immune to the viruses we are exposed to as children.[33] Such a phenomenon reduced risk from disease for subjects born before 1950 during the 2009 influenza virus pandemic.[10,44,45]

Selection in the presence of antibodies to the HA can lead to increased binding of HA protein to cellular receptors, which in turn can reduce antibody titres in HAI assays because more antibody is needed to compete for binding to the HA.[40] Other studies have produced variable results on receptor binding when selecting viruses in the presence of antibodies.[38] In our hands, HA binding avidities of circulating and sera-grown viruses were the same or lower than the vaccine strains. These observations demonstrate that the effects of antibody selection can be variable in how they affect receptor-binding avidity.

H3 HA proteins have progressively reduced receptor binding avidity and altered receptor specificity since the viruses H3N2 emerged in 1968.[46,47] Consequently, cell culture isolation of H3 viruses can select mutations in NA protein that facilitate agglutination.[34,35,48] Similarly, we have found a D151N mutation in the NA protein in viruses isolated in MDCK cells that was not present in viruses from human nasal washes/swabs. This mutation confers strong sialic acid binding and an ability to agglutinate through the NA protein.[34,35,48] In the presence of oseltamivir, HA titres were reduced with these mutated viruses (data not shown and refs 34,35), further corroborating that the mutated NA protein can mediate RBC agglutination. We found strain-dependent differences in the titres obtained in the HAI and MN assays. These differences could reflect the types of antibody activity revealed in the two approaches. For example, in the MN assays, antibodies against the NA protein, not relevant in the HAI assays, could also neutralize the virus.[5,6] In the MN assays, we did not directly investigate the contribution of the mutation in the NA protein found after growing the viruses in MDCK cells (D151N). However, it has been shown that this mutation does not affect the ability to detect HA-specific neutralizing antibodies in the MN assay.[34]

In this study, we show that viruses that resemble circulating immune escape mutants can be identified *in vitro* by culturing vaccine strain viruses in the presence of different immune serum. The observation suggest that this strategy could be used to inform the selection of virus strains for annual vaccine production, along with existing approaches using antigenic cartography and sequence variation. Further, these studies show that, on a person-to-person basis, increased risk of infection can be a result of both a poor response to the vaccine as well as antigenic variation in the circulating strains. The observations suggest that risk from infection is due not only to low antibody titres, but also to limitations in how we respond to immunization, as well as the viruses we are exposed to. The studies highlight the need to better understand the basis of failed vaccine protection and the factors that influence individual responses to infection or vaccination. Such knowledge will lead to improved seasonal vaccines and novel strategies that induce more broadly protective influenza vaccines.

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Disclosures

All authors declare no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phylogenetic tree of the viruses of the viruses sequenced in this study.

Table S1 Demographics of participants included in this study.

Table S2 Clade classification of the haemagglutinin (HA) sequences obtained in this study.

Table S3 Amino acid differences in the haemagglutinin (HA) sequences between the circulating viruses present in the nasal washes of the patients (first letter), and the viruses isolated in Madin–Darby canine kidney cells (second letter).

Table S4 Vaccination histories of the infected patients.

Table S5 Vaccination histories of the non-infected patients.