Influenza virus infection history drives and shapes antibody responses to influenza vaccination

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Influenza virus infection history drives and shapes antibody responses to influenza vaccination

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Summary

Background

The controversial hypothesis that recalled immunological memory limits responses to variant virus strains has been revived by recent reports linking poor vaccine effectiveness against A(H3N2) influenza viruses with prior vaccination. The impact of memory induced by prior infection is rarely considered, and is difficult to ascertain because infections are often sub-clinical. This study investigates influenza vaccine immunogenicity among participants who had been monitored for 9 years for clinical influenza infection or seroconversion.

Methods

In 2007, 269 households from Ha Nam, Viet Nam commenced ongoing monitoring for influenza infection. In 2016, 72 adult participants with documented prior A(H3N2) infection and 28 without infection received trivalent inactivated influenza vaccine for the first time. Serological responses were assessed by hemagglutination inhibition assay against 40 A(H3N2) viruses spanning 1968-2018. Effects of prior infection were determined by comparing geometric mean titres and titre rises. Generalized additive and lowess models were used to fit, and compare, titre landscapes across strains.

Findings

Participants with documented prior A(H3N2) virus infection had higher pre-vaccine titres against strains circulating since 2004 compared to those without prior infection. Moreover, they had higher titre rises on days 7, 14, 21 and 280 post-vaccination against vaccine and subsequently circulating strains. Accordingly, 1/72 versus 4/28 of vaccinees with and without documented prior infection experienced illness due to A(H3N2) in the season after vaccination ($p = 0.021$). The range of A(H3N2) virus clades recognized by vaccine-induced antibodies was associated with the clade that last caused infection, indicating that recalled immunity drove antibody production against shared epitopes.

Interpretation

These results suggest that immunological memory from prior infection drives and shapes antibody production induced by inactivated influenza vaccine, and underpins the capacity for vaccine to induce sufficient antibody for protection.

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Introduction

RNA viruses undergo relatively rapid mutation, which can critically impact vaccination strategies. [1] Influenza viruses are particularly prone to substitutions within the major surface protein, hemagglutinin (HA), as a consequence of viral RNA replication without proofreading,[2] and selection of human antibody escape mutants. This process, termed antigenic drift, facilitates recurrent influenza infection throughout life. In turn, prevention by vaccination, requires repeated administration of vaccine containing regularly-updated virus strains. Vaccine effectiveness (VE) has been poor against A(H3N2) viruses since at least 2010, when VE estimation by subtype became more widely implemented.[3] This could, in part, be due to greater mismatch between vaccine and circulating strains. A(H3N2) viruses have undergone greater antigenic evolution compared to A(H1N1) and B influenza viruses,[4] and more often acquire substitutions within antigenic sites when propagated in eggs to produce vaccine.[4,5] It is further speculated that vaccine immunogenicity and effectiveness may be limited by recall of immunological memory against past strains, a hypothesis that was first proposed in the 1960's and termed original antigenic sin. [6] Interest in this phenomenon has been revived by a series of recent reports that antibody responses, [7] and VE against A(H3N2) viruses [8-11] are attenuated among people who received vaccine in prior year(s). A meta-analysis indicates that while repeat vaccination effects are more pronounced for A(H3N2) than for other subtypes, there is substantial heterogeneity in effects.[12]

The cellular and molecular mechanisms underlying the variable effects of prior vaccination and pre-existing immunity remain largely undefined. The antigenic distance hypothesis postulates that when successive vaccine strains are antigenically similar, existing antibodies or memory B cells attenuate vaccine immunogenicity by masking or clearing vaccine antigen, resulting in attenuated VE if the vaccine and epidemic strains differ, but not if they are also similar.[13] Alternately, it is hypothesized that memory B cells induced by prior vaccination dominate and focus responses on epitopes that are conserved between prior and prevailing vaccine strains, compromising responses against epitopes that have changed.[14] This could enhance antibody responses and VE, if epidemic strains retain those conserved epitopes, but could reduce VE if these epitopes have changed.[14] The epitopes recognized by influenza virus neutralizing antibodies are largely located on the globular head of HA, surrounding the receptor binding site.[15] Up to 131 amino acid positions in the head of HA of A(H3N2) viruses have been associated with antigenic variation and assigned to one of five antigenic sites, designated A to E.[16,17] Antigenic sites A and B are immunodominant,[16] and single amino acid substitutions in these sites can result in escape from vaccine-induced immunity, particularly if glycosylation sites are introduced.[4,18]

Few studies consider how prior influenza infections affect the immunogenicity and protection afforded by influenza vaccines. Understanding infection history is contingent on detecting asymptomatic/subclinical

infection, which may account for up to three-quarters of influenza virus infections.[19,20] To this end, participants of a cohort in northern Viet Nam (Ha Nam Cohort), who were influenza vaccine naïve, and who had been monitored for influenza infection for 9-years, since December 2007, were vaccinated in 2016. The impact of prior infection with A(H3N2) viruses of varying antigenic distance from the vaccine was determined by measuring the titre and strain-coverage of antibodies induced by vaccination, and the capacity of the vaccine to prevent influenza-like illness (ILI) due to A(H3N2) virus infection in the subsequent season.

Methods

Study design and participants

A comprehensive study design is described in the **appendix (p 2-3),** and the full protocol is available via the Australian New Zealand Clinical Trials Registry (ACTRN, 12621000110886). Briefly, this study was part of an ongoing, prospective, population-based unvaccinated cohort study.[19] In 2007, 270 households that initially comprised a population of 945 individuals were enrolled (**figure 1a**). Participants have been monitored for influenza virus infection by active ILI surveillance and by serology on blood samples collected annually or biannually, at times spanning transmission peaks. Infection was defined as having ILI with RT PCR-confirmation of influenza virus infection or a four-fold or greater antibody titre rise (seroconversion) against a circulating strain. In 2016, we selected all 28 adult participants who had no detected A(H3N2) virus infection since 2007, then selected 72 of similar sex and age who had at least one A(H3N2) virus infection (**appendix p 3**).

Trivalent inactivated influenza vaccine (TIV; Vaxigrip, Sanofi Pasteur) was administered to the 100 selected participants in November 2016 (**figure 1b**). The virus strains included in this vaccine were all egg-grown (e), specifically A/California/7/2009 (H1N1)pdm09-like, B/Brisbane/60/2008-like, and A/Hong Kong/4801/2014 (H3N2)-like, hereafter abbreviated to HK14e. Blood samples were collected before and 4, 7, 14, 21, and 280 days after vaccination. Blood samples were also collected 7 and 21 days after confirmed influenza illness occurring in the season after vaccination.

Study protocols were approved by ethics committees of the University of Melbourne (1646470), the National Institute of Hygiene and Epidemiology in Viet Nam (IRB-VN01057 – 08/2016), and the Oxford Tropical Medicine Research Unit (30-16). All participants provided informed consent, conducted in Vietnamese.

Procedures

Sera were tested in hemagglutination inhibition (HI) assay against 40 A(H3N2) viruses that circulated from 1968 to 2018 (**figure 1c**). Viruses were propagated in mammalian cell lines and/or in eggs (**appendix pp 4-5**), and HA and neuraminidase (NA) genes were sequenced. NA can agglutinate erythrocytes if T148I or D151G/N amino acid substitutions arise during virus propagation, and this can interfere with HI antibody detection (**appendix p 5**).[21] Where necessary, viruses were plaque-selected to produce stocks that lacked NA T148X or D151X substitutions, and were more sensitive for detecting HI antibodies (**appendix p8**). Virus HA genes were compared by constructing a phylogenetic tree (**figure 1c)**. HA antigenic site positions, defined by Lee et al,[17] that varied between HK14e and at least one recent prior strain were tabulated to determine whether antigenic variation from HK14e was clustered within particular sites, and if this varied between prior infecting strains (**figure 1e**).

HI assays were performed according to WHO Global Influenza Surveillance Network protocols with minor modifications, and additional quality controls to enable comparison of titres across multiple viruses and time points (**appendix pp 4-8**). HI titres were read using an automated reader (CypherOne, InDevR, **appendix p 4**).

Outcomes

The primary outcome was vaccine immunogenicity, comparing participants who had or lacked recent A(H3N2) virus infection. This included proportions seropositive (defined as a titre of 40 or more) or seroconverting (defined as a four-fold or greater titre rise), geometric mean titres (GMT), and geometric mean ratios (GMR). The strain-coverage of antibodies induced by vaccination was further compared by fitting antibody titre landscapes across all A(H3N2) viruses tested.[22] Titres were determined at a range of time points, but comparison focused on day 14 post-vaccination, when titre peaks were detected, and on day 280, when titre decay plateaues.[23]

In secondary analysis, participants who had been infected with viruses from distinct genetic clades were compared to investigate whether antigenic relatedness between the prior strain and the vaccine strain affects the strain-coverage of antibodies induced by vaccination.

ILI events post-vaccination caused by an A(H3N2) virus were also evaluated.

Statistical Analysis

HI titres were $\log_2$ transformed to estimate GMTs and GMRs, calculated as the mean of post-vaccination minus pre-vaccination differences. Mixed effects linear regression was used to estimate GMTs and GMRs, and to determine the size of the effect of recent infection. The regression model included a random effects term to account for within-person correlations of antibody titres over time, and an interaction term for time of serum collection by recent infection status (**appendix p10**). For ease of interpretation estimated GMTs and GMRs were reported as back-transformed values. Fisher's exact test was used to compare proportions with and without prior infection who seroconverted at day 14; maintained a 4-fold titre rise at day 280; or who became infected post-vaccination.

To construct and compare antibody landscapes, generalized additive models (GAMs) and lowess models were used to fit $\log_2$ titres against A(H3N2) viruses organized antigenically.[22] We used the GAM function from the R package mgcv, and accounted for repeated measurements on each individual through specification of a random effect.[24] Plots were generated with ggplot2.[25] The lowess model has been published online (https://github.com/acorg/ablandscapes).

Results

100 participants who had complete serological and virological assessments to detect influenza virus infections since December 2007 were vaccinated in November 2016 (**figure 1a**). Twenty-eight had no A(H3N2) virus infection detected since 2007 and 72 had at least one infection, hereafter referred to as recent infection. 51/72 had one recent prior A(H3N2) infection, 18/72 had two, and 3/72 had three prior infections. Infection was detected as ILI, confirmed by RT-PCR, for 16/72, and as seroconversion without ILI for 56/72. Age and sex distributions of participants with and without recent infection were similar (**figure 1b**). The proportions having an A(H1N1) virus infection since 2007 were similar among participants with prior A(H3N2) infection (40/72, 55%) and without prior A(H3N2) infection (14/28, 50%). The year that participants were last infected with A(H3N2) virus ranged from 2008 to 2015 (**appendix p9**). Viruses circulating during these years belonged to a range of genetic clades that varied in genetic distance from the 2016 vaccine strain, which was egg-grown A/Hong Kong/4801/2014 (HK14e), belonging to clade 3c2a (**figure 1c, appendix p9**). Twenty-six positions within antigenic sites A-E differed between at least one prior strain and HK14e (**figure 1d, e)**. Differences were clustered within sites A and B for the comparison of HK14e with clade 3c3a viruses (HN14/Sw13). In contrast, differences were clustered in site C for the comparison of HK14e with clade 3c1 viruses (HN12/Vi11) (**figure 1e**).

Confirmed A(H3N2) virus illnesses were detected 275-340 days after vaccination in 5 of 100 vaccinees. A(H3N2)$^+$ ILI was also detected in 5 of 456 (1.1%) unvaccinated adults 249-344 days after the vaccine campaign, indicating that vaccination had little apparent effect on the timing of A(H3N2) virus infections. Vaccine efficacy cannot be estimated because vaccinees were purposefully selected so that most participants who lacked recent A(H3N2) virus infection received vaccine (**appendix p3**). Infecting strains belonged to clades 3c2a1, 3c2a2, and 3c2a1b (**figure 1c**), and contained the K160T substitution in site B that renders them antigenically distinct from the HK14e vaccine strain.[4] A(H3N2)$^+$ ILI was detected in 4/28 (14%) vaccinees who lacked recent A(H3N2) virus infection, but only 1/72 (1·4%) vaccinees who had recent A(H3N2) virus infection (odds ratio 0·084, 95% CI 0·009 - 0·793, p = 0·021). This effect of recent infection was subtype-specific since A(H3N2)$^+$ ILI cases accounted for similar proportions of vaccinees with recent A(H1N1) virus infection (3/54, 5·6%) and without recent A(H1N1) virus infection (2/37, 5·4%). These results suggest that vaccinated adults who lacked recent infection with an A(H3N2) subtype virus were relatively unprotected against A(H3N2) illness.

Vaccination induced robust antibody production by day 7 when 2-fold or greater titre rise was detected in 87% of participants, and 4-fold or greater titre rise was detected in 62% of participants (**figure 2a**). This contrasts with studies showing negligible production of antibody by day 7 after primary exposure to influenza virus,[26] and suggests that recalled memory B cells contributed substantially to the antibody response. Titres were

highest at day 14 and then declined (**figure 2a**). Nevertheless, by day 280, titres were still at least 4-fold higher than at baseline for 54% of participants, indicating that vaccination induced sustained antibody production.

Participants who had recent A(H3N2) virus infection were more often seropositive (titre ≥ 40) against HK14e at all time points, and more often seroconverted (titre rise ≥ 4-fold) compared to participants who lacked recent infection (**table 1**). Notably, 74% of participants with recent infection seroconverted against A/Kansas/14/2017 (Ka17), a clade 3c3a strain, compared to 43% of participants without recent infection, with comparable differences in proportions seropositive. Similarly, seroconversion against A/Brisbane/60/2018 (Br18) from clade 3c2a1b, was more common among vaccinees with recent infection, and 83% remained seropositive 280 days after vaccination compared to 56% of participants without recent infection. Recent A(H3N2) virus infection had little effect on the proportion of participants seropositive against A(H1N1)pdm09 in the vaccine (**table 1**). These results indicate that recent A(H3N2) virus infection enhances the capacity of vaccine to induce A(H3N2)-reactive, but not A(H1N1)-reactive, antibodies. Therefore, effects of recent infection are likely to be mediated by type/subtype-specific memory B cells, rather than by broadly cross-reactive B or T cells.

To further examine the strain-coverage of antibodies induced by vaccination, generalized additive models (GAM) were used to fit titres against 40 strains that circulated up to 46 years before and 4 years after the vaccine strain emerged (**figure 2b-d**). As reported previously,[22] pre-vaccine antibody titres were relatively high against strains encountered early in life (**figure 2b, appendix pp 11-12)**, consistent with hypotheses that immune responses induced against early life strains are recalled upon subsequent encounter of later strains.[6,27]. Vaccine-induced titre rise was greatest against strains proximal to HK14e, and diminished as virus genetic and temporal distance from HK14e increased (**figure 2c, d**), presumably reflecting the degree to which antigenic sites were conserved with the vaccine strain. Vaccine-induced back-boosting of titres was largely limited to strains circulating after participant's birth years (**appendix p 16**), suggesting that back-boosting reflects recall of memory B cells induced by prior infections. Alternately, back-boosting could reflect low-avidity antibody binding to past-strains when antibody concentrations are high since titre rise extended across more strains on day 14 after vaccination than on day 280 (**figure 2c, d, appendix pp 13-14**). Vaccination caused a sustained shift in the peak of the antibody landscape from older strains towards 2011 strains by day 280 after vaccination (**figure 2c**, **appendix pp 11,12,17**).

Pre-vaccination titres were higher across the landscape among participants who had recent infection (**figure 3a, appendix p10**). This was particularly marked for the comparison of participants with no recent infection versus those with RT PCR confirmed prior A(H3N2) infection. Differences were also clearly apparent for the comparison with participants having serologically confirmed recent infection, but were more restricted to strains circulating since 2004. Pre-vaccination titres against strains circulating since 2004 were not detectably associated with participant age (**figure 2b, appendix p 16**). Titres remained higher among participants with

recent infection after vaccination (**figure 3b, appendix p10**). Moreover, titre rises were higher against vaccine and subsequently circulating strains among participants with recent infection, and were at least as high against past strains (**figure 3c-d, appendix pp 13-14**). Effects of recent infection were observed across age groups (**appendix pp 16**), inclusive of the oldest participants born in the 1930's and 1940's (**appendix p 39**). Notably, by day 280, when A(H3N2)$^{+}$ ILI cases had already been detected, GMTs against several circulating strains exceeded 40 among participants who had recent infections, but were lower in those who lacked recent infection (**figure 3e, appendix 10**). We have shown previously that titres of this magnitude can be associated with substantial protection in this cohort.[28] These results indicate that recent infection boosts the titre and breadth of A(H3N2)-reactive antibodies induced by vaccination.

We next investigated whether the strain coverage of vaccine induced antibodies differed between participants who were last infected with clade-3c3a (HN14/Sw13) versus clade-1 or -3c1 (HN09- or HN12) viruses, which are clearly genetically and antigenically distinct (**figure 1e**). To obtain a more detailed comparison of antibody titres across strains, viruses circulating since 2007 were represented on a two-dimensional map of antigenic distances (**figure 4 a-f**). Pre-vaccination titre landscapes differed somewhat between prior infection groups (**figure 4 a-c**) whereas post-vaccination landscapes were markedly different (**figure 4 d-f)**. Landscapes remained relatively focused on clade-1/3c1 viruses among participants with prior clade-1/3c1 virus infection (**figure 4e**) and on 3c3a viruses among participants with prior 3c3a virus infection (**figure 4f**). Notably, participants with prior 3c3a virus infection had higher titres against HK14e than those with prior 1/3c1 virus infection even though HK14e was closer to clade 1/3c1 viruses on the antigenic map. Titre rise landscapes also differed, extending more towards clade-1/3c1 viruses among participants with prior 1/3c1 infection, and more towards the 3c3a viruses among participants with prior 3c3a virus infection (**appendix p 19**). Differences between prior infection groups were apparent by day 7, and persisted until day 280, after vaccination (**appendix p 19-20**). These results suggest that recalled prior strain immunity may drive antibody production towards epitopes that are shared between the vaccine strain and prior strains.

Antigenic site B is the immuno-dominant antigenic site on HA of A(H3N2) viruses, and is well conserved between HK14e and clade 1/3c1, but not 3c3a, viruses (**figure 1e**). To investigate whether this affected antibody production against site B of the HK14e vaccine, sera from participants with prior clade 1/3c1 versus 3c3a infection were titrated against a site B antigenic variant (**figure 4g)**. Reverse genetics was used to change HK14e HA position 159 from Y to S **(appendix p22).** The Y159S substitution was chosen because Sw13e has an S at position 159 (**figure 1e**), and is antigenically distinct from HK14e (**figure 4a, appendix p22**). The antigenic effect of the Y159S substitution was confirmed using ferret antisera and a site B directed mAb: HK14e antisera titres against the Y159S variant were lower than against native virus and reverse genetics virus bearing wild-type HA, and higher than against Sw13e (**appendix p22**). Several participants with prior 3c1 infection had higher pre-vaccination titres against wild-type compared to Y159S virus, indicating the presence of antibodies

against site B of HK14e, vice versa several participants with prior 3c3a infection had higher pre-vaccination titres against the Y159S variant (**figure 4h**). Post-vaccination sera from 9/14 participants with prior clade 1 or 3c1 infection had greater than two-fold higher titres against wild-type compared to Y159S virus indicating that antibodies were induced against site B of HK14e. Only 3/13 participants with prior 3c3a infection, had higher post-vaccine titres against wild-type compared to Y159S virus and differences did not exceed two-fold. These results indicate that antibody was poorly induced against site B of HK14e among people with prior 3c3a infection. It is therefore probable that vaccination induced antibodies against sub-dominant sites among participants with prior 3c3a infection. Since sub-dominant sites, such as site C, are better conserved across past and future strains (**appendix p23**), this could give rise to antibodies with broader strain coverage.

The five vaccinees who developed A(H3N2)$^{+}$ ILI in the season after vaccination had poor antibody responses induced by vaccination compared to participants who did not develop A(H3N2)$^{+}$ ILI (**figure 5a-d**). Titre rise by day 7 was markedly low, and did not increase further by day 21 (**figure 5g-j, appendix p 24)**. However, in these same participants, antibody titres increased between days 7 and 21 post-infection, and were higher than titres detected at the same time points post-vaccination (**figure 5c-l**). Antibody titres detected 7 and 21 days after infection of three unvaccinated participants were equivalent to titres detected after infection of vaccinated participants (**appendix p 25**) suggesting that infection responses were not boosted by prior vaccination, and that infection induced a more potent response than vaccination.

Discussion

In this study, adults who had undergone active investigation to detect influenza virus infections since 2007 were vaccinated for the first time in 2016 with inactivated influenza vaccine containing a new A(H3N2) strain. Vaccination induced robust A(H3N2)-reactive antibody responses that were at least as good among older compared to younger adults, contrasting with studies in more highly vaccinated populations.[29,30] Detailed analysis of the kinetics and breadth of the A(H3N2)-reactive antibody response demonstrated that much of the antibody detected was induced between day 4 and day 7 after vaccination, and was cross-reactive with past strains. These findings indicate that recalled memory B cells contribute substantially to the vaccine response. Moreover, participants with an A(H3N2) virus infection during the 9 years prior to vaccination had higher antibody titres, with faster rises and better-maintained antibody levels against the vaccine virus and future circulating viruses. Similarly, A(H3N2)$^{+}$ ILI was predominantly detected among vaccinees who lacked prior A(H3N2) virus infection indicating that both vaccine immunogenicity and effectiveness are enhanced by immunological memory associated with prior infection.

The boosting effects of prior infection, observed here, contrast with reports of negative effects of prior or repeated vaccination,[7-11] suggesting that the type of prior exposure is highly relevant. Several groups have demonstrated that neutralizing antibodies can become focused on limited virus epitopes that have remained conserved across successively encountered strains.[31,32] It is hypothesized that recalled memory B cells dominate and focus responses on epitopes that are well conserved in successively encountered strains, which could either enhance or compromise protection depending upon whether these targeted epitopes undergo mutation in subsequent strains.[13,14] In the current study, the strain-coverage of antibodies and capacity to generate antibodies against a prominent site B epitope was shaped by the prior infecting clade, consistent with memory B cell dominance. These findings present a paradox whereby memory B cell recall is pivotal for inactivated egg-based influenza vaccine to elicit sufficient antibody for protection, but may also be problematic in terms of the capacity for vaccination to update immunity by generating memory B cells and antibodies against epitopes that have mutated in a new vaccine strain. To generate antibodies and memory B cells against variant epitopes, influenza vaccines must either induce memory B cells to undergo further affinity maturation[33] or induce naïve B cell differentiation. Memory B cells may have a competitive advantage because they have undergone affinity maturation, so may compete more successfully for antigen in order to engage T cell help for further differentiation, and are additionally less reliant than naïve B cells on T cell help for activation.[34,35] Inactivated influenza vaccines deliver antigen transiently, and induce minimal innate co-stimulation, hence may have little capacity to activate naïve B cells and generate new B cell clones and antibodies in the presence of vaccine-reactive memory B cells.

Infection induced higher antibody titres against a broader antigenic range of A(H3N2) viruses than vaccination among individuals who who developed A(H3N2)$^{+}$ ILI in the season after vaccination. This indicates that infection may have greater potential to expand the antibody repertoire than vaccination. In turn, as the epitope range of the memory B cell pool increases, the potential to recognize epitopes in a new vaccine strain will also increase, providing a mechanism for the differential effects of prior infection and vaccination. Similarly, in ferrets and mice, priming with inactivated influenza vaccine induces little to no antibody, and no protection against variant virus strains, whereas priming by infection induces more antibody and substantial protection against variant strains.[36,37] These differences in antibody responses may reflect a greater capacity for influenza virus infection, as opposed to vaccination, to activate both the innate and adaptive immune systems,[38] and in turn activate naïve B cells. Additionally, antigen may be retained for longer periods after infection than vaccination, and may be available to engage naïve B cells after the memory B cell response starts to contract.[39]

In summary, this study demonstrates that prior A(H3N2) virus infection and pre-existing immunity can increase, and extend the breadth of, antibody responses induced by a new A(H3N2) vaccine strain, and thereby enhance protection despite antigenic drift. However, the range of strains against which antibodies were induced was dictated by the strain with which participants were previously infected, suggesting that the vaccine is inducing a memory-dominated response. Such memory dominance may need to be overcome in future vaccine strategies to increase protection against A(H3N2) viruses.

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Contributors

MA assisted with virus propagation, performed serology, assisted with data analysis and co-drafted the manuscript
HVMP co-management of the Ha Nam Cohort including sample collection and processing and diagnostic testing over the course of the vaccination study, and critically reviewed the manuscript.
LC assisted with virus propagation, and serology, sequenced virus HA and NA genes and plaqued viruses, and critically reviewed the manuscript.
LTQM co-conceived and co-designed the study, co-managed the Ha Nam Cohort sample collection and processing and diagnostic testing over the course of 9-years of cohort investigation and over the vaccination study, and critically reviewed the manuscript.
RT performed components of the data analysis and critically reviewed the manuscript.
SW performed components of the data analysis and critically reviewed the manuscript.
PQT co-designed the study, co-managed Ha Nam Cohort field work and data collection over the course of 9-years of cohort investigation and over the vaccination study, and critically reviewed the manuscript.
DP assisted with data analysis and critically reviewed the manuscript.
NTD assisted with study design, managed all activities of the health care workers to collect samples and data, managed communication with participants, and critically reviewed the manuscript.
NLKH, LTT, NTHT, TTKH, NTND, and VTNB processed samples, performed influenza diagnostic testing and virus isolation over 9-years of cohort investigation between 2007 and 2016, and also over the course of vaccination and subsequent follow-up, assisted with data cleaning, and critically reviewed the manuscript.
AK assisted with data analysis and critically reviewed the manuscript.
LH assisted with virus propagation and critically reviewed the manuscript.
TND and DDA co-management of the Ha Nam Cohort over the course of 9-years of cohort investigation and over the vaccination study, and critically reviewed the manuscript.
KK contributed to data interpretation and critical review of the manuscript.
SDB, KG-J, DS, IB co-designed the study and critically reviewed the manuscript.
SS assisted with data analysis and critically reviewed the manuscript.
HRvD co-conceived and co-designed the study, co-managed the Ha Nam Cohort over the course of the vaccination study, and critically reviewed the manuscript.
AF conceived the study, co-managed the Ha Nam Cohort sample collection and processing and diagnostic testing over the course of 9-years of cohort investigation and over the vaccination study, assisted with sample processing, virus propagation, and serology, managed data and data analysis, and co-drafted the manuscript.

Declaration of interests

HRvD was funded by Sanofi (travel and consultancy fee) to present at, and attend a meeting about the potential role of influenza vaccination in AMR in 2019.

Role of the funding source

The funders had no role in the conduct of the study.

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Table 1. Proportions of participants with and without recent A(H3N2) virus infection who were seropositive or seroconverted against vaccine and subsequently circulating strains

Prior H3N2	Test antigen [clade]	Seropositive (HI ≥ 40)										Seroconvert			
		Pre		d7		d14		d21		d280 [a]		d14		d280 [a]	
		n (%)	p	n (%)	p	n (%)	p	n (%)	p	n (%)	p	n (%)	p	n (%)	p
No	HK14e [3c2a]	8 (29)	0·000	25 (89)	0·065	26 (93)	0·076	23 (82)	0·006	21 (78)	0·026	18 (64)	0·058	12 (44)	0·180
Yes		51 (71)		71 (99)		72 (100)		71 (99)		66 (94)		60 (83)		42 (60)	
No	Mi14 [3c2a]	1 (4)	0·001	16 (57)	0·001	20 (71)	0·000	19 (68)	0·000	17 (63)	0·114	20 (71)	0·064	12 (44)	0·656
Yes		25 (35)		64 (89)		71 (99)		69 (96)		56 (80)		64 (89)		36 (51)	
No	NC16 [3c2a1]	3 (11)	0·002	17 (61)	0·003	21 (75)	0·000	19 (68)	0·000	15 (56)	0·008	17 (61)	0·140	11 (41)	0·652
Yes		31 (43)		65 (90)		72 (100)		69 (96)		58 (83)		55 (76)		33 (47)	
No	Br18 [3c2a1b]	5 (18)	0·020	20 (71)	0·003	25 (89)	0·065	25 (89)	0·683	15 (56)	0·008	13 (46)	0·066	7 (26)	0·805
Yes		32 (44)		68 (94)		71 (99)		67 (93)		58 (83)		49 (68)		22 (31)	
No	Ka17 [3c3a]	0 (0)	0·017	9 (32)	0·014	11 (39)	0·000	9 (32)	0·000	3 (11)	0·004	12 (43)	0·005	3 (11)	0·024
Yes		13 (18)		44 (61)		58 (81)		53 (74)		30 (43)		53 (74)		24 (34)	
No	Sw17 [3c2a2]	0 (0)	1.000	1 (4)	0·035	4 (14)	0·082	2 (7)	0·019	1 (4)	0·170	6 (21)	0·235	2 (7)	0·722
Yes		1 (1)		16 (22)		24 (33)		21 (29)		11 (16)		25 (35)		9 (13)	
No	*H1N1pdm09*	*5 (18)*	*1.000*					*23 (82)*	*0·527*			*23 (82)*	*0·756*		
Yes		*14 (19)*						*63 (88)*				*62 (86)*			

a = 27/28 participants without prior H3N2 and 70/72 participants with prior H3N2 provided samples on d280

p = Fishers Exact test

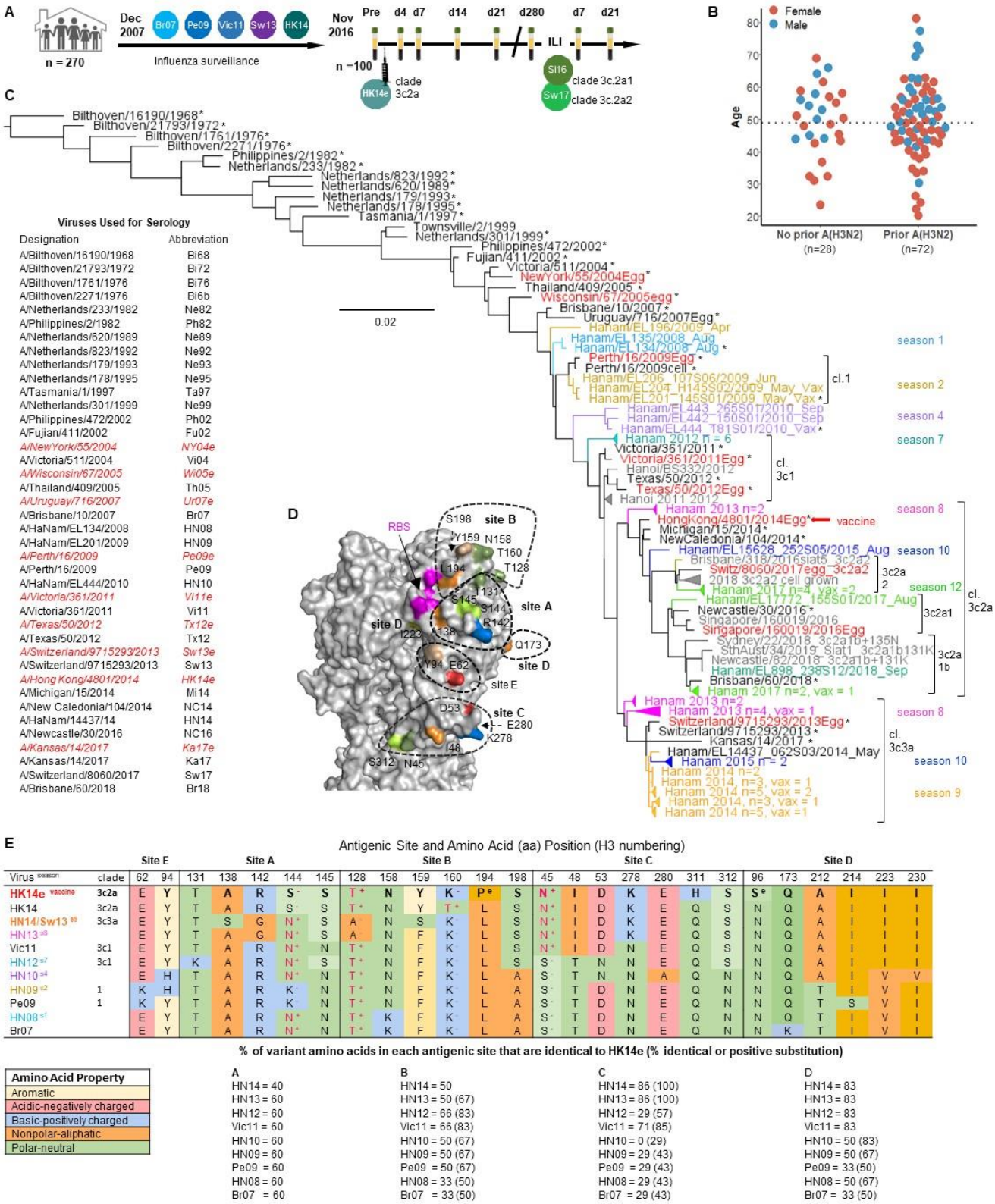

Figure 1. Participant selection and investigation of previously circulating A(H3N2) viruses. (A) Study design and timeline. (B) Age and sex distribution of vaccinated participants with and without recent prior A(H3N2) virus infection. The dotted line indicates the median age. (C) Phylogenetic tree of the HA genes of viruses recovered from Ha Nam Cohort ILI cases (coloured by season), and viruses used to construct antibody

landscapes (coloured black if cell grown or red if egg grown). Viruses from participants of the vaccine study are indicated by the suffix “Vax”. Clades (cl.) and sub-clades are delineated using parentheses. (D) Model of the globular head of HK14e HA (SWISS-MODEL: A0A0K0YAS1), showing amino acid positions within antigenic sites A to E that differed from at least one of the prior infecting strains, and receptor binding site (RBS) residues. (E) Antigenic site positions that varied between HK14e and at least one prior infecting strain are tabulated and shaded according to amino acid properties. Substitutions that result in gain (+) or loss (-) of glycosylation are coloured in pink. Egg adapted substitutions are indicated by a superscript ^{e}. The relative extent of identity or similarity between previously encountered viruses and HK14e, within each antigenic site is summarized below the table.

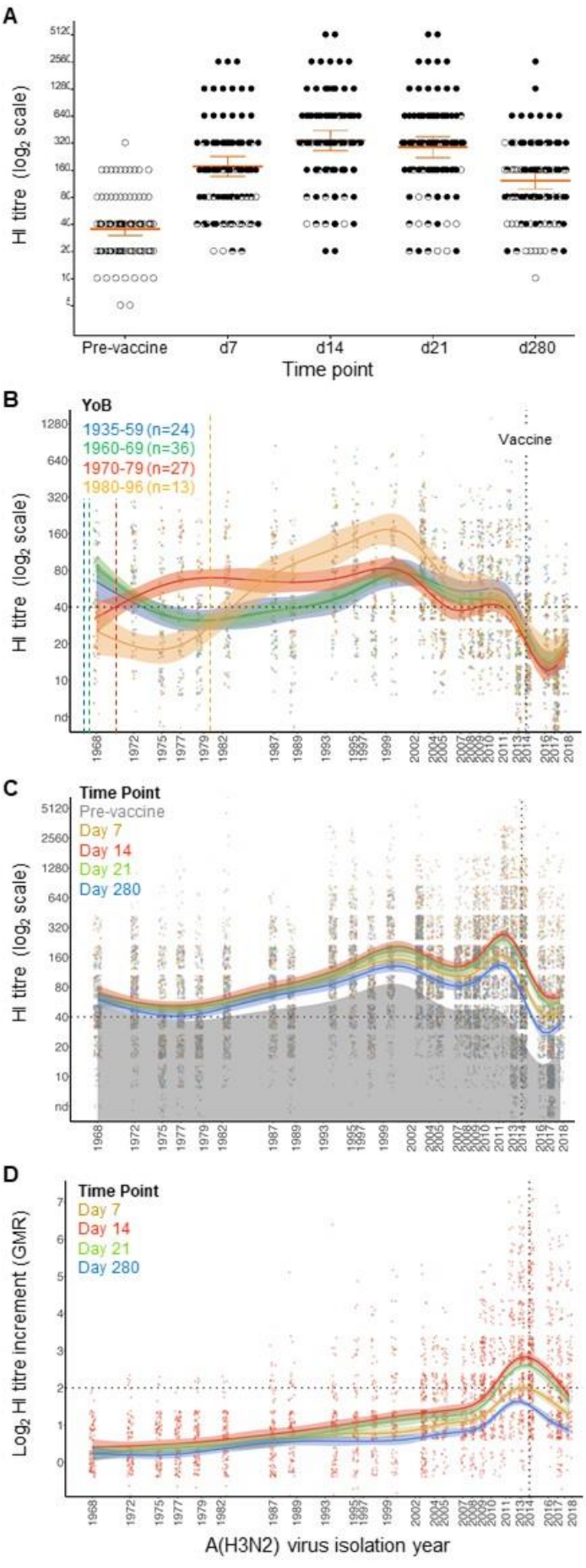

Figure 2. Kinetic and strain coverage of the A(H3N2) virus-reactive antibody response to vaccination. (A) Titres against the HK14e vaccine strain are shown for each participant (n=100) and time-point. Filled circles indicate titres that were at least 4-fold higher than pre-vaccine titres; half circles indicate titres that were only 2-fold higher and open circles indicate titres that were unchanged compared to pre-vaccination. Bars and error bars show geometric means and 95% confidence intervals. (B) Pre-vaccine titre landscapes across strains spanning 1968 to 2018 were estimated using GAMs. Participants are grouped by year of birth (YoB) with dashed and colour-matched vertical lines representing the earliest strain that participants could have been exposed to. (C) Fitted titre landscapes for pre-vaccination (grey-shaded area) and post-vaccination (coloured lines) time-points are compared for all vaccinees. Shading indicates 95% confidence intervals (CI) for the model, and dots show individual participant titres against each antigen. (D) Fitted landscapes of post-vaccination minus pre-vaccination titre increments are shown for all vaccinees. Dotted horizontal lines indicate thresholds for seropositivity or seroconversion. Dotted vertical lines in B-D indicate the position of the vaccine antigen.

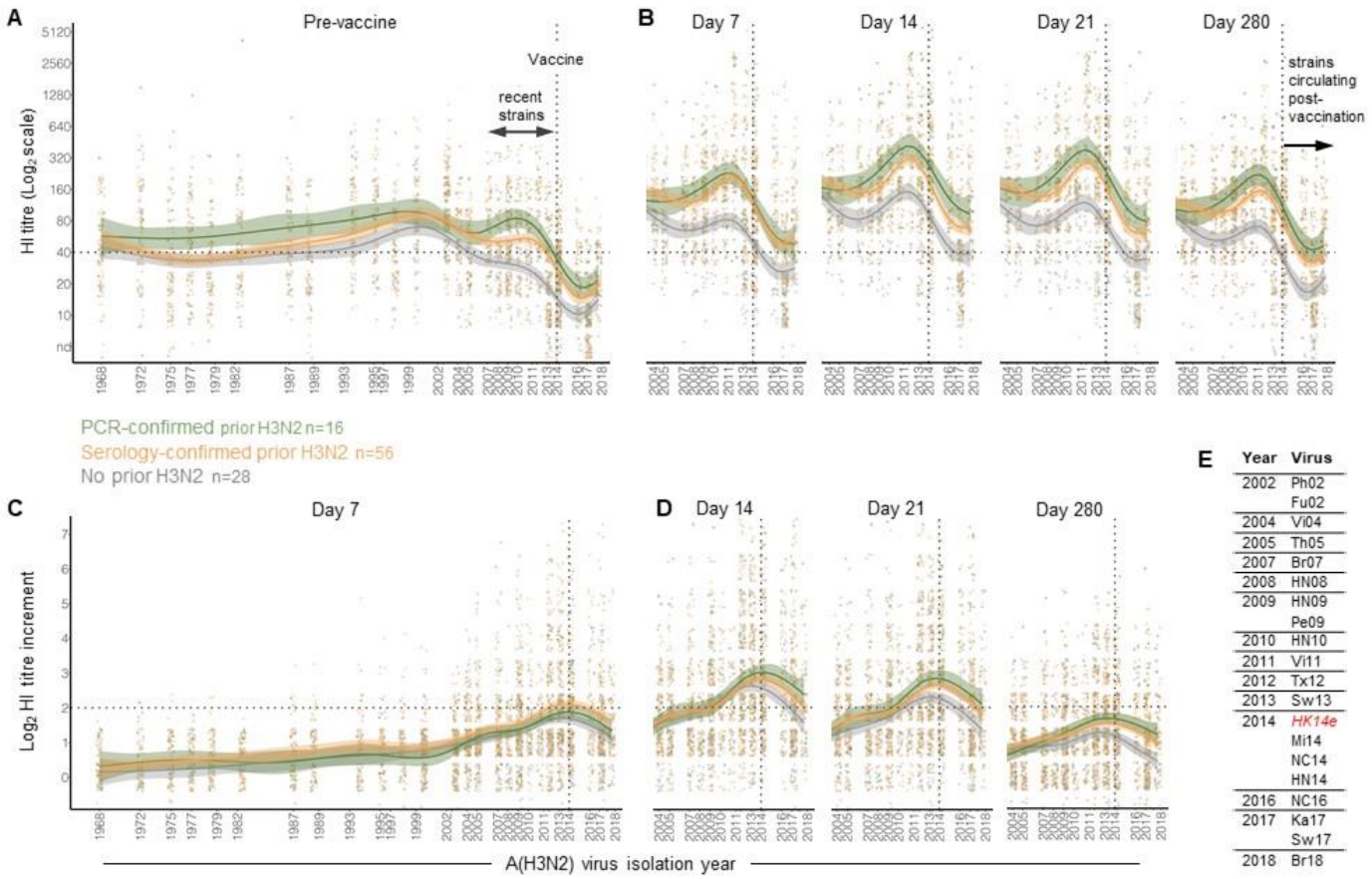

Figure 3. Recent A(H3N2) virus infection enhances the titre and strain-coverage of A(H3N2)-reactive antibodies induced by vaccination. (A) Pre-vaccine titres landscapes against strains spanning 1968 to 2018 were estimated using generalized additive models (GAMs). Line colours correspond to documentation of prior A(H3N2) infection since 2007. Shaded areas indicate 95% confidence intervals, and dots show individual participant titres against each antigen. (B) Post-vaccination titre landscapes against strains spanning 2004-2018. (C-D) Landscapes of titre rise, calculated as post- minus pre-vaccination Log2 titre, were estimated using GAMs. (E) List of viruses used to generate landscapes shown in B and D.

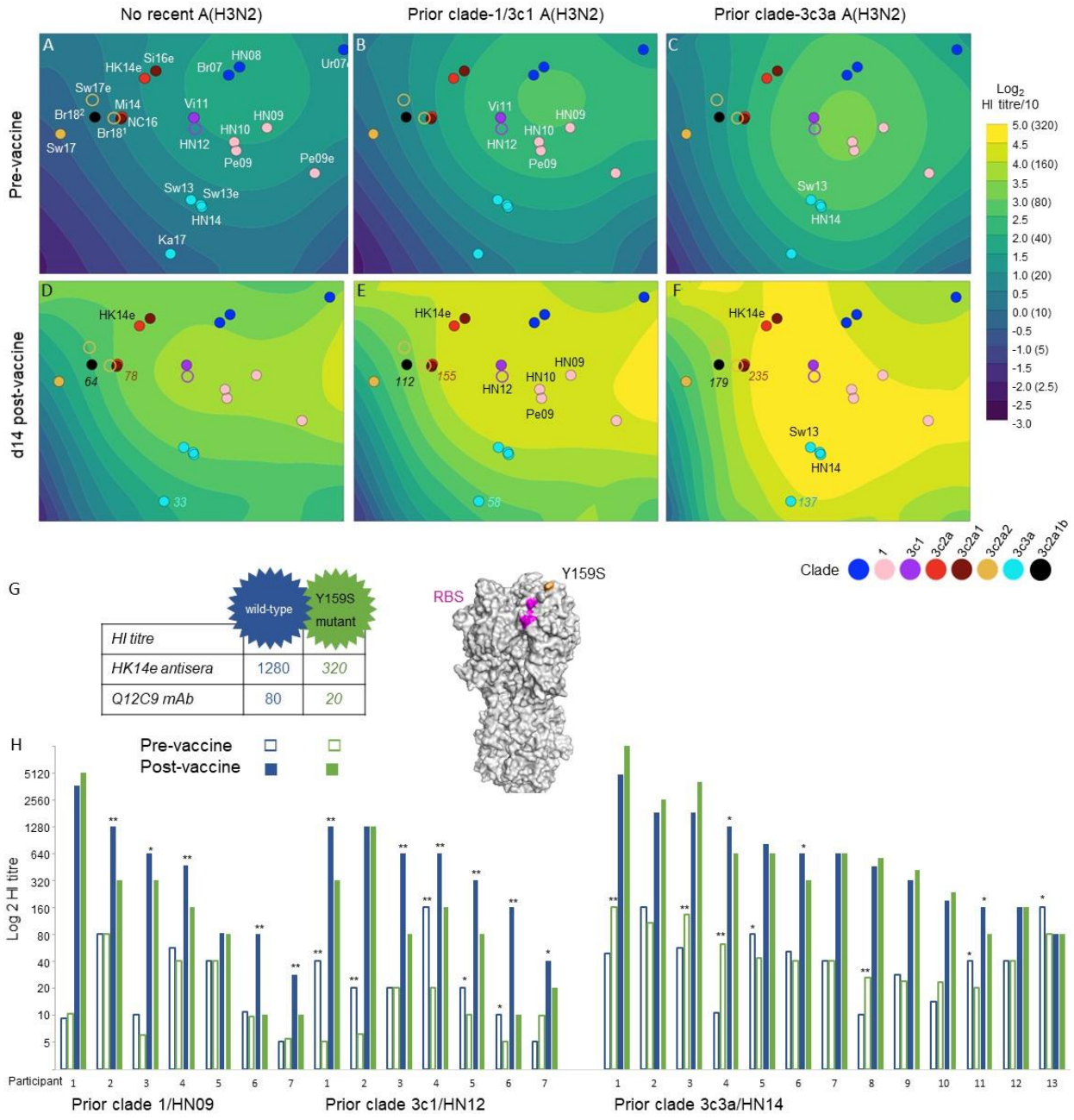

Figure 4. The strain-coverage of antibodies induced by vaccination is influenced by the A(H3N2) virus clade that caused prior infection. (A-F) Serum titres of participants in each prior infection group were modelled against a two-dimensional antigenic map of recent A(H3N2) viruses, and represented as contours. Each circle represents a virus on the map, coloured by (sub)clade, assigned since 2009. Abbreviated virus names are shown for all viruses in panel A, and only for viruses that had been encountered by participants in each group in the other panels. Numbers in italics in panels D-F are *GMTs* for selected viruses. Viruses against which participant sera were titrated are indicated by solid circles, otherwise viruses are indicated by open circles. Results are presented for 28 vaccinees with no recent A(H3N2) infection, 38 with a prior clade 1 or -3c1 infection, and 13 with a prior 3c3a virus infection. (G) Reverse genetics was used to create a HA Y159S variant virus that was antigenically distinct from HK14e in site B, based on titres of HK14e antisera and a site B directed

mAb (Q12C9). (H) Pre and post vaccination titres of individual participants are compared against wild-type versus Y159S virus to examine whether vaccination induced antibodies against site B of HK14e. Titres that were 2-fold or > 2-fold different between viruses having wildtype versus Y159S HA are indicated by * and **, respectively.

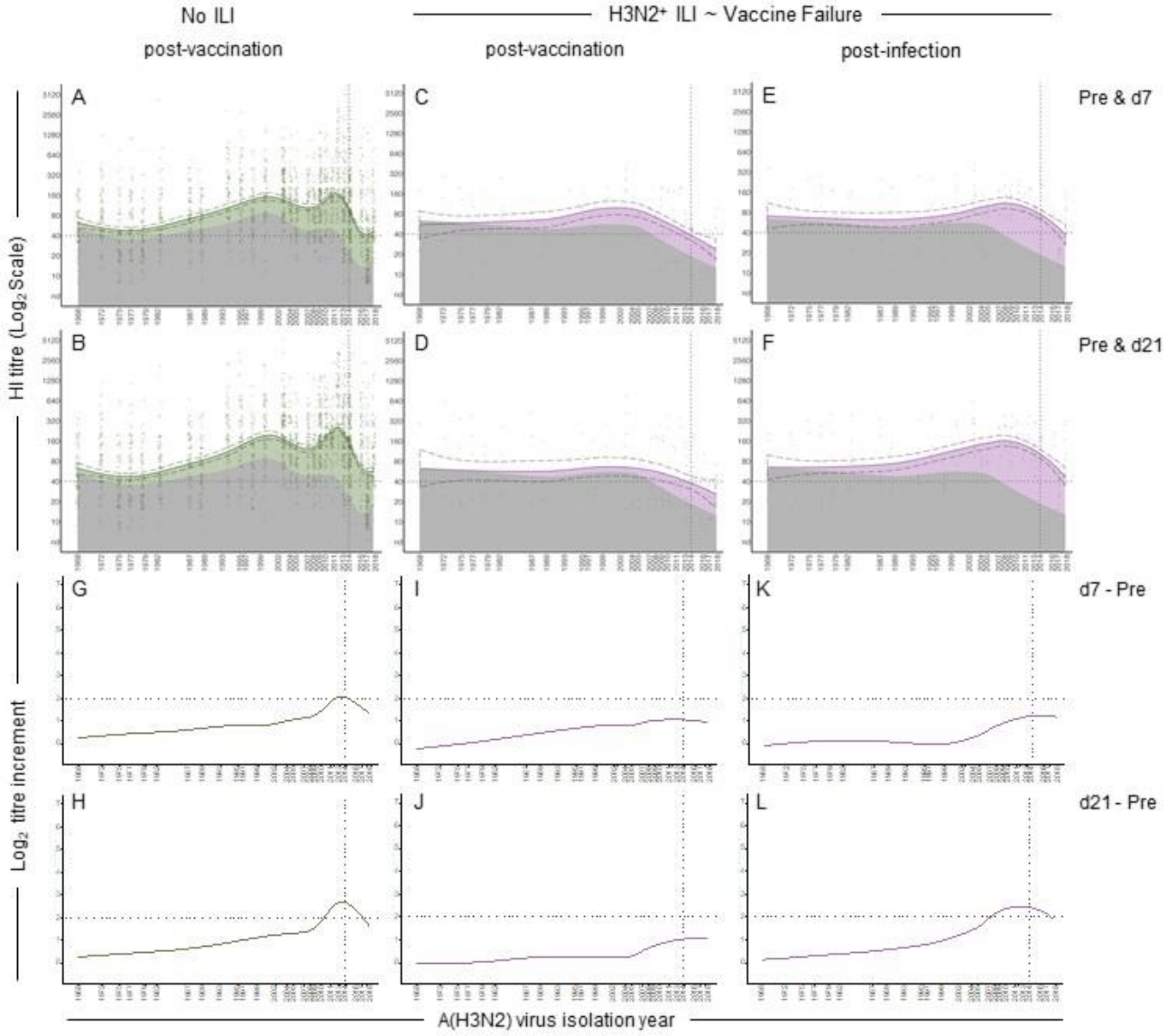

Figure 5. Antibody titre landscapes associated with infections detected after vaccination. Log_2 titres across strains of vaccinees who remained protected (n=95, A-B) or who developed $A(H3N2)^+$ ILI (n=5, C-F) were modelled using GAMS to generate pre- (grey shading) and post-vaccination (A-D) or post-infection (E-F) landscapes (coloured shading). Landscapes on days 7 and 21 post-vaccination or post-infection are shown in comparison to pre-vaccination landscapes (A-F) or as Log_2 titre increments from baseline (G-L). Dashed lines above and below the shaded areas represent 95% CIs, and dots show individual participant titres against each antigen. Dotted horizontal lines indicate thresholds for seropositivity or and seroconversion. Dotted vertical lines indicate the position of the vaccine antigen.

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Supplementary Files

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