DOES REPEATED INFLUENZA VACCINATION CONSTRAIN INFLUENZA IMMUNE RESPONSES AND PROTECTION?

PROTOCOL

4 March 2021

Version 4.3

[redacted]

Sponsor: Melbourne Health

Funding: US National Institutes of Health (Award:1R01AI141534)

Statement of Compliance

This study will be conducted in compliance with all stipulation of this protocol, the conditions of the ethics committee approval, the NHMRC National Statement on ethical Conduct in Human Research (2007) and the Note for Guidance on Good Clinical Practice (CPMP/ICH-135/95).

1 TABLE OF CONTENTS

2 GLOSSARY OF ABBREVIATIONS AND TERMS

Abbreviation	Description (using lay language)
ARI	Acute respiratory illness. Defined as one or more of the following symptoms include fever ≥37.8°C, headache, body aches, cough, sore throat, runny nose, sputum.
FRA	Focus reduction assay. A laboratory assay that measures the ability of antibodies to neutralize virus infectivity.
HI assay	Haemagglutination inhibition assay. Laboratory test which measures anti-haemagglutinin antibodies. These antibodies inhibit attachment of the influenza virus to target cell membrane receptors on red blood cells.
HCW	Health care worker. Any personnel eligible for the free vaccination programme run at participating hospitals or health services. Personnel may include staff, including administrative, research, clinical and support services, employed at the participating hospital. It may also include volunteers, students or honorary staff.
GMT	Geometric mean titre. Arithmetic mean of the logarithms (base 2) of the last positive dilution of each serum.
MN assay	Microneutralization assay. A laboratory test which measures the ability of antibodies to neutralize virus infectivity.
PBMC	Peripheral blood mononuclear cell. A type of white blood cell that contains a single lobed nucleus.
RT-PCR	Real-time reverse transcriptase polymerase chain reaction. A laboratory test used to make many copies of a specific genetic sequence for analysis and can be used to diagnose disease.
Seropositive or seropositivity	Antibody titre of ≥40, as measured using a haemagglutination inhibition assay.
Sero-conversion	4-fold rise in antibody titre, as measured using a haemagglutination inhibition assay.
VE	Vaccine effectiveness. A measure of real-world benefit to patients for whom vaccine is recommended.
WHOCCRRI	World Health Organization Collaborating Centre for Reference and Research on Influenza.

3 EXECUTIVE SUMMARY

Hospitals in a number of countries have introduced annual staff influenza vaccination policies, some with a mandatory requirement. This strategy aims to protect both staff and patients from infection and incurs a considerable cost. Staff may be vaccinated for 10 or more consecutive years. However, some evidence suggests that the antibody response to influenza vaccination subsides with repeated vaccination. This finding is corroborated by epidemiological studies which have indicated that the vaccine's effectiveness (VE) decreases with repeated administration. The possibility of suboptimal protection with repeated vaccination presents a compelling need to evaluate healthcare worker (HCW) influenza vaccination policies. HCWs are an important group in which to study these effects because they differ from most other vaccine target groups in being healthy adults, the group for whom we expect the vaccine should work best.

Our overall goal is to understand the mechanisms underlying observations of reduced immunogenicity and VE among multiply vaccinated persons in the context of their adaptive immune responses to vaccination and how this impacts expected gains from vaccination programs. To achieve this goal, we will recruit a cohort of HCWs working in 6 Australian hospitals. HCWs will represent a range of vaccination experience from unvaccinated to frequently vaccinated. We will follow HCWs for 4 years, document confirmed influenza cases, calculate influenza attack rates, and assess correlates of protection. We will conduct detailed immunological assays among subgroups to evaluate the role of prior exposures and memory B cell responses to vaccination. We will use mathematical modelling to interpret dynamic antibody responses and produce models of the effectiveness of HCW vaccination programs. The rationale for the proposed research is that increasing our understanding of the immunological consequences of repeated vaccination will improve the evidence base for decision making about vaccination policy.

4 PROJECT TEAM

Table 1 lists each team member, their role in the project and responsibilities.

Table 1. Roles and responsibilities

TBN: to be named

4.1 Steering Committee

Table 2 lists the members of the steering committee for the project, their current position and qualifications. The steering committee will be engaged by the project team to provide expertise and advice on the analysis strategies and interpretation of results.

Table 2. Steering committee

[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]

5 STUDY SITES

A list of the study sites and site contact details are provided in Table 3.

Table 3. Study sites and contact details

6 BACKGROUND

Influenza vaccines require annual re-administration both because circulating strains, especially influenza A(H3N2) viruses, undergo rapid antigenic drift demanding re-configuration of the vaccine, and because vaccine-induced immunity against homologous strains may wane. Annual seasonal influenza vaccination is currently recommended for healthcare workers (HCW) to protect themselves against infection, reduce absenteeism and minimize the risk of transmission to patients. Under such policies, HCW may include anyone who works at a hospital, irrespective of patient contact, including administrative, research, clinical and support services staff. Many North American hospitals require HCW who decline vaccine to wear face masks throughout the influenza season (1), and they report vaccination uptake of over 90% in their hospitals (2). Australian hospitals generally do not have such HCW vaccination policies and vaccine uptake is around 60%-70%.

The effectiveness of influenza vaccines is at best moderate. A 2012 meta-analysis estimated pooled efficacy to be around 59% (95% CI 51–67%) and effectiveness around 50% in healthy non-elderly adults (3) (the demographic in which HCW would largely fall). A more recent review observed a pooled VE estimate of only 33% (95%CI: 26–39) for A(H3N2)(4), and low and negative estimates have been reported (5-8). Thus, the expected protection from vaccination afforded to HCW is questionable.

The effects of repeated vaccination are unclear and may reduce effectiveness (9). This was first noted during a vaccine trial in an English boarding school in the 1970s (9). A subsequent study in Texas observed poorer serologic responses in repeat vaccinees in 4/7 seasons (10), and a 1999 review found that roughly half of published serological studies reported poorer post-vaccination antibody titres among vaccine-experienced compared with vaccine-naïve vaccinees (11). Responses may be even poorer when revaccinated with the same formulation if the circulating virus has drifted (12). We have conducted 2 small studies to examine immunological responses to vaccination; [redacted] (13) [redacted]. Both of these studies showed attenuated antibody responses to vaccination among repeatedly vaccinated HCW, especially towards A(H3N2) antigens (Figure 1).

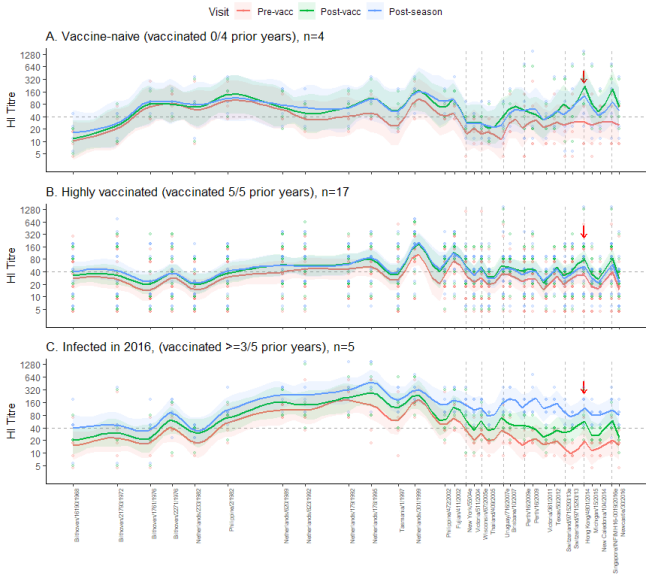
Interest in this phenomenon was reinvigorated when a 2013 household study reported VE=-45% (95%CI:-226-35) among people vaccinated two years in a row compared with people vaccinated only in the prior season (14). Consequently, many groups have begun routinely reporting effectiveness estimates by prior year's vaccination status, often reporting reduced VE among multiply vaccinated patients (8, 15-20). However, no study has yet reported on both vaccine effectiveness and immunological responses to repeated influenza vaccination.

An explanation for this phenomenon, including inconsistencies among studies, has been attempted by mathematical modelling (21). The antigenic distance hypothesis posits that when two vaccine strains (V1 and V2) are antigenically similar, responses to epitopes in V1 dominate such that repeat vaccination impairs VE if the circulating strain (C) has changed from V2, but enhances VE when C has not changed. In contrast, if V1 and V2 are antigenically distant, repeat vaccination has little effect because responses to V2 are not compromised. In Australia during the 2017 season, V1=V2 but the V2-C distance was great and VE for A(H3N2) among repeat vaccinees was poor (3%; 95%CI: -29 to 27)(19). Similar findings were observed in Canada in 2014/15 (16). These effects are not expected each year because of annual differences in V1-V2 and V2-C antigenic distance. However, on average, negative

interference is seen more often for A(H3N2) viruses compared with other influenza types/subtypes (22), probably because of the higher rate of antigenic drift in A(H3N2) viruses (23).

Figure 1. Summary antibody landscapes for 26 HCW

By collating the results of many antibody assays to historical influenza strains, it is possible to visualize the landscape of an individual's responses to vaccination and infection. The plot shows preliminary antibody landscapes for 26 HCW followed in 2016 at the Royal Melbourne Hospital. 31 antigens representing antigenic clusters that circulated from 1968-2016 were assessed (x-axis). Lines are estimated using a loess curve; full landscape analyses uses a nonlinear model (24). These plots suggest: (1) Post-vaccination and post-season HI titres against the vaccine strain (A/Hong Kong/4801/2014e; red arrows) were higher for the vaccine-naïve group compared with highly vaccinated or infected HCW; (2) Post-vaccination titres are higher and better-sustained post-season in the vaccine-naïve compared with the highly vaccinated group; (3) Post-vaccination titres against egg-grown antigens (grey-dashed lines) were generally higher than their cell-grown counterparts (shown to their right); (4) Post-infection titres were high and showed better response to historical cell-grown than egg-grown antigens.



Concepts regarding the underlying mechanisms at play have evolved over many years. First is the concept of original antigenic sin, which suggests that a person's initial influenza infection affects responses to subsequent strains by preferentially orienting antibodies towards priming epitopes that remain in subsequent strains, often as subdominant epitopes (25). Second is the concept of antigenic seniority, which suggests that prior infections have cumulative negative effects on responses to later strains, resulting in antibody titres that are higher to more 'senior' strains encountered earlier in life. As with original antigenic sin, it is suggested that immune boosting and interference may account for antigenic seniority, with successive influenza exposures boosting antibody responses to more senior strains that dominate over responses to new epitopes on the later strain. A similar concept, termed back-boosting, was conceived from studies that developed antibody landscapes to depict how infection and vaccination affect titres to prevailing and past strains in the context of antigenic distance (see Figure 1). Both infection and vaccination induce broad back-boosting of pre-exposure antibody landscapes, suggesting that memory responses are invoked (24, 26). Importantly, effects of vaccination are associated with antigenic distance, with better responses to an antigenically distinct and more advanced vaccine, suggesting that antigenic distance may be an important determinant of a vaccine's ability to escape interference from prior immunity. Several groups have used molecular approaches to demonstrate that antibodies can indeed become preferentially focused on an epitope that is conserved among successively encountered strains (27, 28). Some evidence indicates that memory B cells drive this focused antibody response (28). These earlier studies focused on A(H1N1)pdm09-reactive antibodies so it is important to establish whether antibody focusing also occurs upon successive exposure to A(H3N2) viruses, in which case the range of conserved epitopes may be greater and more complex depending upon the range of strains an individual has encountered. While antibody focusing may not necessarily reduce vaccine titres or effectiveness, it could create a future opportunity cost if the conserved epitopes are subsequently altered in circulating strains (29). Thus, the occurrence and consequences of antibody focusing may be linked to antigenic drift and the antigenic distance hypothesis, in that a series of similar vaccines containing a shared epitope may promote antibody focusing that would provide little protection if the circulating strain drifts.

6.1 Preliminary results and related prior work

Effects of prior vaccination among HCW: [redacted] has run 2 serosurveys in Australian hospitals. In the first, 202 HCW were recruited during the 2015 influenza vaccination campaign, among whom post-season follow-up was 90% (n=183). In 2016, 190 HCW were enrolled, with 157 (83%) included in the final analyses. Haemagglutination inhibition (HI) assays were used to compare post-vaccination serum antibody responses between frequent and infrequent vaccinees. Figure 1 shows the HI antibody landscape for a selection of participants. A blunted response was observed among frequent vaccines (panel B in in Figure 1), with lower post-vaccination geometric mean titres (GMT), lower rises in GMT and lower levels of seroprotection compared with infrequent vaccines (panel A in in Figure 1), both post-vaccination and post-season. In both groups, post-vaccination antibody responses to cell-grown antigens were weaker than to egg-grown antigens (grey-dashed lines in Figure 1), and responses were especially strong against the vaccine strain (red arrow). In contrast, infected HCW showed stronger responses to cell-grown antigens, suggesting that vaccination focuses the response on egg-grown antigens while infection induces broad antibody production against circulating viruses.

Some limitations of these studies warrant mention. First, our sample was too small for extensive subgroup analyses; thus we are proposing to recruit a much larger cohort to identify subgroups for Aim 2. Second, few vaccine-naïve HCW were recruited because of high vaccination uptake among staff (>80%); thus, we have identified hospitals with lower vaccine uptake and will do purposive sampling of vaccine-naïve HCW. Third, the severity of influenza seasons is unpredictable. Our first study was conducted during a moderate-to-severe influenza season(30) and serological evidence of infection (4-fold increases in HI titres post-season) was evident in 25 HCW.(13) Our second study was conducted during a mild season. We actively followed and tested HCW, of which 65 reported an ARI, 6 tested positive, all for A(H3N2), but only 2/6 reported fever and only 1/5 showed further 4-fold rises post-season. To overcome seasonal variations, we are proposing to follow HCW for 4 years to capture a range of severity, and improve the possibility of comparing responses to the same and different vaccine formulations.(12) We will use a sensitive ARI definition that does not require fever, because we and others have shown febrile ARI may miss 50% of influenza-positive cases.(31) Although the role of mild infections in transmission remains unclear,(32, 33) they may be important in hospital settings.

Effect of prior infection on post-vaccination antibodies: [redacted] has followed an established cohort of ~1000 individuals in Ha Nam, Vietnam for 10+ years. 100 adults from this cohort, whose 10-years-prior infection status was known, received influenza vaccine for the first time in 2016. Pre-vaccination GMTs against the 2016 A(H3N2) vaccine component, A/Hong Kong/4801/2014, were higher if subjects had documented prior A(H3N2) infection (Error: Reference source not found), indicating that prior infections induced antibodies against epitopes that were retained in the vaccine strain. Post vaccination GMT and seroconversion rates were also higher amongst the 72 Ha Nam vaccinees who had documented prior A(H3N2) infection compared to 28 lacking recent prior A(H3N2) infection. HI titre rises were positively associated with multiple and more recent infection (Error: Reference source not found). Preservation of antigenic sites should be greatest between recently circulating strains and

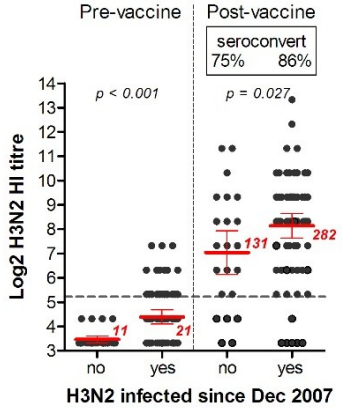


Figure 2. Effect of prior A(H3N2) infection on pre- and post-vaccine HI titres against A/Hong-Kong/4801/2014. *Symbols represent individual log2 HI titres for 72 vaccinees with documented prior A(H3N2) infection and 28 without. P values are shown for the comparison of pre and post-vaccine GMTs in these groups. Red bars show GMT + 95% CI. The dashed horizontal line represents the seropositivity threshold (HI≥40).*

the A(H3N2) vaccine strain. Therefore, higher vaccine HI titres in people with more recent prior infection are likely due to boosting of responses to preserved epitopes. An A(H3N2) epidemic commenced 9 months after vaccination, when A(H3N2) illness was detected in 4/28 vaccinees without recent prior infection versus 0/72 with recent prior infection (p = 0.006). A(H3N2) illness was also more common amongst vaccinees who did not seroconvert (3/17) compared to seroconverters (1/83, p = 0.013). Thus, recent prior A(H3N2) infection was associated with higher HI titres and seroconversion, and both prior infection and seroconversion were associated with protection after influenza vaccination.

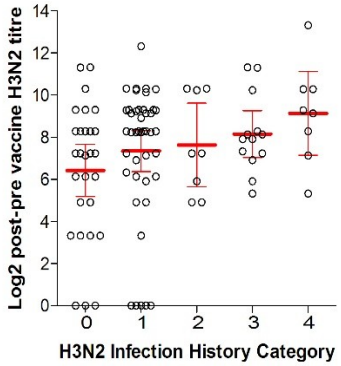


Figure 3. Effect of prior A(H3N2) exposure level on HI titre increase after vaccination.
Log2 titre increments are plotted according to a score based on time and number of prior A(H3N2) infections:
0 = no infections;
1 = 1 infection 4-9 Y prior
2 = 2 infections 4-9 Y prior
3 = 1 infection 1-3 Y prior
4 = 2 infections 1-3 Y prior.

It is notable that recent prior infection in the Ha Nam cohort was associated with enhanced vaccine antibody responses yet prior vaccination in the HCW cohort was associated with attenuated responses. We hypothesize that infection generates responses across a greater breadth of epitopes and antigenic sites compared to vaccination, such that even though baseline titres against that vaccine strain were relatively low in Ha Nam participants (Error: Reference source not found) compared to HCW (Figure 1) boosting across multiple epitopes could result in greater titre rises. Antibody focusing (described above (27)(28)) may occur because memory B cells and/or antibody monopolize antigen, interfering with the development of responses to altered epitopes in drifted strains. We propose that memory responses are more likely to monopolize antigen following vaccination, when antigen is limited, than following infection, and therefore vaccination is more likely to promote antibody focusing.

Modeling influenza infection and immunity: Understanding these complex dynamics, across a range of antigens and vaccination histories, is difficult to parameterize using standard statistical methods. Thus, [redacted] has developed several mathematical modeling tools to quantify the processes that shape influenza serological dynamics. It has been challenging to estimate influenza infection and vaccination history from observed HI titres because observed titres against specific influenza strains are the result of three main processes: (1) prior infection and vaccination history of that participant; (2) cross-reactive antibody responses against antigenically similar strains, which may vary over time; and (3) measurement variability in the assay itself. This leads to several sources of uncertainty; variation in the shape of antibody landscapes between two different participants may be the result of different exposure histories, differences in antibody responses, variability in the titre measurement, or a combination of all three.

Using a Bayesian modelling approach, it is possible to jointly estimate individual-level infection histories, temporal dynamics of antibody responses, and assay variability using serological data (Figure 4). This approach works because certain features of the antibody response, such as boosting following infection, and subsequent waning, exhibit a degree of consistency between participants with identical exposure histories.(34) Across a study population, it is therefore possible to estimate how much of the variation in titres is explained by differences in individual exposure histories, dynamic antibody responses or assay variability. Recent work has shown that although cross-sectional serological data cannot reliably estimate the shape and magnitude of short- and long-term dynamic antibody responses, with longitudinal samples it is possible to distinguish a short-term antibody response that wanes from a longer-term persistent response.(35) There can be a substantial rise in observed titre post-infection or

vaccination as a result of transient boosting;(24) such models can adjust for this dynamic process, and hence provide a more reliable estimate of the true exposure history.

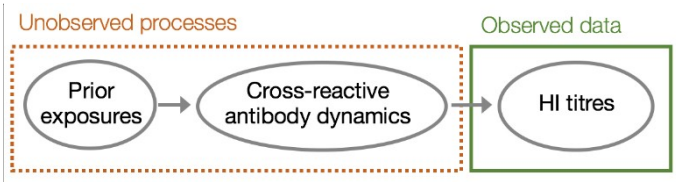


Figure 4 Schematic of Bayesian model of serological dynamics
Each participant in the study population has a set of unknown prior influenza exposures; these feed into a mechanistic model of cross-reactive antibody dynamics, which includes flexible parameters such as boosting, waning, and cross-reactivity. With sufficient observed titre data, it is possible to infer the unobserved processes and hence obtain estimates of exposure history for each individual.

Because such a model separately estimates individual-specific exposures and accompanying antibody dynamics, once it has been fitted to available data, it can simulate the likely antibody responses generated by any hypothetical combination of infection/vaccination. This makes it possible to forecast the temporal evolution of individual-level responses: antibody landscapes immediately following infection/vaccination may have a very different shape to landscapes months or years later. The model is not limited to simulating the sequence of exposures that occurred in the study population, it can also show what immune responses would be generated if influenza exhibited different epidemiology (e.g. in another setting) or under a different vaccination schedule.

To understand how individual-level antibody responses influence infection risk, and hence the effectiveness of vaccination, it is necessary to combine estimates of immunity with models of exposure risk. Previous work has shown that prior immunity may interact with person-to-person contact patterns in a non-linear manner to shape transmission during an outbreak.(36) Transmission models stratified by key epidemiological factors, including social contact patterns and prior immunity, have previously been used to estimate the direct and indirect effects of vaccination in different groups.(37, 38) Individual-based models have also been used to examine the indirect benefits of health care worker vaccination on patient morbidity in different health settings.(39, 40) To account for such heterogeneity in infection, we will combine all our data, including surveillance and serological data, to infer individual-level infection risk for a given immunological profile and prior exposure history, and hence evaluate the potential impact of different vaccination strategies.

6.2 COVID-19

Given the similarities between cases presenting with influenza and cases presenting with COVID-19, our funding agency has requested that we add on surveillance research activities to enhance our understanding of the COVID-19 pandemic. Our cohort presents a number of opportunities for epidemiological, virological and immunological investigations of COVID-19. Other novel coronavirus outbreaks, including SARS-CoV and MERS-CoV, have been characterized by nosocomial transmission,[1-3] and while this does not appear to be the main driver of transmission for COVID-19,[4] it is likely that our ARI surveillance will detect some COVID-19 cases in HCWs. We are already collecting sera around April and November, which will enable assessment of the asymptomatic infection rate. We intend to follow up HCW experiencing ARI to document the duration of symptoms and the illness outcome (e.g. GP visit, hospitalization, days absent from work). With the availability of COVID-19 vaccines (CoVax), studies comparing COVID-19 vaccines are needed and likely to be numerous. Studies that investigate the cellular and molecular basis for any differences in antibody responses against the CoVax brands may be rarer. In addition, there may be limited analysis of responses to CoVax and influenza vaccination, whether the sequence of vaccines matters, and if so why. Importantly, we already have the infrastructure and teams in place to conduct follow up of HCW experiencing an influenza illness, which

can be utilized for the follow up of HCW experiencing a COVID-19 illness or a HCW who has been vaccinated for COVID-19.

7 SIGNIFICANCE OF THE PROPOSED RESEARCH

The nature and impact of prior immunity is largely ignored during the implementation and evaluation of influenza vaccines, despite the requirement for frequent vaccine update and re-administration. Apparent attenuation of effectiveness with repeated administration calls this practice into question. Prior attempts to understand repeated vaccination effects have limitations. For example, the antigenic distance hypothesis (21) considered only the immediate prior vaccination and not prior infection or earlier vaccinations. Prior immunological studies, including our own, have been able to include only limited evaluation of the potential influence of prior exposures (e.g. through landscapes), and have not searched for evidence of antibody focusing that is enhanced among highly vaccinated persons (e.g. through quantifying the memory B-cell response). Earlier studies of the effects of repeated vaccination included obsolete vaccines (e.g. whole cell), were methodologically weak (10, 11, 22), or focused on adjuvanted A(H1N1)pdm09 vaccine (41). Finally, no previous mathematical model of the effectiveness of influenza vaccination for preventing infection in HCW and patients has taken into account prior vaccination status or individual serological responses to vaccination, and their associated cost-effectiveness analyses may therefore overestimate the benefits of annual vaccination. Advances in comprehensive immunologic measurements and computing power mean it is now possible to better understand the vaccine's mechanism of action with respect to key immunologic concepts, such as original antigenic sin (25), back-boosting (24) and antibody focusing.

Completion of the proposed research will define influenza exposures and cellular and molecular processes that contribute to attenuating vaccine antibody responses. This in turn will inform measures to improve VE, including, for example, the potential utility of adapting annual strain selection, annual recommendations for which vaccine formulations to use (e.g. inactivated vs. LAIV), vaccination schedules (e.g. annual or not), and investment decisions for novel types of influenza vaccines. The outputs of our cohort surveillance will be used to inform new mathematical models of vaccination and infection dynamics relevant to this frequently vaccinated population. HCW represent a target group for vaccination largely comprised of healthy adults (i.e. the group for whom we believe the vaccine should work best) and who may have higher influenza infection rates (42). The findings from our study in Australia will be relevant to HCW vaccination policy in all nations and can be used to optimize vaccination schedules and re-evaluate the cost-effectiveness of HCW vaccination programs. Improving the methodology for HCW studies lays a foundation for the future: established HCW cohorts, like the one proposed, can be leveraged for the evaluation of novel vaccines and vaccination strategies for which HCW will likely be a key target. Our findings will also be relevant to other highly vaccinated populations, such as the elderly, and to the evaluation of novel vaccines and novel applications of existing vaccines.

8 AIMS AND OBJECTIVES

We propose to establish a longitudinal cohort of healthcare workers (HCW) to understand why immunogenicity and effectiveness appear to attenuate with repeated administration of the influenza vaccine. To do this, we will focus on three specific aims:

1. To study how the immunogenicity and effectiveness of influenza vaccination is influenced by prior vaccination experience.

2. To characterize immunological profiles following infection and vaccination
3. To evaluate the impact of immunological profiles on vaccination effectiveness

8.1 Aim 1: To study how the immunogenicity and effectiveness of influenza vaccination is influenced by prior vaccination experience

8.1.1 Objectives:

1. To compare immunological responses to vaccination by vaccination history
2. To compare influenza attack rates by vaccination history

8.1.2 Outcomes

1. Seropositivity post-vaccination; i.e. proportion of post-vaccination titres >40 (1)
2. Seropositivity post-season; i.e. proportion of HCW with antibody titres >40 at the end of the season (~November each year) (1)
3. Fold-rise in geometric mean antibody titre (GMT) per- to post-vaccination (1)
4. Fold-change in geometric mean antibody titre (GMT) post-vaccination to post-season (1)
5. Seroconversion fraction post-vaccination; i.e. proportion of samples with 4-fold increases in HI titre (1)
6. Proportion of HCW PCR-positive for influenza at the end of each season (2)

Completion of this aim will result in immunogenicity data, as well as attack rates to potentially calculate VE, and confirm the existence of differences in immune responses by vaccination history and, as the study progresses, by infection history.

Characterization of the cohort in this Aim will inform the selection of sera for further assessment in Aim 2, and immunogenicity data and attack rates will inform Aim 3.

8.2 Aim 2: To characterize immunological profiles following infection and vaccination

8.2.1 Objectives:

1. To evaluate whether repeated vaccination leads to more focused or narrow antibody response profiles
2. To evaluate whether the breadth of the antibody response is associated with influenza susceptibility in HCW
3. To explore the cellular and molecular mechanisms that shape the antibody response

8.2.2 Outcomes

1. HA antibody landscapes for vaccine-naïve and highly vaccinated HCWs (1)
2. HA antibody landscapes for infected versus uninfected HCWs (2)
3. Enumeration of influenza haemagglutinin (HA)-reactive B cells, and of subsets with phenotypic markers indicative of activation, and of memory versus naïve status, for vaccine-naïve, highly vaccinated and infected HCWs (2,3)
4. B cell receptor gene usage by influenza HA-reactive B cells recovered post-infection from selected vaccine-naïve, highly vaccinated and infected HCWs with distinct antibody response profiles. In depth characterization of HA antigenic sites recognized by serum antibodies from selected HCW including vaccine non-responders who lack seroprotection, and vaccine serological responders who fail to be protected (2,3)

Qualitative insights from this Aim will inform the development of mechanistic mathematical models of antibody responses in Aim 3.

8.3 Aim 3: To evaluate the impact of immunological profiles on vaccination effectiveness

8.3.1 Objectives:

Quantify the impact of immune profiles on effectiveness of vaccination using mathematical models fit to data from Aims 1 and 2:

1. To estimate the key immunological parameters that shape observed antibody landscapes
2. To determine how antibody responses generated by prior exposures correlate with protective immunity
3. To determine how different epidemiological scenarios, antigenic variation, vaccination schedules and vaccine compositions influence vaccination effectiveness
4. Estimate the potential benefits of different HCW vaccination programs

8.3.2 Outcomes

1. Quantify biological mechanisms that shape the antibody response
2. Estimate protective titres
3. Estimate vaccine effectiveness
4. Optimal vaccination strategy for HCW under different vaccine availability

8.4 Aim 4: To increase our understanding of the epidemiological, virological and immunological characteristics of SARS-CoV-2 infections among HCWs

Note: activities relevant to this aim appear in the addendum to this protocol

8.4.1 Objectives

1. To estimate risk factors and correlates of protection for SARS-CoV-2 infection
2. To characterise SARS-CoV-2 viruses infecting HCWs
3. To characterise immunological profiles following infection by SARS-CoV-2

8.4.2 Outcomes

1. Estimated attack rates among symptomatic and asymptomatic HCWs
2. Risk factors for asymptomatic, mild and severe infection
3. Estimated antibody titre associated with protection
4. Estimated antibody kinetics over time
5. Estimated duration of viral shedding and viral load over time
6. Exploratory immunological findings to characterize the response to SARS-CoV-2 infection, including enumeration of SARS-CoV-2-reactive B and T cells and identification of dominant epitopes

8.5 Aim 5: To increase our understanding of the immunological characteristics of SARS-CoV-2 vaccination among HCWs

Note: activities relevant to this aim appear in the addendum to this protocol

8.5.1 Objectives

1. To measure and compare immunological responses to Adeno or RNA vaccines versus influenza protein vaccine

2. To characterise immunological profiles following vaccination for SARS-CoV-2

8.5.2 Outcomes

1. Estimated post-vaccination serum antibody titres over time
2. Enumeration of SARS-CoV-2-reactive B and T cells and identification of dominant epitopes
3. Exploratory immunological findings to characterize the response to SARS-CoV-2 vaccination

9 STUDY DESIGN

9.1 Overview of Study Design

The proposed study is a longitudinal cohort study (Figure 5) that includes 2 nested studies (Figure 6 and Figure 7). This study will collect data, blood and respiratory specimens from approximately 1500 HCWs to understand responses to influenza vaccination.

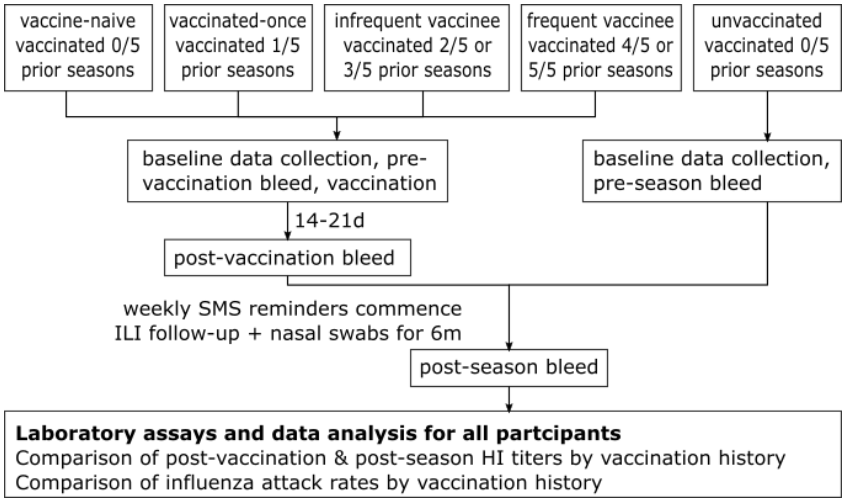


Figure 5. Study flowchart for recruitment of the primary cohort and main analyses proposed in Aim 1.
We plan to recruit around 1500 HCWs, and expect that this will consist of at least 100 HCWs in each of the vaccination groups; the majority are likely to be frequent vaccinees. The expected proportion of each group is described in 12.1.

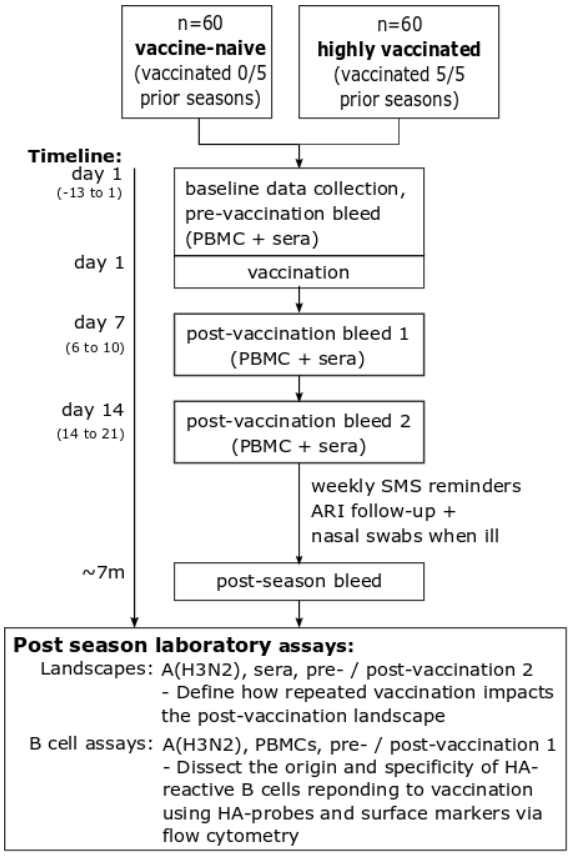


Figure 6. Study flowchart for the nested study comparing highly vaccinated and vaccine-naïve HCWs

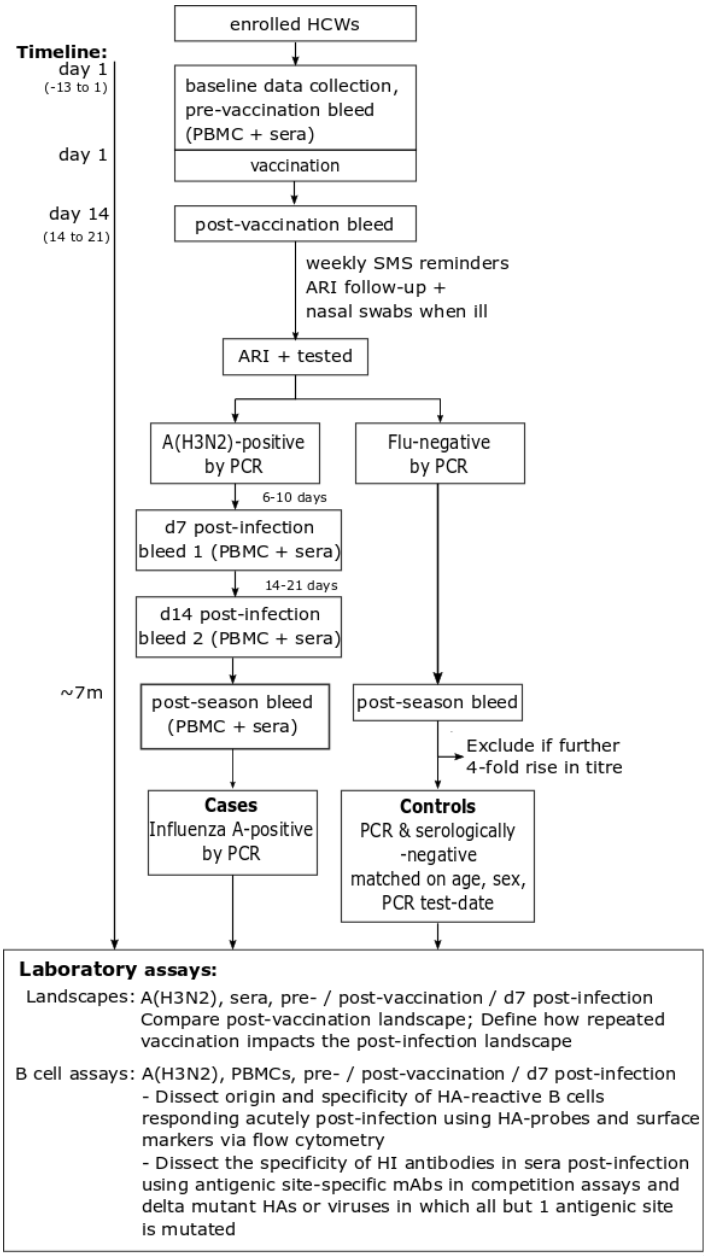


Figure 7. Study flowchart for the nested case-control study comparing A(H3N2)-infected and uninfected HCWs

Note, unvaccinated HCW will also be eligible for post-infection follow and would not be vaccinated or have the post-vaccination blood draw.

9.2 Study Schedule

The study will run for 5 years (Figure 8).

For Aim 1, recruitment of HCWs will begin in April 2020 to coincide with the usual timing of hospital influenza vaccination campaigns. Follow-up of HCW enrolled in year 1 will continue for 4 years (2020-2023). The cohort will be open to recruitment of new HCW in years 2-4, with a preference for vaccine-naïve HCW, to replace HCW lost to follow-up. Year 5 will be devoted to laboratory, statistical and mathematical analyses. Post-season blood samples will be collected by November or the end of the influenza season of each follow-up year. Samples collected in any study year will be analysed as described for Aim 1 at the end of each study year; i.e. starting around December (e.g. comparison of

post-vaccination HI titres; calculation of annual attack rates). We will allow 12 months in year 5 for final analyses and preparation of manuscripts reporting the final results for Aim 1.

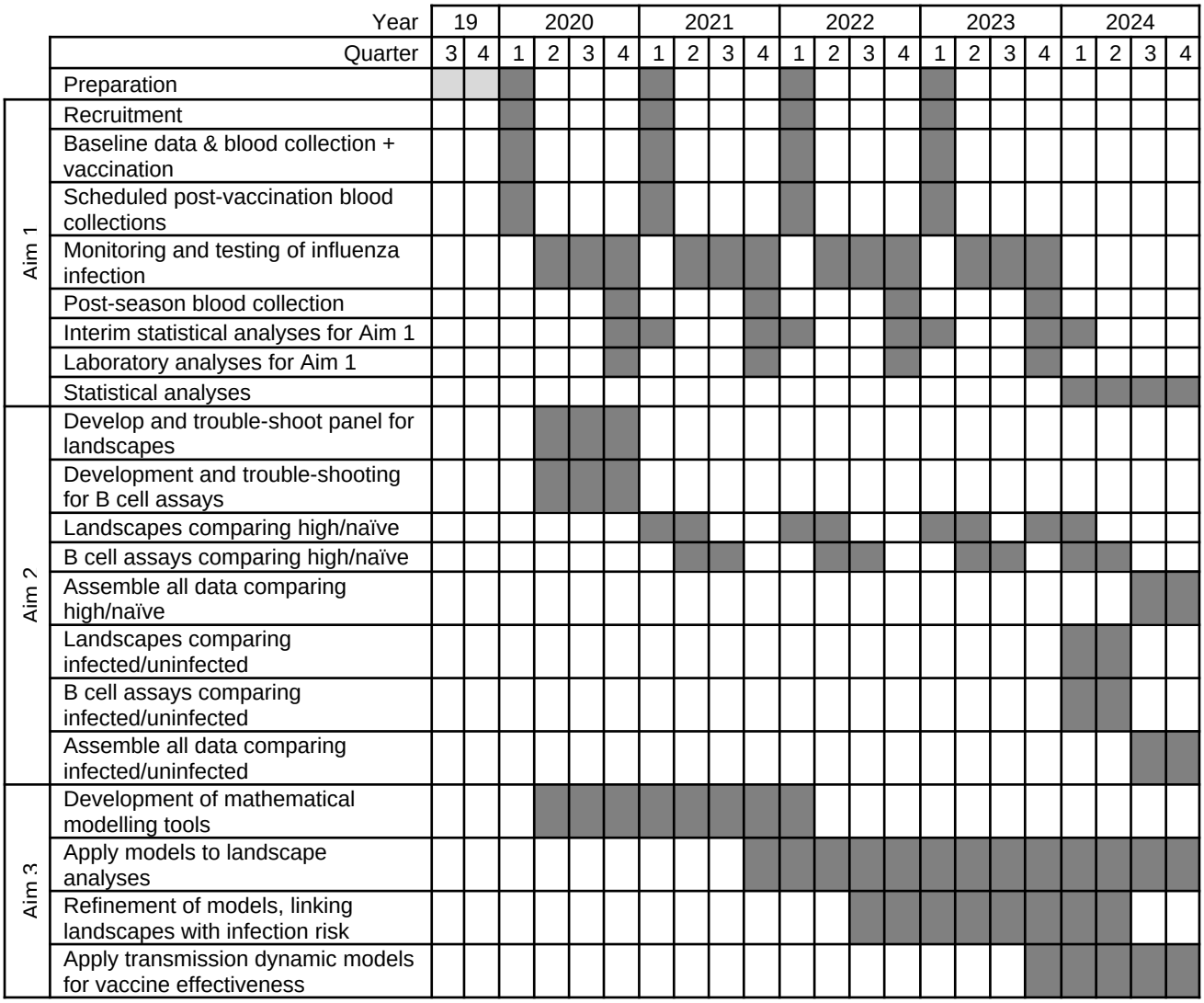


Figure 8. Study timeline, by year and quarter

For Aim 2, development and trouble-shooting of the panel to be used for landscape analyses will commence in year 1. Comparison of the immune responses for the nested study comparing highly vaccinated and vaccine-naïve HCWs will commence in year 2 when all sera from year 1 are available. This will include landscapes and memory B cell assays. Assays comparing infected and uninfected HCWs will be conducted in year 5 once the full set of infected HCWs is known. If, however, sufficient A(H3N2)-infected HCWs are identified sooner, these assays can commence sooner.

For Aim 3, preliminary development of the mathematical models will commence in year 1. Models comparing the antibody landscapes can commence in year 2 once early data are available, and can be updated as new data become available. Models for vaccine effectiveness will be worked up during the life of the project, with final models developed in year 5.

10 STUDY POPULATION

10.1 Eligibility

10.1.1 Inclusion criteria

Eligible participants will be recruited from 1 of 6 participating hospitals (Table 5) and will meet the following criteria:

- Personnel (including staff, honorary staff, students and volunteers) located at a participating hospital or healthcare service at the time of recruitment who would be eligible for the hospital's free vaccination programme
- Be aged ≥18 years old and ≤60 years old;
- Have a mobile phone that can receive and send SMS messages;
- Willing and able to provide blood samples;
- Available for follow-up over the next 7 months;
- Able and willing to complete the informed consent process.

There are no restrictions on the type of HCW that can be recruited into the study in terms of their job role. HCW will be any hospital staff, including clinical, research, administrative and support staff.

Table 4. Participating hospitals

Site	Hospital	Approx. number of staff	Approx. vaccination uptake in 2017	Site PI

10.1.2 Exclusion criteria

- Immunosuppressive treatment (including systemic corticosteroids) within the past 6 months;
- Personnel for whom vaccination is contraindicated at the time of recruitment

10.2 Cohort Recruitment

10.2.1 Recruitment strategies

Participants will primarily be recruited from the staff vaccination clinics, which are temporarily set up around April each year for the influenza vaccination campaign. As per our previous HCW studies (see 6.1), a member of the study team (e.g. site manager, phlebotomist) will wait outside the clinic to ask approaching staff members whether they are interested in participating in the study. If the staff members indicate their interest, the study team member will go through the eligibility criteria (Appendix A) and informed consent procedures. Baseline data collection and blood draws will take place in a private location located in or adjacent to the clinic. To facilitate recruitment, the baseline blood draw may occur up to two weeks prior to vaccination.

The study will be promoted via the staff newsletters, the staff influenza vaccination campaign webpage, the study website, email circulation to Heads of Department and with flyers, as permitted according to

each hospital's policy. A copy of the advertising materials can be found in Appendix B. HCWs will be able to contact the site manager or site PI to make an appointment for their baseline bleed.

Participants who are interested in participating in the study may register their name and contact information on the study website for follow up from the site manager.

We expect that a high proportion of frequently and infrequently vaccinated HCWs will be willing to participate in the study and there is unlikely to be any risk associated with recruiting that target sample through the staff vaccination clinics.

For vaccine-naïve and unvaccinated HCWs we will employ active, targeted recruitment strategies to ensure we meet our target sample size. This is expected to be the most difficult group to recruit. There is a possibility that people who are unwilling to be vaccinated have a dislike for needles which would also make them reluctant to participate in a study which involves blood draws. This is also a possibility for the other vaccination groups. HCWs will not be pressured into participation.

Recruitment will be open, with new HCW recruited in years 2-4. Although we will make efforts to retain HCWs throughout the 4 years of active recruitment and follow-up, we expect some attrition and there is a need to recruit new vaccine-naïve and unvaccinated HCW to ensure there is a comparison group for annual analyses.

Participants will be reimbursed for their time, [redacted]:

- Participants in the main study will receive [redacted].
- Participants in the nested cohort will receive [redacted]
- Participants testing positive for influenza will receive [redacted]
- Participants testing positive for SARS-CoV-2 will receive [redacted]

10.2.2 Retention strategies

HCW will be enrolled for up to 4 years. We do not expect high attrition [redacted] The latter is a larger hospital with higher staff turn-over and thus for the proposed study we have tried to select hospitals with a smaller staff populations.

To retain HCWs we will use the following strategies:

1. Weekly reminders through SMS or email to report ARI symptoms during the influenza season (however disruptions to usual seasonality might mean that a different period is more appropriate.). Frequent reminders [redacted] has been associated with reduced attrition in cohort studies;
2. MS Outlook calendar invites and SMS reminders for scheduled follow-up appointments, which worked well in our previous HCW studies;
3. [redacted] at each follow-up appointment involving a blood draw;
4. Monitor completion of weekly ARI surveys to identify participants who are not responding and identify communication issues;

5. Provide prompt feedback about the results of influenza testing;
6. Provide annual feedback on antibody responses to vaccination (e.g. HI titre responses to the 4 vaccine components);
- [redacted]
- [redacted]
9. Study website with copies of publications or presentations arising from the study.

10.2.3 Follow-up

At enrolment, participants will be requested to provide a mobile phone number and email address. Follow-up appointment reminders will be sent via email and/or SMS (Appendix D).

In addition, participants will be given an appointment card at enrolment (Appendix G). The dates of visits will be completed as each visit happens. This is so that participants will have at hand their participant ID, the timeline of visits that they will have to do (specific to them) and contact numbers for questions/concerns.

10.2.4 Informed consent

HCWs will be recruited at the staff vaccination clinic or via study marketing materials. HCWs who express an interest in participation will be provided with a copy of the participant information and consent form, either in person (if recruited at the staff vaccination clinic) or by email (if recruited via study marketing materials). Written or electronic informed consent will be sought from all HCWs prior to enrolment in the study. Participation in the study will be voluntary. HCWs will be informed that they are free to withdraw from the study at any time. The concept of voluntary participation will be clearly explained and ample time will be given to ensure that participants understand the content and have all of their questions answered.

Willing HCWs will sign the informed consent to indicate assurance of their understanding and voluntary participation in the research. [redacted]

Personal information will not be released without written permission of the participant, except as necessary for monitoring by the Human Research Ethics Committee. The study coordinator and site PI's contact details will be made available to participants should they have any queries or concerns during the study. [redacted]

For the unvaccinated group of HCWs, the importance of vaccination will be explained and they will be offered the option of receiving the vaccine. If they still decide that they do not wish to be vaccinated, but do wish to participate in the study, we will enrol them and collect data and biological specimens as described above for the unvaccinated group. Those who decide to be vaccinated will be referred to the vaccination clinic, as per hospital protocols.

10.3 Data Collection

10.3.1 Overview of data collection

The data collected from participants includes:

1. Baseline questionnaire
2. Social contacts questionnaire
3. Additional information – participant contact information and influenza vaccination documentation
4. Symptoms diaries (during the influenza season or during inter-seasonal period if there is an increase in COVID activity)
5. Blood samples
6. Respiratory swab samples

At each recruitment site, the same general procedures for obtaining informed consent, collecting baseline data and collection of specimens will be followed. Tables summarising the procedures at each visit are found below.

Table 5. Scheduled and unscheduled visits for participants in the main study. Visits shown are for 1 year only.

	Assessment/ Procedure	Screening & recruitment	Visit 1 (14d)*	Visit 2 (7m)	Visit 3 (ARI)	Visit 4 (7d post-infA$^+$ ARI)	Visit 5 (14d post-infA$^+$ ARI)	Weekly (during influenza season)
Procedures	Informed consent	x						
	Demographic information	x						
	Vaccination history	x						
	Risk factors	x						
	Height/weight measurement	x						
	Vaccination	x						
	Sera collection	x	x	x		x	x	
	PBMC collection			x**		x	x	
	Symptoms diary							x
	Respiratory swab				x			

*as more data become available the exact timing of the post-vaccination bleed may change; however the number of bleeds will remain the same. Blue shading indicates unscheduled visits. infA$^+$ ARI = influenza A-positive ARI.

**if influenza A positive, PBMC collection to occur post-season

Table 6. Scheduled and unscheduled visits for participants in the nested case control study comparing highly vaccinated and vaccine-naïve participants. Visits shown are for 1 year only.

	Assessment/ Procedure	Screening & recruitment	Visit 1 (7d)*	Visit 2 (14d)*	Visit 3 (7m)	Visit 4 (ARI)	Visit 5 (7d post-infA⁺ ARI)	Visit 6 (14d post-infA⁺ ARI)	Weekly (during influenza season)
Procedures	Informed consent	x							
	Demographic information	x							
	Vaccination history	x							
	Risk factors	x							
	Height/weight measurement	x							
	Vaccination	x							
	Sera collection	x	x	x	x		x		
	PBMC collection	x	x	x	x		x	x	
	Symptoms diary								x
	Respiratory swab					x			

*as more data become available the exact timing of post-vaccination bleeds may change; however the number of bleeds will remain the same. Blue shading indicates unscheduled visits. infA⁺ ARI = influenza A-positive ARI.

10.3.2 Baseline questionnaire

A baseline questionnaire will be used to collect information about HCW's including demographics, height, weight, vaccination history, and risk factors for influenza infection such as workplace exposures and relevant medical history (39)(Appendix C). The past 5-years' vaccination history of the HCW will be determined through self-report and corroborated with staff influenza vaccination records, where available.

Participants will provide their email and mobile phone number, to allow a unique survey link to be sent for the baseline questionnaire. If paper questionnaires are being used, this information will be collected on the form and entered into the study database at a later time.

10.3.3 Social contacts questionnaires

To help inform our understanding of transmission dynamics, HCW will be requested to complete a social contacts questionnaire. These are detailed questionnaires designed to inform contact networks analysis. A contact is defined as conversational, involving a two-way face-to-face conversation of 3 words or more; or physical, involving physical touch.

For each contact, HCW will be asked:

1. Age of contact. *Enter age*
2. Gender of contact. *Choose from: Female, Male, Other, Unspecified*
3. Type of interaction. *Choose from: Conversational (two-way face-to-face conversation of 3 words or more) OR Physical (handshake, hug, kiss, etc)*
4. Location of interaction. *Choose from: Home, School, Work, Other*
5. Have you spoken to this person before? *Choose from: Yes or No*

Questionnaires will be requested at baseline and at a later time coinciding roughly with the peak of influenza activity.

10.3.4 Vaccination documentation

The 5 year influenza vaccination history will be determined through self-report and corroborated with staff vaccination records, where available. The brand and batch number of vaccine received at enrolment will be recorded. Only 3 vaccines are licensed for adults aged 18-60 years in Australia and all are quadrivalent.

COVID vaccination documentation may also be collected should a HCW be vaccinated for COVID. The date, brand and batch number of the vaccine received will be recorded.

10.3.5 Blood collection

10.3.5.1 Schedule of bleeds

The frequency of blood sample collection will vary depending on whether the participant consents to collection of additional samples for recovery of peripheral blood mononuclear cells (PBMCs) for the nested case control studies. All blood samples will be collected in a private room.

Main study: For the majority of vaccinated participants, serum samples will be collected according to the following schedule:

1. 3 visits at pre-specified times: baseline, post-vaccination and post-season
    a. 1 blood sample will be collected at each visit in a 9ml serum tube

For unvaccinated participants, the post-vaccination blood sample is irrelevant and visit will be scheduled as follows:

1. 2 visits at pre-specified times: baseline and post-season
    a. 1 blood sample will be collected at each visit in a 9ml serum tube

Nested cohort study of highly vaccinated and vaccine-naïve HCWs: For a subset of roughly 60 highly vaccinated and 60 vaccine-naïve participants, samples will be collected according to the following schedule:

1. 4 visits at pre-specified times: baseline, 2 post-vaccination times and post-season
    a. 3 blood samples will be collected at each visit:
        i. 1 × 9ml serum tube
        ii. 2 × 9ml samples in sodium heparin tubes (for recovery of PBMCs)

Nested case control study of infected and uninfected HCWs: For HCWs who report a respiratory illness and are identified to have tested influenza-A positive, further blood samples will be requested. Where the subtype is known, follow up sampling will only be performed for H3N2-positive participants. The exact number is difficult to predict but is expected to be around 60 per year:

1. 2 additional visits post illness onset
    a. 3 blood samples will be collected:
        i. 1 × 9ml serum tube
        ii. 2 × 9ml samples in sodium heparin tubes (for recovery of PBMCs)
2. Post-season, 2 additional blood samples will be collected (if not already being collected):
    a. 2 × 9ml samples in sodium heparin tubes (for recovery of PBMCs)

For all groups, the exact timing of the post-vaccination bleed(s) and post-infection bleed(s) may change as more data about optimal timing become available. Where the number of days is specified, some leeway is permitted. For example, blood samples taken 7 days post-vaccination or infection may be taken 6-12 post; samples taken 14 days post-vaccination or infection may be taken 14-21 days post. However, the number of bleeds will remain the same.

10.3.5.2 COVID vaccination

If the timing of the COVID and influenza vaccines do not coincide, HCWs who are vaccinated for COVID may be requested to provide additional blood samples. Schedules outlined in Tables 5 & 6 will be used to guide timing. Extra blood draws will be minimized as much as possible.

10.3.5.3 Blood collection procedures

Blood collection will be performed in line with each hospital's phlebotomy policy. The use of a butterfly needle connected to a vacutainer tube is recommended as the best collection method to minimize haemolysis and to reduce the risk of needle stick injury. Blood collection will take place in a private room or behind a screen.

10.3.5.4 Blood sample processing

Blood samples will be processed and stored [redacted]

10.3.6 Active surveillance for acute illness

HCWs will be asked to complete symptoms diaries in the form of a simple, weekly online questionnaire (Appendix D). Weekly email and/or SMS reminders will include a link to the online survey. Surveys will be sent during the influenza season, however disruptions to usual seasonality might mean that a different period is more appropriate. Weekly symptom surveys may recommence for a particular site during the inter-seasonal period, if there is an increase in community transmission of influenza or SARS-CoV-2.

A range of symptoms commonly used in influenza symptom severity questionnaires will be graded by participants as absent, mild, moderate or severe. HCWs will have the opportunity to complete previous weeks' reports, which has been found greatly improves the completeness of reporting. Frequent reminders should minimize recall bias and missing data.

HCWs reporting ≥1 respiratory symptom (e.g. cough, sore throat, stuffy nose, chest pain, difficulty breathing) and ≥1 systemic symptom (e.g. feverishness, temperature ≥38°C, chills, headache) or ≥2 respiratory symptoms will be requested to self-collect a respiratory swab. Participants may be provided with a thermometer and will monitor their temperature for 3 days at enrolment to establish their own baseline for normal temperature and fever.

Participants will be requested to complete daily symptoms diaries until their illness resolves, which will include only the symptoms questions from the weekly diary. Site staff will follow-up HCW to determine for how long they remained unwell and whether additional medical attention was required, if this information has not been provided in the weekly diaries.

10.3.6.1 Respiratory specimen collection

Participants will be provided with swab kits (swab plus vial of universal transport medium) at enrolment with instructions on how to use them (). Respiratory swabs in universal transport medium

can be stored in the home refrigerator if the HCW is on sick leave. The site manager will be alerted when a HCW has ARI symptoms and will follow up with the HCW to arrange for respiratory swabs to be forwarded to the hospital laboratory for testing. Depending on state government Department of Health and/or hospital requirements at the time, HCWs may be required to attend a COVID-19 screening clinic for a COVID-19 test. If so, study staff may retrieve swabs collected at a COVID-19 screening clinic to ensure the sample is tested for influenza and other respiratory viruses. Positive swabs will be forwarded to [redacted] for virus characterization.

11 LABORATORY PROCEDURES

11.1 Laboratory procedures relevant to Aim 1

11.1.1 Sero-response to vaccination

Pre-vaccination, post-vaccination and post-season serum samples will be tested for antibodies to the 4 vaccine strains (cell- + egg-derived) in the current year's quadrivalent influenza vaccine. We will primarily use haemagglutination inhibition (HI) assays to measure antibody titres. This assay measures the ability of antibodies in the blood to prevent haemagglutination—the attachment of influenza virus particles to red blood cells. Samples are serially diluted (titrated) and the highest dilution of serum that prevents haemagglutination is the HI titre. Reciprocal titres of 40 or higher are generally accepted as indicating protection against infection ("seropositivity"), with very high titres suggestive of recent infection (43). The HI assay is the standard assay used to measure antibody response to both infection and vaccination, and to assess the sensitivity of circulating influenza viruses to the vaccine.

For A(H3N2) viruses, we may use a microneutralisation (MN) to assess A(H3N2) antibody responses to vaccination. This is a functional assay that measures the ability of antibodies to neutralize virus infectivity. Serum is mixed with virus and residual infectivity of the virus is assessed by adding the mixture to cells. The highest dilution of serum that neutralizes virus infectivity is the MN titre. Concordance between the HI and MN is high (44).

Other antibody assays may be performed as well to understand the breadth of the antibody response. These might include but would not be limited to neuraminidase antibody assays.

Participants will receive results of their antibody responses to vaccination (e.g. HI titre responses to the 4 vaccine components) ().

11.1.2 Influenza testing and virus characterization

Respiratory swabs collected from HCWs reporting ARI symptoms will be tested using reverse transcription real-time polymerase chain reaction (RT-PCR), according to standard operating procedures [redacted] Influenza-positive samples will be forwarded [redacted] for virus characterization. The virus subtype (for influenza A) or lineage (for influenza B) will be identified. Viruses will be isolated and tested by HI/MN or similar assay to assess antigenic match to vaccine, and sequenced to assess genetic match to the vaccine and to identify any genetic clusters within hospitals.

11.1.3 Reporting of influenza RT-PCR results

The site manager will receive RT-PCR results from their relevant diagnostic laboratory and enter the results into the study database. Participants will be informed of their test results and provided with the standard results sheet provided by each testing laboratory. Participants will be informed that:

- False positive and negative results are possible;
- Participants should consult their personal healthcare provider if they have questions, concerns, or any medical needs related to their illness;
- They should follow their employer's guidelines for reporting illnesses and returning to work.
- Participants will be reminded that they will be contacted to schedule post-infection follow-up bleeds.

11.2 Laboratory procedures relevant to Aim 2

For aim 2, samples collected from HCW in the two nested studies will be further assessed. These include:

1. Highly vaccinated HCWs
2. Vaccine-naïve HCWs at the time of recruitment
3. Infected HCWs

Samples from other participants may also be assessed. For example, if after initial comparisons of highly vaccinated and vaccine-naive HCW, it becomes necessary to understand how responses might differ in HCWs vaccinated 2 or 3 times prior to recruitment, we may select sera from such participants for further assessment.

11.2.1 Antibody landscapes

We will build antibody landscapes (24) by testing sera in HI assay (as described in 11.1.1) against influenza A(H3N2) strains that have circulated since 1968, including currently circulating strains and candidate vaccine viruses to assess "future" protection (see Figure 1). The panel will probably include 3-4 viruses from each antigenic cluster. We have already established a panel of ~33 A(H3N2) viruses (see Figure 1), to which we will add additional viruses as the study progresses.

11.2.2 HA-reactive B cell response

PBMCs will be used to enumerate influenza haemagglutinin (HA) reactive B cells and their phenotypic subsets. Analysis on day 7 will focus on B cells that have actively participated in the response, indicated by high expression of proliferation (CD71) markers and/or by expression of plasmablast markers ($CD38^{hi}$,$CD27^{hi}$)(45).

A mixture of fluorescent-labeled HAs representing HAs of vaccine and past strains will be used to differentiate B cells that react only against the vaccine strain from those that cross-react against vaccine and past-strains. This analysis will define whether there is greater dominance of cross-reactive memory B cells over vaccine strain-only-reactive B cells in highly vaccinated HCW compared to previously vaccine naïve HCW, and whether differences in the composition of the B cell response underlie differences in the breadth of the serum antibody response.

To further validate whether B cells that react with vaccine HA only originate from the naïve B cell pool while HA cross-reactive B cells originate from the memory pool they will be individually sorted for selected HCW. B cell receptors (BCR) will be amplified, bar-coded and sequenced using high-throughput Illumina. The selection of HCW and time-points (e.g. d7 or 14) will be based on frequencies observed during the initial analyses described above.

To examine whether repeated vaccination is associated with focusing of B cell HA responses BCR diversity will be compared amongst cross-reactive memory B cells from selected vaccine naïve and highly vaccinated HCWs who have highly expanded clones.

11.2.3 Antibody focussing

Antigenic site-specific monoclonal antibodies (mAbs)(28) and reverse engineered viruses containing single substitutions in HA antigenic sites will be used to investigate antibody focusing (28, 29). Sera from HCWs exhibiting a range of responses will be assessed, including, but not limited to:

1. Pre-vaccination sera from HCWs who did not appear to respond to the vaccine
2. Pre- and post-vaccination and post-infected sera from HCWs who appeared to respond to the vaccine but were subsequently infected
3. Pre- and post-vaccination sera from HCWs who appeared to respond to the vaccine and did not get infected despite probable exposure. Probable exposure will be defined exposure to colleagues or patients with confirmed influenza.
4. Additional groups selected as further information about the antibody responses become available during the study.
5. Preference will be given to sera from HCWs in the nested study to facilitate comparison of B cell probe reactivity and serum antigenic site reactivity

mAbs procedures: competitive ELISAs utilizing biotinylated mAbs will be used to detect epitope specific antibodies in human sera. The mAb panel will gradually be augmented as B cell receptors (BCRs) from sorted HA-reactive and cross-reactive B cells from our vaccine studies are expressed. We will only use mAbs that inhibit HI at concentrations ≤ 1ug/ml.

Reverse-engineered viruses: Viruses with engineered point mutations within HA antigenic sites (28) will be use to compare serum HI titres against viruses containing wild-type versus engineered HA. The extent to which antibodies are focused on a given antigenic site in a vaccine virus will be indicated by the extent to which titres are reduced by substitution(s) within that site. We will focus on introducing substitutions within an antigenic site(s) that has been preserved in successive strains, and/or that appears to be the focus of serum antibody binding based on mAb studies. Where possible we will introduce substitutions that subsequently occur in circulating strains, and also examine whether this contributes to any loss in titre.

12 DATA CONSIDERATIONS

12.1 Sample Size

We aim to recruit 1,500 HCWs. This is based on the number of HCW we reasonably expect to recruit and retain (~250 per site), based on our previous studies of HCW (see 6.1) and detection of meaningful effects.

We expect that there will be relatively more highly vaccinated HCW willing to participate. We will encourage recruitment of unvaccinated and vaccine-naïve HCW to ensure there are adequate numbers of these groups for comparisons with HCW vaccinated repeatedly. The target samples size by vaccination group is approximately:

- 600 frequently vaccinated(vaccinated 4/5 or 5/5 prior years),
- 300 infrequently vaccinated (2/5 or 3/5 prior)
- 200 vaccinated once (1/5 prior)
- 200 vaccine-naïve (0/5 prior)
- 200 unvaccinated (0/5 prior and not vaccinated in current year).

Enrolment will be open for recruitment of new vaccine-naïve and unvaccinated HCWs in each study year. Annual analyses will be separated because our underlying hypothesis presupposes that the repeat vaccination effects will not be apparent in all years. Samples sizes were calculated in R 3.4.1 using the TrialSize package.

12.1.1 Primary objective

For the primary objective of Aim 1 (immunogenicity), the power analysis considers expected post-vaccination seropositivity among vaccinated HCWs in any one study year (46). A recent US study (47) observed a trend in post-vaccination seropositivity against A(H3N2) from 49% in frequently vaccinated, to 57% in infrequently vaccinated and 69% in vaccine-naïve HCWs; i.e. a roughly 10 percentage point difference, which we consider meaningful. In our study [redacted] sero-positivity among frequent vaccinees was 76% and the ratio of frequent: infrequent vaccinees was 2.5:1; thus the sample needed to see a trend in sero-positivity from 76% to 86% at α=0.05, β=0.2 is at least 162 vaccine-naive and 405 frequently vaccinated HCWs.

12.1.2 Secondary objectives

For the secondary objective of Aim 1 (attack rates), with a sample of 1,500, we expect ARIs to be reported among ~40% of HCWs, among whom ~20-25% will test positive, for an annual attack rate of ~3.5% (n≈50/ year; 200 total). [redacted] Published data suggest attack rates will be around 1.2% among vaccine naïve and 5.44% among the unvaccinated (42), and that the odds of infection will follow a trend, with the odds lowest for vaccine-naïve, followed by unvaccinated, then the repeat vaccinees (22). If we assume a monotonic trend within levels of repeated vaccination, with the odds of infection highest for the highly vaccinated (and comparable with the unvaccinated), with at least 159 HCW per vaccination group, at α=0.05, this study will have 80% power (β=0.2) to detect a trend in attack rates from 1.2 in vaccine-naïve, to 5.4 in frequently vaccinated/unvaccinated. We therefore aim to recruit at least 200 HCWs per vaccination group (to account for potential attrition). Based on surveillance data, >50% of infections are expected to be A(H3N2), and if we are able to collect day 14 post-infection sera from at least 50%, we will have a feasible number of infections for the immunological analyses proposed in Aim 2 (n≈50).

The total staff population at the 6 hospitals is 25,500, with vaccination uptake in 2017 at 50-80%. Thus, we expect to meet our target sample size of 1,500, which represents just 6% of staff.

12.2 Data Analysis

All statistical analyses will be performed in the statistical software package R.

12.3 Data cleaning

The data collected will be reviewed and cleaned prior to any statistical analyses. Cross-tabs will first be run for categorical variables to identify any unusual associations. The paper/electronic record will be checked to identify the data entry error. For continuous variables, histograms and boxplots will be generated to potential identify outliers. All outliers will be checked against the original data collection records.

12.4 Missing data

Missing data patterns will be explored and the method of multiple imputation will be used for handling missing data and to assess the sensitivity of the results to the missingness (48).

12.5 Analyses relevant to Aim 1

12.5.1 Baseline comparisons

Descriptive statistics will be calculated to summarise the data collected at baseline, following STROBE guidelines. Histograms will be generated to examine the distribution of continuous variables. Descriptive statistics to be reported will be frequency (%) for categorical variables, and mean (standard deviation) (or median, 25^{th}-75^{th} percentiles) and range for continuous variables. The baseline distribution of $\log_2$ antibody titre, age, sex, number of years worked at the hospital, occupation type, employment status and presence of high-risk conditions, will be presented by vaccination groups.

12.5.2 Serological endpoints

We will compare day post-vaccination HI titres among vaccination groups each season:

1. Seropositivity among vaccination groups will be calculated and compared using logistic regression, with seropositivity coded as 1 if the titre ≥40, and 0 if the titre is <40. We will test for trend among vaccination groups, assuming seropositivity will be lowest in the most highly vaccinated.
2. Seroconversion post-vaccination will be calculated and compared among vaccination groups by logistic regression, with seroconversion coded as 1 if the fold-rise in titre is ≥4 and 0 if the fold-rise in titre is <4. We will test for trend, assuming seroconversion will be lowest in the most highly vaccinated.
3. The post-vaccination fold-rise in antibody titres will be assessed using linear regression. The Jonckheere-Terpstra test will be used to test for a trend, assuming the $\log_2$ antibody titres in the highly-vaccinated group will be lower than the rarely vaccinated group, which in turn will be lower than the vaccine-naïve group.

The main exposure of interest is the vaccination experience of HCWs (categorical). All models will be adjusted for potential confounders and factors that may influence immune response; e.g. baseline titre, age, sex, BMI.

12.5.3 Influenza attack rates and VE

Evidence of influenza infection will be based on RT-PCR-confirmed infection, only, as serological evidence may be biased in vaccinees who elicit a good antibody response to vaccination (22). Attack rates will be calculated for each vaccination group as the number of cases during the person-time at risk.

VE will be estimated using a Cox proportional hazards regression model comparing the risk of influenza infection (coded as 1 for infected or 0 for uninfected) among HCWs by vaccination status: VE = $(1\text{-}HR_{adj}) \times 100\%$. If there are sufficient cases, the model will be adjusted for potential confounders (e.g. age group), and factors that may modify the risk of infection. Using virus characterization data, we will assess if failures are associated with antigenic mismatch.

12.5.4 Duration of illness

The number of days ill with influenza (count) will be compared among vaccination groups, adjusted for age. Because of the excess of 0 counts (people who never get infected), zero-inflated negative binomial regression will be used.

12.6 Analyses relevant to Aims 2 and 3

The laboratory analyses conducted in Aim 2 are relatively new and exploratory and there are therefore no well-established or well-accepted statistical methods for their comparison. [redacted]. For analysis of memory B cell data, only a small number of samples assayed will be assessed and it is likely that the sample will be too small for meaningful statistical analyses. This is due to the high cost of these assays. Thus, these data will be qualitatively assessed.

Aim 3 specifically addresses the parameterisation and interpretation of antibody data generated from Aim 2 by building mathematical and statistical models. Once parameterised, the immunological data will be used in inform mathematical and statistical models to understand risk of infection and the expected benefits of HCW vaccination programs.

Although several analyses are proposed herewith, as modelling tools are developed during the life of the project additional and/or modified analyses of these data will be conducted.

12.6.1 Interpreting the landscapes

Landscapes will initially be plotted for different groups of interest (i.e. vaccine-naïve, highly vaccinated and infected) and compared qualitatively. These initial plots will be based on predicted titres output from a random effects model that estimates the titre for each antigen, separately by time of blood collection. Some further exploratory statistical analyses of the antibody landscapes may be performed, such as using dimension reduction techniques and latent-class models to classify the shape of the antibody dynamic over time.

As part of Aim 3, models of antibody dynamics and individual-level exposures will be develop to quantify the different aspects of the antibody response that generated observed immunological profiles. These models will have linked components incorporating individual-level infection and vaccination history, cross-reactive antibody dynamics, as well as an observation process to account for noisy titre measurements (

). We will include parameters to control for each feature of the antibody response such as antigenic seniority, back-boosting, cross-reaction and waning of responses. These will be separately modelled to capture the relative contributions of prior infection and vaccination to underlying titres (Error: Reference source not found). We will also include observation error and censoring to convert the continuous model of expected titres into discrete serological measurements.

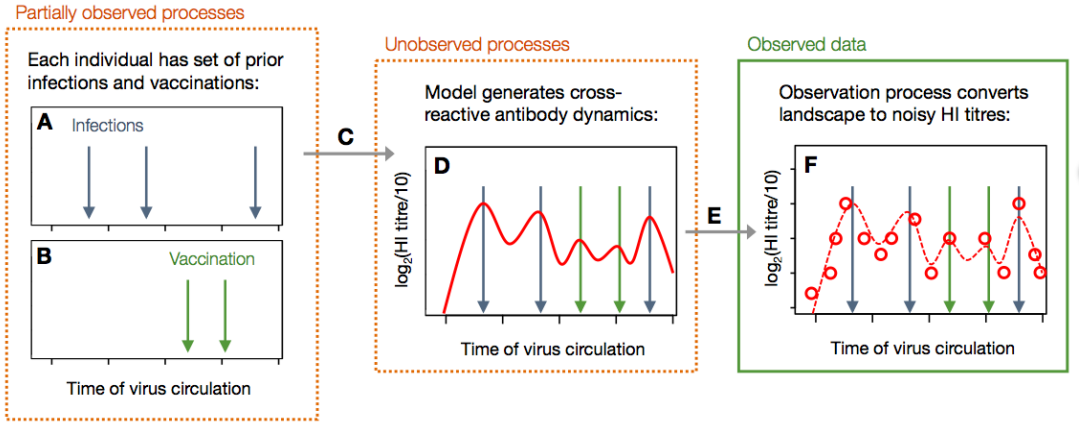


Figure 9. Antibody dynamic model

The model includes three components that reflect processes that are generally unknown or only partially observed: (A) individual-level infection or (B) vaccination history; via a model cross-reactive dynamics (C), these are linked to the third component (D), a 'smooth' underlying antibody landscape that results from a given history. An observation process (E) converts the three partially observed components into the final component, a set of noisy observed titres (F). By iteratively resampling model parameters using Markov chain Monte Carlo, it is possible to infer the unobserved processes from observed titre data.

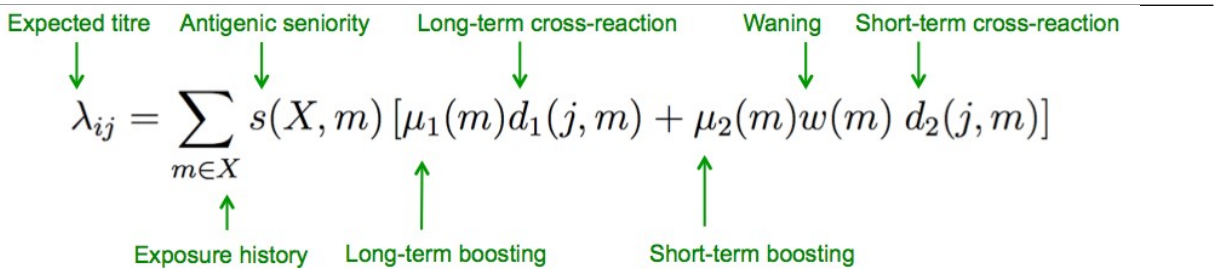


Figure 10. Model of antibody processes

The model translates exposure history into expected antibody titres, as indicated by arrow (C) in Figure 8. Here λ_{ij} denotes expected titre against strain j for individual i, with other parameters described in

.

With this model, we will be able to calculate the likelihood of observing a particular set of titres against the test strains, given a specific infection history and set of antibody response parameters. This will make it possible to estimate infection and vaccination histories as well as antibody response parameters in a statistically robust way, using Bayesian tools such as Markov chain Monte Carlo. As well as HI titre data, the inference process will include observed data on individual infections and vaccinations as informative priors, to reduce uncertainty about prior influenza exposures. We will use simulation studies to ensure that the inference methods can provide reliable parameter estimates given the size and structure of the available datasets. Using this modelling approach, we will be able to quantify the differences between immune responses following natural infection and vaccination, and obtain estimates of prior infections and vaccination in the study population.

Table 7. Parameters to be estimated in the antibody dynamic model, as defined in Figure 9

Letters in the 'process' column correspond to the labels in

. Although we will initially use simple functions to model processes such as antigenic seniority, these will be refined based on the findings from the B cell dynamics measured in Aim 2.

Process	Parameter to be fitted	Functional form in model
A. Infection history	Set of times at which infections occurred	Vector of timings
B. Vaccination history	Set of times at which vaccination occurred	Vector of timings
C. Model of antibody dynamics	Long-term boosting $\mu_1(m)$ following exposure to strain *m*	Constant value, fitted separately for infection and vaccination exposures
	Long-term cross-reactive response against strain *j* following exposure to strain *m*	Linear decline in titre based on antigenic map distance between strains, scaled by a cross-reaction parameter σ_1
	Short-term boosting $\mu_2(m)$ following exposure to strain *m*	Constant value, fitted separately for infection and vaccination exposures
	Short-term cross-reactive response against strain *j* following exposure to strain *m*	Linear decline in titre based on antigenic map distance between strains, scaled by a cross-reaction parameter σ_2
	Waning	Linear decline in titre with time, scaled by a waning rate ω
	Antigenic seniority	Linear decline with number of infections, scaled by parameter α
E. Observation	Observation error	Normally distributed with standard deviation parameter

model		ε, with censoring at discrete values]

12.6.2 Using dynamic antibody landscapes to predict infection risks

To investigate the implications of antibody landscapes for vaccination effectiveness, we will translate individual-level immune responses into landscapes that reflect protective immunity against specific strains. We will combine antibody responses with empirical data on correlates of protection, as well as observed infections in our cohort, to estimate infection risk against current and future influenza strains. In particular, the model will include a process parameter to adjust for individual HCW exposure risk, as infections will depend both on the extent of protective immunity and the potential for exposure. This will produce a generalizable model that can simulate individual-level immunity against any given antigenic variant, and show how this immunity is expected to change as antibody responses are boosted via infection or vaccination, and wane afterwards. As the model is refined we will identify a minimum set of titres against past or forward strains that capture the underlying 'smooth' antibody landscape and provide a reliable correlate of protection.

12.6.3 Evaluating current influenza vaccine effectiveness

We will use our validated model to refine estimates of the reduction in infection and disease resulting from HCW vaccination. The mechanistic nature of our mathematical model means we will be able to estimate immune responses following any hypothetical combination of infections or vaccination, not just those actually observed in our study population. Our model will therefore be able to account for the prevalent subtype or lineage; vaccination coverage, including different levels of HCW coverage and the mix of vaccine experience among HCWs; VE factors, including the expected VE dependent on the vaccine experience of each HCW; and vaccine composition, including whether the vaccine strains have been updated between seasons. We will also consider the influence of pre-existing immunity – both natural and vaccination-induced – based on dynamic antibody responses parameterized from antibody titre data. Using these data, we will use our model to explain variation in vaccination response among HCWs in light of their prior infection histories, vaccination experience, and risk of infection to understand why VE might differ by vaccination experience.

12.6.4 Evaluating alternative influenza vaccine scenarios

With our model in place, we will also compare the performance of current vaccination programs with simulated alternatives to predict the impact of repeated vaccination and circulating virus on VE under different scenarios. In particular, we will examine the potential impact of: highly-valent vaccines, which include more than a single strain for each subtype; universal vaccines that generate a broadly cross-reactive response against conserved influenza epitopes; and near-universal vaccines that produce a broader response, but still have potential to generate effects such as antibody focusing or seniority, which could reduce effectiveness.

13 STORAGE OF BIOSPECIMENS

13.1 Serum samples storage and shipping

Blood samples collected in serum tubes will be centrifuged within 24 hours of collection to separate the clotted blood from the serum and the serum removed to a clean tube. The clotted blood may be discarded. Sera will be aliquoted into separate tubes, the number of which will depend on the volume recovered, but is expected to be around 3 aliquots of approximately 200-500µl each. Each aliquot will be labelled with the study identification number and stored at -20°C.

[redacted], 1 serum aliquot will be stored at -20°C, while the remaining aliquots will be stored in a -70°C freezer or in liquid nitrogen. All freezers are locked and located in secured areas. [redacted]

13.2 Peripheral Blood Mononucleocytes (PBMCs) storage and shipping

Blood samples collected in heparin tubes will be processed for recovery of PBMCs on the same day or within 24 hours of collection. The recovered cells will be aliquoted in to ~4 tubes. Each aliquot will be labelled and cryogenically stored in liquid nitrogen [redacted]

[redacted]

13.3 Respiratory swabs

Respiratory swabs will be tested for influenza [redacted] The laboratories are all NATA accredited to provide diagnostic testing for influenza. Media from swabs that test positive for influenza virus will be aliquoted into two tubes and stored at -70C until shipping on dry ice [redacted] Samples will be stored at 4°C if shipping is planned within 2-3 days of specimen collection; otherwise samples will be stored in -70°C freezers. Remaining influenza virus negative swabs/media will be stored at -70°C in on-site freezers.

Influenza positive samples received at [redacted] On arrival, one aliquot will be inoculated into established cell lines to obtain influenza isolates for antigenic and genetic testing. Isolates will be assessed using antigenic assays such as the HI assay (see 11.1.2). Both original specimens and isolates may be genetically sequenced (note that viral RNA will be sequenced, not human DNA). Both the original specimen and any isolates recovered will be stored in -70°C freezers, according to NATA-approved SOPs [redacted]. All freezers are locked and located in secured areas. Samples will be stored indefinitely using study numbers.

13.4 Consent to biobanking of specimens

Samples will be stored indefinitely and may be retested as new technologies for understanding immunological responses to vaccination and infection become available. During the informed consent process participants will be asked if their samples can be stored for future studies.

14 DATA ENTRY AND MANAGEMENT

14.1 Data security

14.1.1 Study database

[redacted]

[redacted]

[redacted]

14.1.2 Data entry

[redacted]

[redacted]

14.2 Data storage

[redacted]

[redacted] All research data will be stored for a minimum of 15 years after completion of the project.

14.3 Participant identification and confidentiality

[redacted]

15 PARTICIPANT SAFETY AND WITHDRAWAL

15.1 Risks to participants

Study investigators and institutions are committed to protecting personal health information through the maintenance of privacy and security of each subject's personal information in this study. To protect

confidentiality, [redacted] If information from this study is presented publicly or published in a medical journal, results will be presented using aggregate statistics; individuals will not be identified by name or by any other personally identifiable information. [redacted]

Collection of respiratory swabs is not typically associated with pain or discomfort. However, if the lower nostril is sore or irritated due to illness, rotating a swab in this area may cause minor and very brief discomfort. Collection of blood will be completed by trained healthcare staff and should only be associated with minor and brief discomfort associated with the insertion of the needle. Any complications related to collection of respiratory swabs and/or blood should be reported to the site manager, and will be investigated by study staff, including a medical doctor.

15.2 Risk management and safety

15.2.1 Definitions

Adverse Event (AE): Any untoward medical occurrence in a patient enrolled into this study regardless of its causal relationship to study treatment.

Adverse events are classified as serious or non-serious.

Serious Adverse Event (SAE): An SAE is defined as any event that:

- results in death; or
- is immediately life threatening; or
- requires inpatient hospitalisation; or
- results in persistent or significant disability/incapacity.

Important medical events may be considered an SAE when, based upon appropriate medical judgement, they may jeopardize the participant and may require medical or surgical intervention to prevent one of the outcomes listed in this definition.

15.2.2 Eliciting adverse event information

Adverse events will be recorded from the time the HCW signs the Informed Consent Form until 7 days after each visit

15.2.3 Assessment and documentation of adverse events

The study coordinator is responsible for recording all adverse events, regardless of their relationship with the exposure, with the following exceptions:

- Conditions that are present at screening and do not deteriorate will not be considered adverse events.
- Abnormal laboratory values will not be considered adverse events unless deemed clinically significant by the investigator and documented as such.

Adverse events will be recorded using the Adverse Events Report Form, which will record:

- A description of the AE;

- The onset date, duration, date of resolution;
- Severity (mild, moderate or severe);
- Seriousness (i.e. is it an SAE?)
- Any action taken (e.g. treatment, follow-up tests);
- The outcome (e.g. recovery, death, continuing, worsening);
- The likelihood of the relationship of the AE to the exposure being investigated (e.g. Unrelated, Possible, Probable, Definite).
- Whether and when reported to the HREC

All AEs will be followed to adequate resolution.

15.2.4 Serious adverse event reporting

Any SAE occurring in a study participant will be reported to the Human Research Ethics Committee of the relevant hospital within 24 hours of the investigator becoming aware of the event, in accordance with the hospital policy. The SAE reporting form will be completed, signed and submitting by an investigator.

15.3 Participant withdrawals

HCWs may withdraw from the study at any time. The site PIs or site managers may withdraw a HCW from the study if s/he is showing significant distress towards the blood draw procedures. Information collected to that point will be kept unless the HCW explicitly requests otherwise. This information will be used for comparison with HCWs who complete all study procedures.

15.3.1 Follow up of withdrawn participants

All participants will be included in the study analyses, so it is important to have information on as many participants as possible. If a participant wishes to withdraw from the study, every reasonable effort will be made to complete a final evaluation of participants who exit the study early and the reason(s) for withdrawals will be recorded in the participant's study records.

15.4 Replacements

Recruitment to the cohort will be open, with preferential recruitment of new vaccine-naïve and unvaccinated HCWs in years 2-4.

15.5 Incidental findings

As part of participation in the study, HCWs will submit respiratory swabs for influenza testing. Testing is usually done using a multiplex PCR respiratory virus panel. These panels include from 5 to 17 viruses, and include, at a minimum: influenza A, influenza B, and respiratory syncytial virus. HCWs will receive the results of their test [redacted] Conditions which require additional treatment (e.g. influenza) or booster vaccination (e.g. pertussis) will be treated as per standard policy at each hospital. The antiviral drug Oseltamivir is available to HCWs through state health department programs to protect HCWs.

15.6 Vulnerable subjects

Pregnant women will not be excluded from the study. There are no special risks to pregnant women and they are identified as a target population for influenza vaccination in Australia and eligible for free vaccination under the National Immunisation Program (http://www.immunise.health.gov.au/internet/immunise/publishing.nsf/Content/Handbook10-

home). However, because this study requires long follow-up, pregnant women may be ineligible if they cannot meet the follow-up requirements; e.g. if they take extended parental leave.

15.7 Potential benefits of the proposed research to participants and others

Participants will not personally benefit from participating in this study. This study may provide information that will be useful to local study sites and facilities which may improve operations and infection prevention and control for all HCP. For example, local sites will receive information on occupational groups at increased risk for influenza illness and information. This study will provide additional understanding of immune responses to influenza vaccination and add to the existing knowledge on antibody focusing and enhancement that influences vaccination responses. This study will update models of the potential benefits of HCW vaccination. This information is useful for re-assessing influenza vaccination programs in hospitals.

16 RESOURCE SHARING PLAN

16.1 Data Sharing Plan

We intend the share the data generated by this research in several ways.

Publications: It is expected that at least 2-4 publications per aim will arise from this research. Publications will be formatted according the STROBE statement, where appropriate. We will target key journals in epidemiology, immunology and computational biology.

Presentations at Scientific Meetings: We intend to share our findings at international meetings and symposia, and will look for opportunities to collaborate and share resources with other investigators. We will submit abstracts to both local meetings and to key research meetings for influenza, immunology and epidemiology, including, for example, *Options for the Control of Influenza*, the *International Congress of Immunology*, and the *World Congress of Epidemiology*.

Nested studies: Nested studies that utilize the cohort will be encouraged. Any nested studies proposed will be reviewed by the project steering committee and will of course require IRB approval.

Sharing original data: The proposed study will collect demographic and clinical information, as well as blood and respiratory specimens from participants. Because we will be conducting longitudinal follow-up, we will be collecting identifiable information. Any data shared will be stripped of identifiers prior to release for sharing. However, there remains the possibility of deductive disclosure of participants with unusual characteristics. Thus, data will only be shared with new collaborators under a data-sharing agreement that provides for: (1) a commitment to using the data only for research purposes and not to identify any individual participant; (2) a commitment to securing the data using appropriate computer technology; and (3) a commitment to destroying or returning the data after analyses are completed.

Sharing of study protocols and SOPs: We will share our study protocols, SOPs, data collection tools and other study materials with other researchers/potential collaborators, upon request. The study protocol will be registered with https://clinicaltrials.gov/.

Sharing of code: All code for statistical analyses and mathematical models will be developed in R. All code will be shared upon request. Code for the mathematical models behind the analysis in each paper will be published simultaneously on GitHub under a GPLv3 license. This allows anyone to reuse and modify the code as long as any changes are made publicly available. For some components of the

analysis, we will develop packages in R which will be downloadable from the Comprehensive R Archive Network, https://cran.r-project.org/.

Study website: Information about the study, copies of any publications or presentations arising from the research and links to relevant external resources containing code will be shared via a study website.

16.2 Genomic Data Sharing

This study will not generate human genomic data. However, all virus sequencing data generated will be uploaded to the Global Initiative on Sharing All Influenza Data (GISAID) website, as part of standard surveillance practices [redacted]

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18 APPENDICES

Appendix A Screening form

RECRUITMENT SCRIPT

"Hello, my name is _____ and I am from the [*INSERT DEPARTMENT NAME*]. We are asking healthcare workers who work at [INSERT HOSPITAL NAME] to consider joining a research study on influenza vaccination. This study will follow a group of healthcare workers to better understand how repeat influenza vaccination impacts the immune response. Would you be interested in learning more about this study?"

a. YES → Complete screening instrument
b. NO → SAY: "Thank you for your time."
   i. INTERVIEWER NOTE: If offered, select reason for decline:
      - ☐ Too busy
      - ☐ Timing is inconvenient for other reason
      - ☐ Person is not feeling well
      - ☐ Not interested
      - ☐ Other reason, specify: _____

SCREENING INSTRUMENT

Date: _____	Affix PID here if eligible to participate	
A. Inclusion criteria		
1. Are you between 18 and 60 years of age?	☐ Yes	☐ **No → exclude**
2. Are you a staff, volunteer, student, honorary personnel at [*insert hospital name*] eligible for the hospital's free vaccination program?	☐ Yes	☐ **No → exclude**
3. Are you willing and able to provide follow up blood samples?	☐ Yes	☐ **No → exclude**
4. Are you available for follow-up over the next 7 months?	☐ Yes	☐ **No → exclude**
5. Are you willing to provide a mobile phone that can send and receive SMS	☐ Yes	☐ **No → exclude**
B. Exclusion criteria		
6. Have you had immunosuppressive treatment (e.g. systemic corticosteroid treatment or cancer therapy) within the past 6 months?	☐ **Yes → exclude**	☐ No
7. Are you currently ill? Or do you have a fever (>38°C)?	☐ **Yes → exclude**	☐ No
C. If eligible for enrolment, check vaccination history		

HCW Cohort Study – Screening form, Version 2.1, Date: 13 March 2020 1

8. Indicate year(s) influenza vaccine received and whether vaccine was received overseas:

	Yes - Australia	Yes - overseas	No	Don't know
2019	☐	☐	☐	☐
2018	☐	☐	☐	☐
2017	☐	☐	☐	☐
2016	☐	☐	☐	☐
2015	☐	☐	☐	☐

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Appendix B Advertising materials

Health Care Worker Cohort study recruitment notice

The *[insert name of department at hospital site]* is conducting a research study in collaboration with the [redacted].
The study aims to examine the immune response to flu vaccination among hospital workers by measuring antibody levels in blood before and after vaccination. The study will also examine the risk of infection after vaccination by collecting nasal swabs and blood samples from health care workers who develop flu-like illness.

- All staff are invited to participate, especially those not intending to be vaccinated and those who will be getting the flu vaccine for the first time.
- Participation will involve an initial visit before your flu vaccine, with up to 3 follow up visits approximately 7 days, 14 days and 7 months after vaccination. At each of these visits you will have a blood sample taken.
- If you develop flu-like illness during the study, you will be asked to collect a nasal swab for influenza testing. If you test positive for flu, you will be asked to give blood samples approximately 7 and 14 days after your illness started.

[redacted]

- The total time commitment for participating in this study is approximately 4 hours per year. The study will run for 4 years. Participation is voluntary and you will be free to withdraw at any time.

If you are interested in taking part in the study, or would like more information, please contact [*insert name of contact person*] on [*insert contact phone number*] or [*insert contact email address*].

Health Care Worker Cohort study recruitment in years 2-4

Are you planning to get the flu shot this year for the first time?

Even if you're not planning to get the flu shot, you may be interested in our study.

In 2020, the [*insert name of department at hospital site]* initiated a research study in collaboration with the [redacted]. The study aims to examine the immune response to flu vaccination among hospital workers by measuring antibody levels in blood before and after vaccination. The study will also examine the risk of infection after vaccination by collecting nasal swabs and blood samples from health care workers who develop flu-like illness.

- Enrolment in the study is open so we are looking for new volunteers
- All staff are invited to participate, especially those not intending to be vaccinated and those who will be getting the flu vaccine for the first time.
- Participation will involve an initial visit before your flu vaccine, with up to 3 follow up visits 7 days, 14-21 days and approximately 6 months after vaccination. At each of these visits you will have a blood sample taken.
- If you develop flu-like illness during the study, you will be asked to collect a nasal swab for influenza testing. If you test positive for flu, you will be asked to give blood samples 7 and 21 days after your illness started.

[redacted]

- The total time commitment for participating in this study is approximately 4 hours per year. The study will run for another [*number of years left*] years. Participation is voluntary and you will be free to withdraw at any time.

If you would like to hear more about this study, including our progress to date, and are interested in taking part in the study, please contact [*insert name of contact person*] on [*insert contact phone number*] or [*insert contact email address*].

Email to Heads of Department

To: Heads of Department
Subject: Invitation to participate in research study
From: [*insert email of site PI*]
cc: [*insert email of site coordinator*]

Dear [*head of department*],

We are conducting a study to understand the immune response to flu vaccination among hospital workers. We would appreciate it if you would please forward the attached notice to your staff.

Yours sincerely,

[*Site PI*]

Attachment: Health Care Worker Cohort study recruitment notice

cc: [*Site Manager, Project Manager, study PI*]

Appendix C Baseline questionnaire

Date: _____

Email: _____ **Mobile:** _____

A. Demographics

A1. Sex: ☐ Female ☐ Male ☐ Other

A2. Date of birth: ___/___/_____ (DD/MM/YYYY)

A3. Do you identify as an Aboriginal or Torres Strait Island person? ☐ Yes ☐ No

A4. Are any children under 12 years of age living in your household? ☐ Yes ☐ No

A5. Height: _____ cm **A6.** Weight: _____ kg

B. Medical history

B1. Do you have any of the following? (Tick all that apply)

- ☐ Cardiac disease
- ☐ Renal disease
- ☐ Chronic respiratory condition
- ☐ Haematological disorder
- ☐ Chronic neurological condition
- ☐ Pregnancy
- ☐ Immunocompromising condition
- ☐ Diabetes or other metabolic disorder
- ☐ Smoker

C. Occupation and Work Responsibilities

C1. How many years have you been employed at [*site name*]: _____ months _____ years

C2. Employment status: ☐ Full time ☐ Casual ☐ Part time

C3. Occupation type

- ☐ Medical
- ☐ Allied Health
- ☐ Administrative
- ☐ Ancillary
- ☐ Nursing
- ☐ Laboratory
- ☐ Other:_____

C4. In what departments, wards, or parts of your health facility do you regularly work? Check all that apply.

- ☐ Emergency Department
- ☐ Critical Care or Intensive Care Unit
- ☐ General Medicine and/or Medical Specialties
- ☐ Pediatrics and/or Pediatric Specialties
- ☐ Surgery and/or Surgical Specialties
- ☐ Gynecology and/or Obstetrics
- ☐ Oncology and/or Hematology
- ☐ Radiology
- ☐ Outpatient clinic
- ☐ Pharmacy
- ☐ Laboratory
- ☐ Nutrition
- ☐ Social Work
- ☐ Physiotherapy
- ☐ Occupational therapy
- ☐ Other:

C3. Do you provide hands-on clinical care to patients? ☐ Yes ☐ No

---

D. Influenza Vaccination

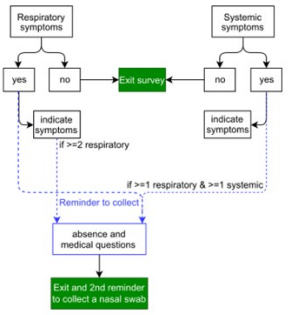
D1. Do you intend to get vaccinated next year? ☐ Yes ☐ No ☐ Don't know

HCW Cohort Study – Baseline questionnaire, Version 2, Date: 7 February 2020

Appendix D Weekly symptom diary

This weekly survey is based on the Flutracking online syndromic surveillance tool (https://info.flutracking.net/). Many HCWs will be enrolled in Flutracking and so questions are aligned with theirs, albeit a little more detailed, to permit assessment of representativeness of the cohort.

The decision tree for this survey is depicted below:



[For the first survey of the year, the following additional questions (Sections A-B) will be added:]

A. Demographics

A1. Are there any children under 12 years of age living in your household? ☐ Yes ☐ No

B. Medical history

B1. Do you have any of the following? (Tick all that apply)

- ☐ Cardiac disease
- ☐ Renal disease
- ☐ Chronic respiratory condition
- ☐ Haematological disorder
- ☐ Chronic neurological condition
- ☐ Pregnancy
- ☐ Immunocompromising condition
- ☐ Diabetes or other metabolic disorder
- ☐ Smoker

C. Occupation and Work Responsibilities

C1. Employment status ☐ Full time ☐ Casual ☐ Part time

C2. Occupation type

- ☐ Medical
- ☐ Allied Health
- ☐ Administrative
- ☐ Ancillary
- ☐ Nursing
- ☐ Laboratory
- ☐ Other:_____

C3. In what departments, wards, or parts of your health facility do you regularly work? Check all that apply.

- ☐ Emergency Department
- ☐ Critical Care or Intensive Care Unit
- ☐ General Medicine and/or Medical Specialties
- ☐ Pediatrics and/or Pediatric Specialties
- ☐ Surgery and/or Surgical Specialties
- ☐ Gynecology and/or Obstetrics
- ☐ Oncology and/or Hematology
- ☐ Radiology
- ☐ Outpatient clinic
- ☐ Pharmacy
- ☐ Laboratory
- ☐ Nutrition
- ☐ Social Work
- ☐ Physiotherapy
- ☐ Occupational therapy
- ☐ Other: _____

C4. Do you provide hands-on clinical care to patients? ☐ Yes ☐ No

COVID vaccination

[Branching logic will be used to hide the question form the survey if participant has received both doses of vaccine or has indicated they are declining COVID-19 vaccination.]

Have you received a COVID-19 vaccine within the last 7 days: ☐ Yes ☐ No – not yet received ☐ No – declined vaccination

[If participant answers yes, s/he will be asked to provide vaccination details:]

Vaccine ☐ Pfizer ☐ AstraZeneca ☐ Other, describe:_____

Vaccine dose ☐ Dose 1 ☐ Dose 2

Date of vaccination

Acute respiratory illness symptoms For the week of: [*start date*] to [*end date*]

Did you have any respiratory symptoms: ☐ No ☐ Yes

[If participant answers yes, s/he will be asked to indicate which symptoms:]

Cough?	☐ Mild	☐ Moderate	☐ Severe
Sore throat	☐ Mild	☐ Moderate	☐ Severe
Stuffy/runny nose	☐ Mild	☐ Moderate	☐ Severe
Chest pain	☐ Mild	☐ Moderate	☐ Severe
Difficulty breathing	☐ Mild	☐ Moderate	☐ Severe

Loss of taste or sense of smell	☐ Mild	☐ Moderate	☐ Severe

Or any systemic symptoms (fever, fatigue, muscle aches): ☐ No ☐ Yes

[If participant answers yes, s/he will be asked to indicate which symptoms:]

Fever or feverishness	☐ Mild	☐ Moderate	☐ Severe
Chills	☐ Mild	☐ Moderate	☐ Severe
Headache	☐ Mild	☐ Moderate	☐ Severe
Myalgia	☐ Mild	☐ Moderate	☐ Severe
Malaise	☐ Mild	☐ Moderate	☐ Severe
Fatigue	☐ Mild	☐ Moderate	☐ Severe

[If participant meets case definition or Acute Respiratory Illness, the questions above will be sent daily until symptoms resolve]

How long have you had these symptoms: _____ days

Have you had any direct contact with patients who have tested positive for influenza, COVID-19 or another respiratory virus? ☐ No ☐ Yes - Influenza ☐ Yes - COVID-19 ☐ Yes - other

Have you had any direct contact with any non-patients who have tested positive for influenza or COVID-19 or another respiratory virus? ☐ No ☐ Yes - Influenza ☐ Yes - COVID-19 ☐ Yes - other

[If participant indicates they have no respiratory or systemic symptoms, end survey]

IF YOU DEVELOP SYMPTOMS BEFORE THE NEXT SURVEY, PLEASE REMEMBER TO CONTACT YOUR SITE COORDINATOR AND COLLECT A SWAB

[If participant indicates they have 2 respiratory or 1 respiratory and 1 systemic, proceed with further questions]

YOU HAVE INDICATED THAT YOU MIGHT HAVE A RESPIRATORY INFECTION. PLEASE REMEMBER TO COLLECT A SWAB. THE SITE MANAGER, *<INSERT NAME>*, WILL BE IN TOUCH TO ARRANGE COLLECTION.

Absence from duties

Have you had to take any days off work? ☐ No ☐ Yes, how many? _____days

Medical attention

Did you seek health advice from a nurse or doctor because of this illness? ☐ Yes ☐ No

From which type of medical service?

- ☐ Hospital inpatient
- ☐ Emergency department
- ☐ General practitioner (GP)
- ☐ 24 hour health advice hotline
- ☐ Other medical professional

What was the diagnosis?

- ☐ Had flu
- ☐ Had COVID-19
- ☐ Other illness
- ☐ Don't know

[Upon closing the webform, the following reminder will appear if relevant:]

PLEASE REMEMBER TO COLLECT A SWAB

Other illnesses

Have you had any other non-respiratory illness or new diagnoses? ☐ No ☐ Yes, Describe: _____

[For the last survey of the year an additional item will be added:]

Intention to vaccinate

Do you intend to get vaccinated next year? ☐ Yes ☐ No ☐ Don't know

THANK YOU FOR YOUR PARTICIPATION IN OUR STUDY. THIS IS THE LAST WEEKLY SURVEY FOR THE YEAR. WE WILL BE IN TOUCH AGAIN IN APRIL [*INSERT YEAR*].

Appendix E Instructions for collection of respiratory swabs

HCW Cohort Study

Respiratory Swab Collection Instructions

For each acute respiratory infection, collect a nasal and throat swab AS SOON AS POSSIBLE after your illness begins. You may take a swab using the home kit provided and either return it when you come to work or send via mail.

Acute Respiratory Infection

An Acute Respiratory Infection (ARI) is defined as:

- At least 2 respiratory symptoms (cough, sore throat, difficulty breathing, runny nose), OR
- At least 1 respiratory symptom (cough, sore throat, difficulty breathing, runny nose) AND 1 systemic symptom (fever ≥38°C, chills, headache, myalgia, malaise)

Included with this kit

- Swabs
- Transport tube
- Specimen bag
- Participant ID labels

Before starting

- Try not to blow your nose before taking the nostril swabs
- Wash your hands before taking the specimens
- Peel open the package and remove the swabs and transport tube
- Label the transport tube with your name, date of birth, participant ID number (use label provided) and the specimen collection date

Nasal swab

1. Peel the package containing the swabs and remove the **narrow swab**. Be sure not to touch the tip or lay the swab down

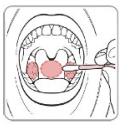
2. Tilt your head back
3. Insert swab into the nostril until a slight resistance is met

4. Gently rotate the swab against the inner wall of the nostril to capture a good sample of mucous if you have a runny nose
5. Remove the swab from the nostril and place in the other nostril and swab as before
6. Remove the cap on the transport tube
7. Place the swab in the transport tube and bend to snap at the breakpoint

Throat swab

1. Remove the **larger swab** from the plastic and be sure not to touch the tip or lay the swab down

2. Open your mouth wide, and stick your tongue out. You will see an arch at the back of your mouth



3. Rub the swab several times across the very back of your throat, behind the arch
4. Ensure you also swab the sides of the arch where your tonsils protrude
5. Try to avoid swabbing your tongue and teeth
6. Place the swab in the transport tube, as before. Bend to snap at the breakpoint

What to do next?

1. Tightly screw the cap onto the tube
2. Place the specimen transport tube in the specimen bag
3. Wash your hands thoroughly using soap and warm water
4. Store in the fridge until ready to deliver or post it to the study staff, preferably within 72 hours
5. Phone or email the study team to let them know you have collected the specimen

Remember, if you have any questions or problems with collecting the specimen, call your study team on [*enter contact number*]

Appendix F Template for antibody results for participants

Influenza Serology Analysis
Summary of Sera Received and Haemagglutination Inhibition Assay Results

Participant ID: _____ **Report date:** _____

Thank you for your participation in this study. Below are the results of your response to vaccination with the [*insert year*] quadrivalent influenza vaccine.

Reference strain	Subtype or lineage	Baseline titre[1]	Post-vaccination titre[1]	End of season titre[1]
		Sera date: dd/mm/yy	Sera date: dd/mm/yy	Sera date: dd/mm/yy
[*insert strain name*]	A(H1N1)pdm09			
[*insert strain name*]	A(H3N2)			
[*insert strain name*]	B/Victoria			
[*insert strain name*]	B/Yamagata			

1. Antibody titres measure the amount of antibodies in your blood. The titres reported here are for each of the four strains of influenza (reference strains) included in the [*insert year of vaccination*] quadrivalent influenza vaccine.

   Commonly, antibody titres of at least 40 indicate protection against the reference strain. This is sometimes called *seroprotection* or *seropositivity*. The degree of protection against the reference strain can vary by a number of factors including the level of exposure to influenza.

These results are provided for research/surveillance information only and should not be used for clinical management.

If you have any queries regarding this report please contact the study coordinator or principal investigators.

HCW Cohort Study – Template of antibody results, Version 2, Date: 7 February 2020
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Appendix G Participant cards

G.1 Vaccinated in the main study

[*insert hospital logo*]

Study Title	**Does repeated influenza vaccination constrain influenza immune responses and protection?**
Vaccinated participant	**Main study**
Participant ID	
Visit	Date completed
Consent and baseline blood	
Vaccination	
Day 14-21 blood	
End of season blood	
Additional visits may occur	
Nasal swab (self-swab)	
6-10 days post-flu blood	
14-21 days post-flu blood	

If you have any questions or concerns you can contact: [*study coordinator*] on [*insert phone number*] or [*insert email*]

G.2 Vaccinated in the nested cohort study

[*insert hospital logo*]

Study Title	**Does repeated influenza vaccination constrain influenza immune responses and protection?**
Vaccinated participant	**Nested study**
Participant ID	
Visit	Date completed
Consent and baseline blood	
Vaccination	
Day 6-10 blood	
Day 14-21 blood	
End of season blood	
Additional visits may occur	
Nasal swab (self-swab)	
6-10 days post-flu blood	
14-21 days post-flu blood	

If you have any questions or concerns you can contact: [*study coordinator*] on [*insert phone number*] or [*insert email*]

HCW Cohort study Participant card – vaccinated cohort, Nested study, version 1, 07/02/2020 Page **1** of **2**

G.3 Unvaccinated in the main study

[*insert hospital logo*]

Study Title	**Does repeated influenza vaccination constrain influenza immune responses and protection?**
Vaccinated participant	**Unvaccinated**
Participant ID	
Visit	Date completed
Consent and baseline blood	
End of season blood	
Additional visits may occur	
Nasal swab (self-swab)	
6-10 days post-flu blood	
14-21 days post-flu blood	

If you have any questions or concerns you can contact: [*study coordinator*] on [*insert phone number*] or [*insert email*]

HCW Cohort study Participant card – vaccinated cohort, Nested study, version 1, 07/02/2020 Page **2** of **2**