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Quantifying homologous and heterologous antibody titre rises after influenza virus infection

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SUMMARY

Most influenza virus infections are associated with mild disease. One approach to estimate the occurrence of influenza virus infections in individuals is via repeated measurement of humoral antibody titres. We used baseline and convalescent antibody titres measured by haemagglutination inhibition (HI) and viral neutralization (VN) assays against influenza A(H1N1), A(H3N2) and B viruses to investigate the characteristics of antibody rises following virologically confirmed influenza virus infections in participants in a community-based study. Multivariate models were fitted in a Bayesian framework to characterize the distribution of changes in antibody titres following influenza A virus infections. In 122 participants with PCR-confirmed influenza A virus infection, homologous antibody titres rose by geometric means of 1·2- to 10·2-fold after infection with A(H1N1), A(H3N2) and A(H1N1)pdm09. Significant cross-reactions were observed between A(H1N1)pdm09 and seasonal A(H1N1). Antibody titre rises for some subtypes and assays varied by age, receipt of oseltamivir treatment, and recent receipt of influenza vaccination. In conclusion, we provided a quantitative description of the mean and variation in rises in influenza virus antibody titres following influenza virus infection. The multivariate patterns in boosting of antibody titres following influenza virus infection could be taken into account to improve estimates of cumulative incidence of infection in seroepidemiological studies.

Key words: Antibody, epidemiology, influenza, serology.

INTRODUCTION

Influenza virus infections are largely associated with mild, acute, self-limiting respiratory diseases, while there are many other causes of acute upper respiratory tract infections apart from influenza viruses. It can therefore be challenging to ascertain all influenza virus infections in a cohort by prospective identification of illnesses, even with laboratory testing to confirm aetiology [1, 2]. One approach to determine the cumulative incidence of infections of a particular influenza type or subtype in a cohort of individuals is to measure the humoral antibody titres against a representative virus strain before and after periods of influenza activity, since infection by influenza virus generally leads to a rise in humoral antibodies after

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7–14 days [1–4]. Conventionally, the proportion of individuals whose antibody titres against the same virus (i.e. the homologous titres) rise by more than a threshold amount is taken to be the incidence of infection [5, 6]. Because twofold differences in antibody titre measurements can occur simply due to variability in the laboratory assay, a $\geqslant$fourfold rise in titre has traditionally been used as an indication of recent infection [7].

However, it is well-known that not every infected person experiences a $\geqslant$fourfold rise in homologous antibody titre, and some studies have attempted to correct this imperfect sensitivity when estimating cumulative incidence of infection [4, 6]. However, these attempts have not considered the potential variability of antibody changes between different people, influenza strains and types of assay, nor the possibility of antibody titre rises against other viruses of the same type/subtype or different type/subtype which can occur, known as cross-reactions [8].

In this paper we used a multivariate Bayesian model to study the extent, variability and correlation of antibody titre rises after influenza virus infection confirmed by reverse transcription–polymerase chain reaction (RT–PCR). We also explored the extent to which covariates such as age, sex, vaccination history, antiviral treatment, case status (infections in index cases *vs*. infections in household contacts), and baseline antibody titres affected these quantities.

METHODS

Participants

A study of influenza virus transmission in households was conducted in Hong Kong from 2009 to 2013 [9–11]. We recruited patients presenting to outpatient clinics with symptoms of acute respiratory illness who lived with at least two other people, and we included in further follow-up those who tested positive for influenza A or B by rapid antigen test. The follow-up included additional laboratory tests of the index patient and their household contact via home visits. The infection status of index cases and their household contacts were determined by collecting nose and throat swabs at baseline, after 3 days, and 6 days, regardless of illness, and testing for influenza A or B viruses by PCR. A subset of study participants provided baseline and/or convalescent serum specimens.

Baseline sera were collected from both index cases and household contacts at the first home visit, <72 h after illness onset for index cases and before illness onset for household contacts. The antibody titres of these participants were measured by haemagglutination inhibition (HI) and viral neutralization (VN) against up to eight different viruses, with some titres of a subset of participants measured twice.

A small number of participants were confirmed by PCR to be uninfected with any influenza A subtype but were not tested for influenza B; we assumed these participants were not infected with any influenza virus, because influenza B had generally low prevalence during our study period. Only participants with at least one baseline titre measurement were included in the analyses.

Laboratory methods

HI assays were used to measure antibody responses to the 2009 pandemic influenza virus A/California/4/2009(H1N1), the circulating seasonal A(H1N1) virus A/Brisbane/59/2007(H1N1), A/Brisbane/10/2007(H3N2)-like virus A/Uruguay/716/2007 (H3N2) which was included as the A(H3N2) component in the 2008–2009 and 2009–2010 northern hemisphere seasonal influenza vaccines, and the A/Perth/16/2009(H3N2)-like seasonal A(H3N2) virus A/HK/1985/2009 that circulated in Hong Kong in 2009–2011. Because of changes in the prevalent A(H3N2) viruses after 2011, sera collected in 2012–2013 were tested against A/Victoria/361/2011(H3N2) virus instead of A/HK/1985/2009. In addition, sera were tested against two influenza B viruses, B/Florida/4/2006 (Yamagata lineage) and B/Brisbane/60/2008 (Victoria lineage), which were included as the influenza B component in the 2008–2009 and 2009–2010 northern hemisphere seasonal influenza vaccines, respectively. The HI tests were performed in 96-well microtitre plates using reagents provided by the WHO Collaborating Centre for Reference and Research on Influenza, Melbourne and the WHO Collaborating Centre, Centres of Disease Control, Atlanta, GA using standard methods as detailed in the WHO reagent kit and elsewhere [6, 12].

Sera collected in 2009 were also tested with VN assays for antibody responses to the 2009 pandemic influenza virus A/California/4/2009(H1N1) and A/HK/1985/2009(H3N2) that was the circulating strain in 2009. The VN tests were performed in microtitre plates using neutralization of virus cytopathogenic effect (CPE) in Madin–Darby canine kidney (MDCK) cells. Serial serum dilutions in quadruplicate were

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mixed with 100 tissue culture infectious dose 50 ($TCID_{50}$) for 2 h and added to MDCK cells. One hour after infection, serum virus mixtures were removed and serum-free minimum essential medium with 2 μg/ml trypsin was added to each well. The plates were incubated and CPE was observed to determine the highest serum dilution that neutralized $\geqslant 50\%$ of the wells. A virus back-titration and positive and negative control sera were included in each assay [10].

Statistical analysis

We specified a statistical model to describe the changes in antibody titre levels to homologous and heterologous influenza virus infections, similar to a model we previously used to describe antibody responses following vaccination [13]. Under the model, the logarithms of the pre-infection antibody titres X_{i1} of subject i follow a multivariate Normal distribution (denoted as N_J) with mean vector μ_1 of length J (the number of antibody titre measurements across all virus subtypes and assays, which here is 8) and variance-covariance matrix Σ_1 of dimension $J \times J$. The variance-covariance matrix reflects how deviations from the average baseline titre levels are correlated between pre-infection antibody titre measurements, so that, for example, if a subject has a higher than average antibody titre by HI against the circulating A(H1N1)pdm09 virus then he/she might also have a higher probability of having a higher than average antibody titre measured by VN against the same virus. Conditional on having infection with virus k confirmed by RT–PCR, the subject's convalescent titres X_{i2} are modelled to rise on average by the J-length vector δ_k (on the logarithmic scale), with variance-covariance matrix $\Sigma_1 + \Sigma_2$, reflecting an additional variation due to infection beyond the natural variation of titres. Uninfected participants had δ_k and Σ_2 set to zero.

Measurement error was taken into account by allowing the observed antibody titres, denoted Y_{i1} and Y_{i2} for the observed baseline and convalescent titres, respectively (of which there were up to two values each for each subject due to repeated measurements), be normally distributed around the true, unobserved titres on the log scale. The variance due to measurement error, Φ, is on the log scale because the titres are determined through doubling concentrations. Data from uninfected participants were also included to improve precision in the estimate of Σ_1. The model is therefore described by the following equations:

$$\log(X_{i1}) \sim N_J(\mu_1, \Sigma_1),$$
$$\log(X_{i2}) \sim N_J(\log(X_{i1}) + \delta_k, \Sigma_1 + \Sigma_2) \text{ for infection } k,$$
$$\log(X_{i2}) \sim N_J(\log(X_{i1}), \Sigma_1) \quad \text{uninfected participants},$$
$$\log(Y_{i1}) \sim N_J(\log(X_{i1}), \Phi),$$
$$\log(Y_{i2}) \sim N_J(\log(X_{i2}), \Phi).$$

The model was also used to estimate covariate effects on δ_k using linear decomposition. This was achieved by setting δ_k equal to an intercept δ_{0k} plus the following dichotomous covariates: age (>40 years or not); sex (male *vs*. female); recent vaccination or not; prescription of oseltamivir or not; index cases **vs**. household contacts; and high (>1:40) *vs*. low ($\leqslant$1:40) baseline titres.

For each parameter, we specified uninformative prior distributions which were flat across the range of possible values. The posterior distributions were estimated with a No-U-Turn sampler using the package 'rstan' in R (R Foundation for Statistical Computing, Austria). For each model and dataset, two chains of 5000 iterations each were simulated, with the first 1000 iterations of each chain used for burn-in, followed by 4000 iterations used for estimation. Convergence was assessed using the potential scale reduction statistic [14].

RESULTS

The analyses in this paper were based on data from 306 participants (age range 4–92 years, 75 index cases, 231 household contacts) who provided sera, including 122 participants (73 index cases, 49 household contacts) who had influenza A or B virus infection confirmed by PCR. These participants were enrolled from 2009 to 2013, and the majority (254/306, 83%) were enrolled in 2009. Table 1 shows the characteristics of the participants. Convalescent sera were collected a median of 23 (range 16–49) days after illness onset of participants with PCR-confirmed infection, including both index and household contacts. Sera from 83 (27%) of the 306 participants were tested twice, providing information on intra-assay variability. Only seven influenza B virus infections were found and they were excluded in further analysis. Convergence was achieved for all parameters.

For each subtype of influenza A virus infection, the strongest rise occurred for the homologous titres (Fig. 1). The homologous geometric mean titre rises

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Table 1. *Characteristics of participants*

	Overall	Uninfected	Pandemic A(H1N1)	Seasonal A(H1N1)	Seasonal A(H3N2)	Seasonal B
Number of participants	306	184	39	24	52	7
Type of participant						
Index cases	75 (24·5)	2 (1·1)	25 (64·1)	10 (41·7)	33 (63·5)	5 (71·4)
Household contacts	231 (75·5)	182 (98·9)	14 (35·9)	14 (58·3)	19 (36·5)	2 (28·6)
Sex						
Female	171 (55·9)	113 (61·4)	20 (51·3)	10 (41·7)	25 (48·1)	3 (42·9)
Male	135 (44·1)	71 (38·6)	19 (48·7)	14 (58·3)	27 (51·9)	4 (57·1)
Age, years						
4–18	34 (11·1)	12 (6·5)	9 (23·1)	5 (20·8)	7 (13·5)	1 (14·3)
19–39	111 (36·3)	66 (35·9)	18 (46·2)	11 (45·8)	12 (23·1)	4 (57·1)
40–59	137 (44·8)	91 (49·5)	10 (25·6)	7 (29·2)	27 (51·9)	2 (28·6)
⩾60	22 (7·2)	13 (7·1)	2 (5·1)	1 (4·2)	6 (11·5)	0 (0)
Prescription of oseltamivir treatment						
Yes	46 (10·5)	1 (0·5)	17 (43·6)	6 (25)	18 (34·6)	4 (57·1)
No	140 (45·8)	81 (44·0)	14 (35·9)	11 (45·8)	32 (61·5)	2 (28·6)
Recent influenza vaccination (for current season)						
Yes	32 (10·5)	17 (9·2)	4 (10·3)	0	11 (21·2)	0
No	273 (89·2)	166 (90·2)	35 (89·7)	24 (100)	41 (78·8)	7 (100)

Values given are *n* (%).

for the influenza A virus infections varied between 1·2- and 10·2-fold, with pandemic A(H1N1) infections showing the greatest average rises and seasonal A(H3N2) the smallest. After seasonal A(H1N1) infections, both of the A(H1N1)pdm09 titres rose marginally significantly on average, while there were marginally significant rises in seasonal A(H1N1) titres after an A(H1N1)pdm09 infection. In most patients, heterogeneous VN titres did not rise substantially after an A(H3N2) infection, including in both HI and VN assays against the H3N2 virus.

Figure 2*a* shows the geometric standard deviations of the multiplicative titre rises, along with their 95% credibility intervals. The VN titres generally varied less after infection than the HI titres for the same antibodies, but not significantly so. In Figure 2*b*, the estimates are shown of the measurement errors of the titres, expressed as the geometric standard deviation of the observed titres relative to the underlying titre. The HI titre for the A/HK/1985/2009 influenza virus had the highest estimated measurement error.

Figure 3 shows the pairwise correlations between the titre rises. The most closely correlated titre rises are those for the A(H1N1)pdm09 assays, followed by the HI titres for A(H1N1)pdm09 and seasonal A(H1N1), and then the titres for seasonal A(H3N2) A/Uruguay/716/2007 and the seasonal A(H1N1) HI titres. The titres for the two A/HK/1985/2009 A(H3N2) viruses, as well as these two titres each with the A/Uruguay/716/2007 virus, had relatively low correlation.

Figure 4 shows the estimated distributions of homologous rises following infection with influenza A subtypes after taking into account natural titre variability as well as measurement error. It is clear that sometimes a substantial proportion of those infected experience <fourfold homologous titre rises, with an estimated 40% of those infected with pandemic A(H1N1) influenza exhibiting <fourfold rises in the homologous HI titre, while the observed proportion was 31%. Up to 50% of those infected with seasonal A(H3N2) were predicted to exhibit <fourfold titre rises in the A/HK/1985/2009 HI assay, while the observed proportion was 44%. Conversely, in 140 patients with no PCR-confirmed infection and complete influenza A virus titres on their first test, 33 (23·6%) had at least one ⩾fourfold rise in an influenza A antibody titre.

Figure 5 shows the adjusted geometric mean ratios, along with their credibility levels, in titre rises between different groups of participants for the homologous rises, i.e. the rises in antibody titres against the homologous influenza virus infection. Several covariates had differential effects on post-infection homologous titre rises. There was a marginally significant increase of 3·9 (95% CrI 0·9–16) in pandemic A(H1N1) HI titre rise after confirmed infection with pandemic A(H1N1) influenza virus between those who had recently been

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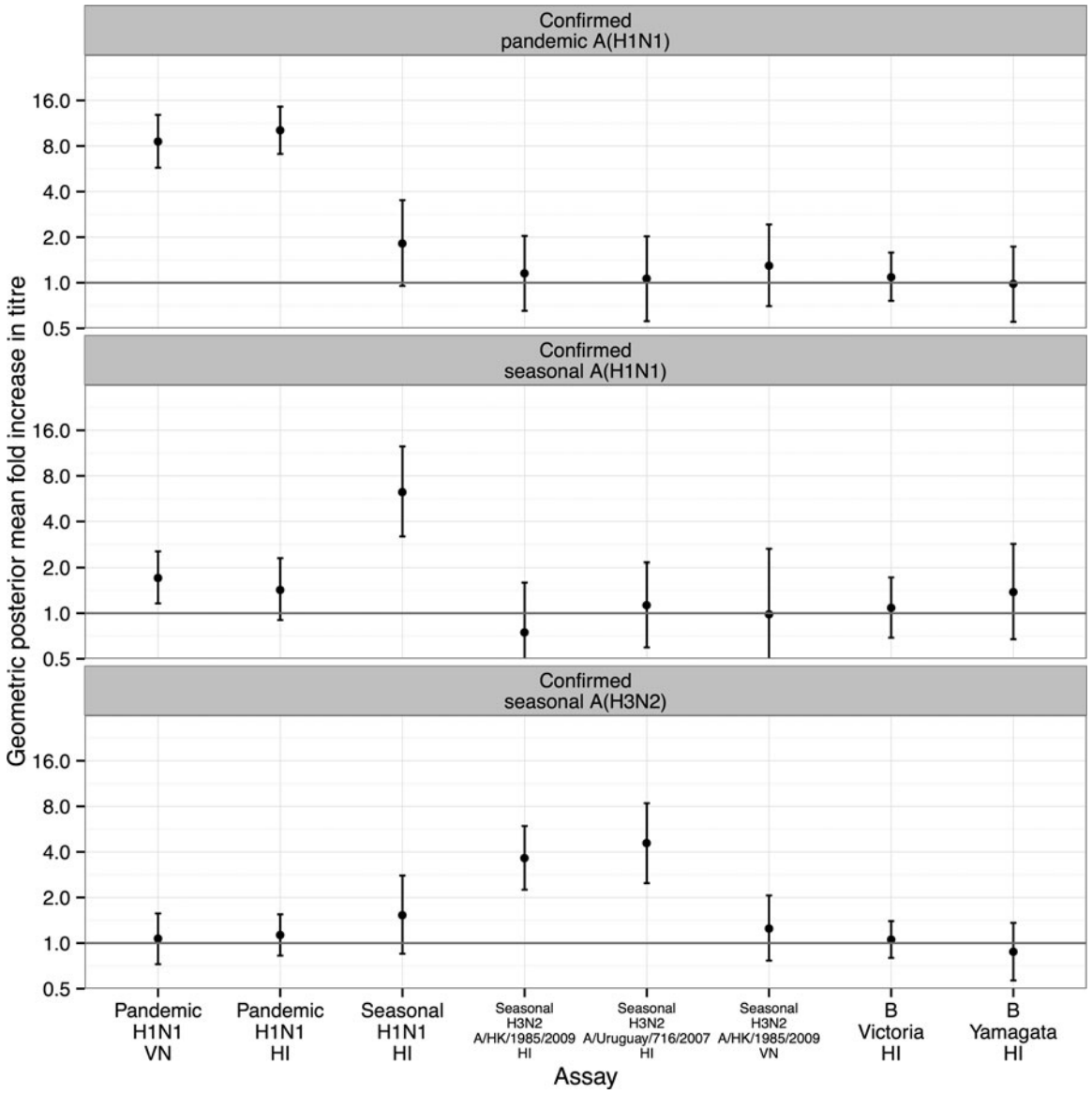

Fig. 1. The geometric mean fold (with 95% credibility intervals) of antibody titre rises after infection with different influenza A subtypes. Each column represents one titre and each row an infection scenario. The means and intervals are shown on a logarithmic scale. HI, Haemagglutination inhibition assay; VN, viral neutralization assay.

vaccinated versus those who had not. There was also significant observed differences in antibody titre behaviour between those who were prescribed oseltamivir treatment and those who were not, after A(H1N1) pdm09 infection, with significant reductions in the rises of the A(H1N1)pdm09 titres by VN. In addition, the model allowed us to explore the associations of these factors with non-homologous antibody titres, i.e. cross-reactions. The only statistically significant non-homologous effects (not shown in Fig. 5) were for the effects of baseline titres, where participants with higher baseline pandemic A(H1N1) HI titres had significantly greater rises in that titre following seasonal A(H3N2) infections (titre ratio 2·1, 95% CI 1·0–4·7), and participants with high baseline A(H3N2) HI titres against the prevalent virus had greater rises in that titre following pandemic A(H1N1) infection (titre ratio 10, 95% CI 2·3–44).

DISCUSSION

We identified substantial variability in antibody titre responses following confirmed influenza virus infections. Part of the variability could be attributed to

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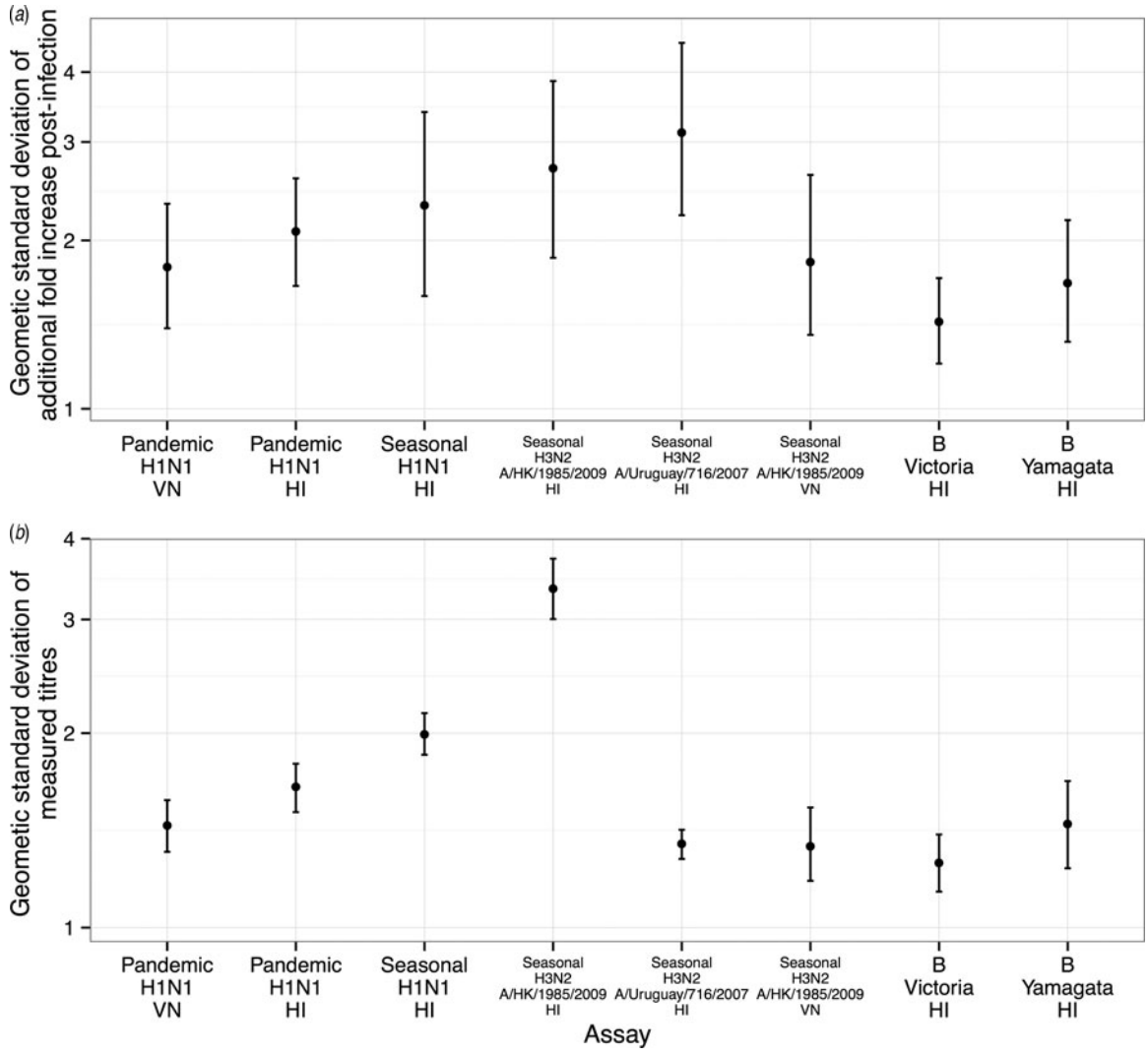

Fig. 2. (*a*) The geometric standard deviation (with 95% credibility intervals) of each titre's rise after influenza infection, estimated by the square root of the diagonal elements of Σ_2, which is the variance-covariance matrix of antibody rises after accounting for natural variation. (*b*) The estimated measurement errors in the titres, represented by the geometric standard deviations of the observed titres, and estimated by the square root of the diagonal elements of Φ, the variance-covariance matrix of antibody rises for observed titres.

covariates including the age of the subject and the receipt of antiviral treatment or prior vaccination (Fig. 5). These findings suggest that the proportion of a cohort that achieves a $\geqslant$fourfold rise in antibody titres to a prevailing virus may be an underestimate the cumulative incidence of infection, particularly for older adults. On the other hand, measurement errors in titres (Fig. 2*b*) suggest that cumulative incidence may be overestimated for strains that are not prevalent. Improved estimation of the cumulative incidence of infection might be achieved by using a multivariate model, such as the one presented here, which captures the pattern in expected rises of homologous and heterologous titres after infection as well as the variability of these titres due to subject characteristics, measurement error and residual noise.

We made estimates of the behaviour of antibody titres against various influenza types/subtypes, including cross-reactions, after boosting by PCR-confirmed natural influenza virus infections. However, our approach has some limitations. First, the sample consisted of index cases presenting with respiratory illness at outpatient clinics in Hong Kong and their household contacts. If subclinical infections have a lower probability of confirmation by PCR, and generally lower antibody responses, we may have overestimated the characteristics of boosting following natural infection on average in this study.

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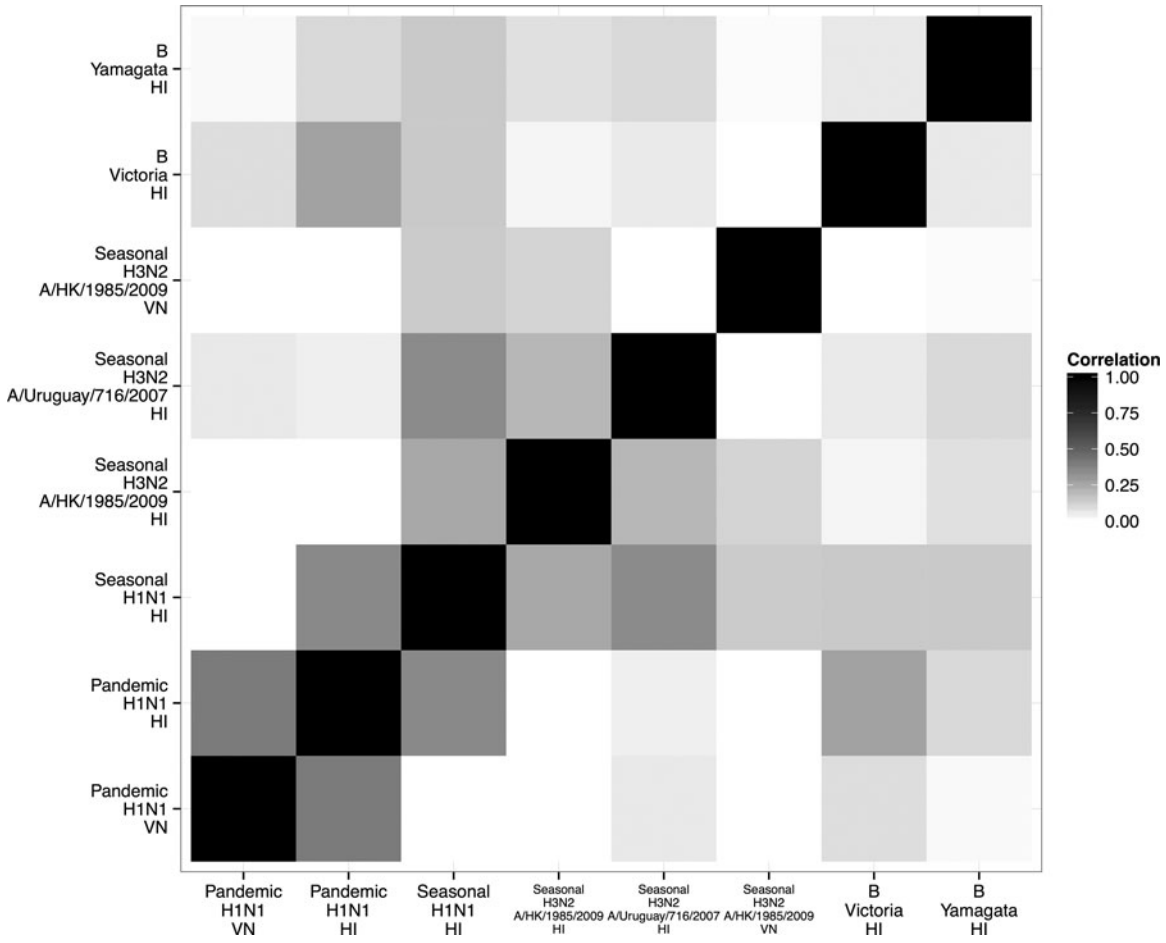

Fig. 3. The pairwise correlations between different antibody titre rises. The darker squares indicate higher correlations. HI, Hamagglutination inhibition assay; VN, viral neutralization assay.

Furthermore, we assumed that household contacts who tested negative for influenza by PCR were not infected with influenza, but some of those contacts may have been infected leading to overestimation of measurement error in our model. Second, the assays used for this study determined antibody levels by the standard method of doubling the dilution of serum until the relevant signal (haemagglutination or neutralization) ceases to be detected. The exact titre levels were therefore not available and the titre levels used in the analysis must be considered to be approximations. Third, we used PCR-confirmed infection as an indicator of infection. While PCR is now the gold standard approach for virological confirmation of influenza virus infections, some infections may have been missed due to imperfect sensitivity. It is also possible that some infections by influenza virus exhibit very low levels of viral shedding but nonetheless cause a rise in humoral antibody titres. Finally, we collected convalescent sera a median of 23 (range 16–49) days after illness onset, and titres may still be increasing up to 28 days after infection in some persons [15, 16], potentially resulting in an underestimation of the magnitude of response on average.

It has been suggested that there is an 'antibody ceiling' such that titres cannot rise much further if they are already higher than average, for example because of vaccination [17]. We did not find evidence of an effect of baseline titres on the amount of boosting following infection, examining differences between participants with baseline titres above or below 1:40 (Fig. 5). If a larger dataset were available, it would be valuable to examine potential ceiling effects associated with high baseline titres which were relatively rare in our dataset. However, we did find a significant difference in titre rises between those recently vaccinated and those not recently vaccinated in homologous titres after pandemic A(H1N1) infection, although it is unlikely this is due to differences in baseline titres as vaccination against the seasonal strains of

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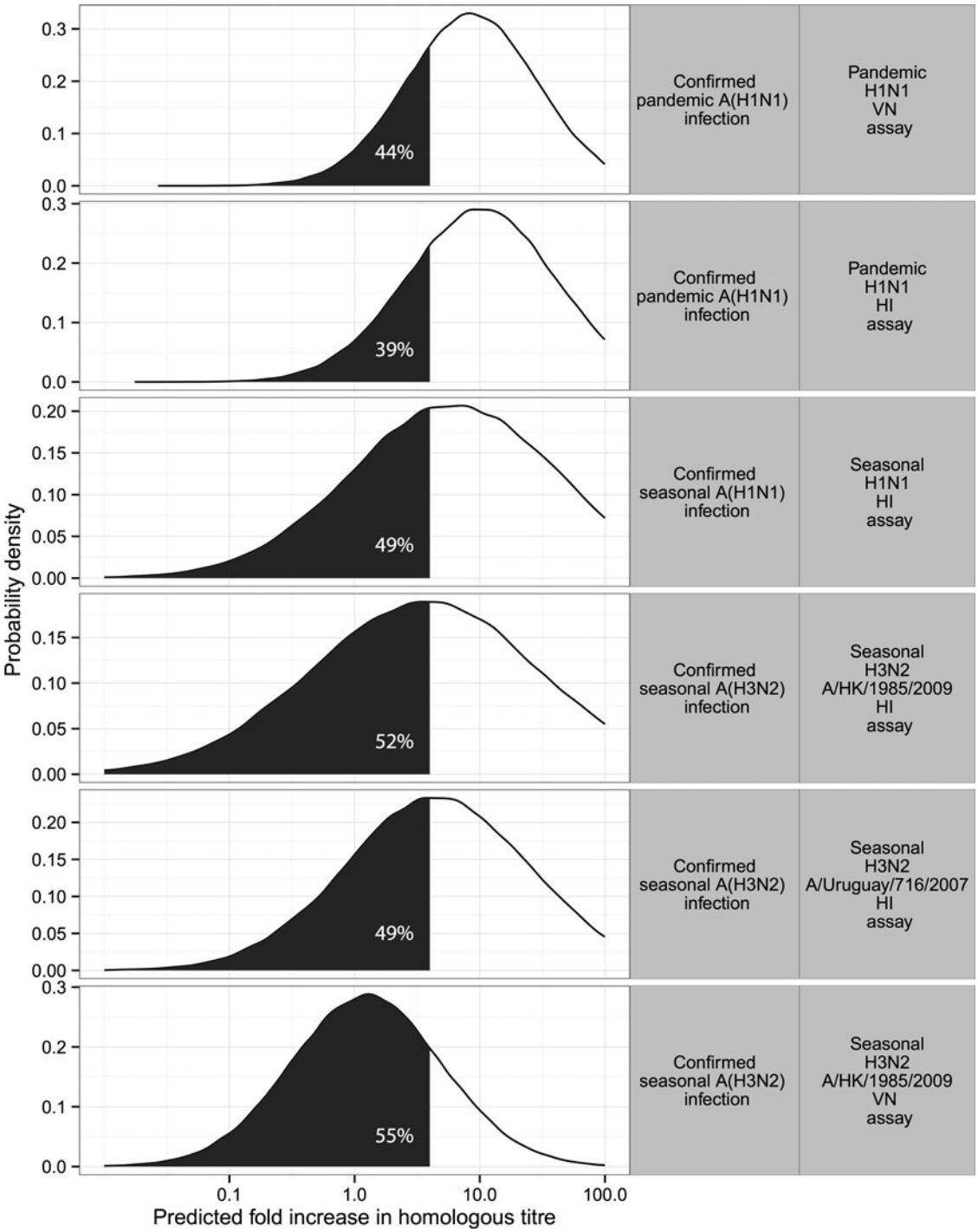

Fig. 4. The predicted rises in homologous titres following PCR-confirmed influenza virus infection, with the shaded areas indicating the proportions of rises that are predicted to be less than a factor of 4. HI, Haemagglutination inhibition assay; VN, viral neutralization assay.

influenza had minimal cross-reaction with 2009 pandemic A(H1N1) influenza titres in that season [18]. This suggests that prior seasonal influenza vaccination may have primed for a stronger serological response when pandemic infection occurs. This observation could have implications for interpretation of vaccine trials [19].

One interesting finding of our analyses is the association of oseltamivir treatment with reduced antibody responses following infection with influenza

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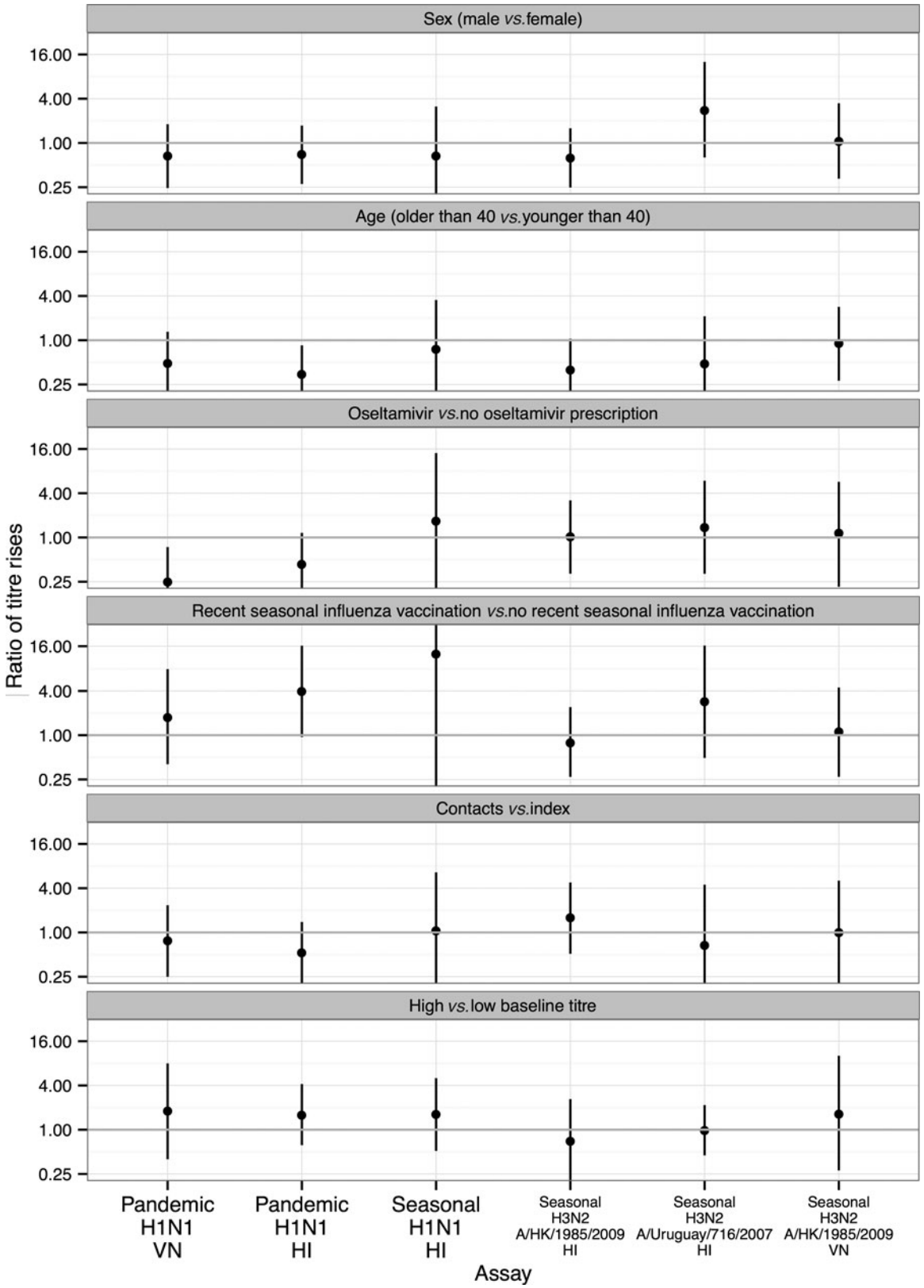

Fig. 5. The posterior geometric mean ratio in homologous antibody titre rises associated with different factors. The first two columns indicate the estimates for participants with PCR-confirmed H1N1pdm09 infection, the third column indicate the estimates for participants with PCR-confirmed seasonal H1N1, and the final three columns indicate the estimates for participants with PCR-confirmed H3N2.

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A(H1N1)pdm09. We previously reported this observation in a subset of the data included in the present analysis [10]. The effect was also reported in a re-analysis of randomized placebo-controlled trials of oseltamivir [20] but not in some other studies [21, 22]. The reason for this phenomenon is not clear.

Our results extend previous empirical quantitative investigations of the antibody responses to influenza virus infection. Chen *et al*. [23] described the change in cross-reactive antibodies to three seasonal influenza A viruses after RT–PCR-confirmed infection with influenza A(H1N1)pdm09, and found evidence of rises in the geometric mean titres of all three, one significantly so. Other studies have reported titres associated with cross-reactions as well as homologous reactions to various influenza virus infections [10, 24–27]. Whereas the proportion of laboratory-confirmed cases with $\geqslant$fourfold rises after infection has been reported as 82% in one study [23], other studies have found evidence that a substantial fraction of infections may not lead to $\geqslant$fourfold increases in antibody titres measured by HI [28, 29]. In our study we estimated that 39–55% of infected persons would not have a $\geqslant$fourfold rise in antibody titre after infection, with some variation by subtype (Fig. 4).

The approach developed here can be extended to incorporate as many virus types and assays as desired – for example new assays that were not available during this study, such as the protein microarray developed by Koopmans and colleagues [30–32], in order to increase the information concerning sera and therefore lead to less uncertainty as to the cumulative incidence of infection in populations. However, determining infections in individual persons using serology will likely remain problematic. Robust models for antibody titre kinetics that build upon our findings here could improve estimates of the cumulative incidence of influenza as well as providing bounds on levels of uncertainty. Some necessary extensions include understanding the behaviour of the titres over a longer period of time, such as the rate of waning after the initial boost [33, 34], both of the homologous titres and heterologous cross-reactions, and the boosting effect of vaccination [13]).

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DECLARATION OF INTEREST

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